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Pattern- and growth-linked cell cycles in *Drosophila* development

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Abstract. During *Drosophila* development the cell cycle is subject to diverse regulatory inputs. In embryos, cells divide in stereotypic patterns that correspond to the cell fate map. There is little cell growth during this period, and cell proliferation is regulated at G2/M transitions by patterned transcription of the Cdk1-activator, Cdc25/String. The *string* locus senses pattern information via a >40 kb *cis*-regulatory region composed of many cell-type specific transcriptional enhancers. Later, in differentiated larval tissues, the cell cycle responds to nutrition via mechanisms that sense cellular growth. These larval cell cycles lack mitoses altogether, and are regulated at G/S transitions. Cells in developing imaginal discs exhibit a cycle that is regulated at both G1/S and G2/M transitions. G2/M progression in disc cells is regulated, as in the embryo, by *string* transcription and is thus influenced by the many transcription factors that interact with *string*'s 'pattern-sensing' control region. G1/S progression in disc cells is controlled, at least in part, by factors that regulate cell growth such as Myc, Ras and phosphatidylinositol-3-kinase. Thus G1/S progression appears to be growth-coupled, much as in the larval endocycles. The dual control mechanism used by imaginal disc cells allows integration of diverse inputs which operate in both cell specification and cell metabolism.

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Animal development involves highly regulated programs of cell proliferation. In developing *Drosophila* the proliferation program includes free-running cell cycles that lack gap phases, G2 regulated cycles, reiterated stem cell divisions, G1 regulated cycles and cycles of DNA endoreplication. The regulatory inputs controlling these cycles vary according to stage and cell type, and impinge upon cell cycle controls in different ways. Here we compare three very different modes of cell cycle control used in *Drosophila*. In the first, cell cycle progression is regulated at G2/M transitions by the patterning system. In the second, progression is regulated at G/S transitions in response to nutrition and cellular growth. The third mode of regulation, which may be more akin to that used in human cells,

cleverly combines both of the first two strategies, and can thus integrate both patterning and growth cues.

Embryonic cell cycles: regulation at G2/M by patterning cues

Mitosis in most *Drosophila* cells is triggered by bursts of transcription of *string* (*stg*), a Cdc25-type phosphatase that activates the mitotic kinase, Cdk1. During the post-blastoderm embryonic cell cycles (14–16) *string* is transcribed in dynamic, invariant patterns that correspond to mitotic patterns, but which precede mitoses by 10–20 minutes (Edgar et al 1994). During this period all other factors required for cell cycle progression, including *cdks 1* and *2* and *cyclins A*, *B* and *E*, are expressed constitutively and are not limiting (for references see Edgar & Lehner 1996). This effectively eliminates G1 phases from these cycles, making *string* the de facto limiter of cell proliferation (Edgar & O'Farrell 1990).

Embryonic cells with the same developmental fate express *string* RNA and protein at the same times and divide synchronously, whereas different cell types have distinct temporal programs of *string* expression and division (Foe 1989, Edgar et al 1994). *string* transcription is altered in highly specific ways in embryos mutant for pattern formation genes of all types, including axis, gap, pair rule, segment polarity, homeotic, neurogenic and proneural genes (Arora & Nüsslein-Volhard 1992, Edgar et al 1994). Since many of these genes encode transcription factors that exhibit spatially restricted expression domains, these are the likely *trans*-regulators of *string*. Although the expression patterns of *string* partially align with those of these putative regulators, they are substantially more complex. This is almost certainly because *string* is regulated combinatorially, rather than by a single transcriptional activator in each cell type.

We mapped the *cis*-regulatory region of *string* using an *in vivo* gene-fusion/reporter approach and found that it consists of >40 kb of modular transcriptional enhancers (Fig. 1; Edgar et al 1994, Lehman et al 1999). Isolated fragments of the control region ranging from 0.5 to ~ 7 kb drive *string* transcription in distinct sets of cells in embryos, the larval brain and the imaginal discs. Many DNA fragments display tight cell-type specificity (i.e. mesoderm, tracheal and epidermal elements were identified), and virtually all the fragments show correct temporal expression in the cells where they are active. Elements within the ~ 40 kb mapped control region account for much of the normal *string* expression program, but our attempts to 'rescue' *string* mutants with coding transgenes as large as 31.6 kb failed, indicating that the functional *string* locus must be larger than this. Consistent with this, regulatory alleles of *string* that delete expression in the developing eye have been mapped farther than 30 kb upstream of the *string* transcription start site (Mozer & Easwarachandran 1999).

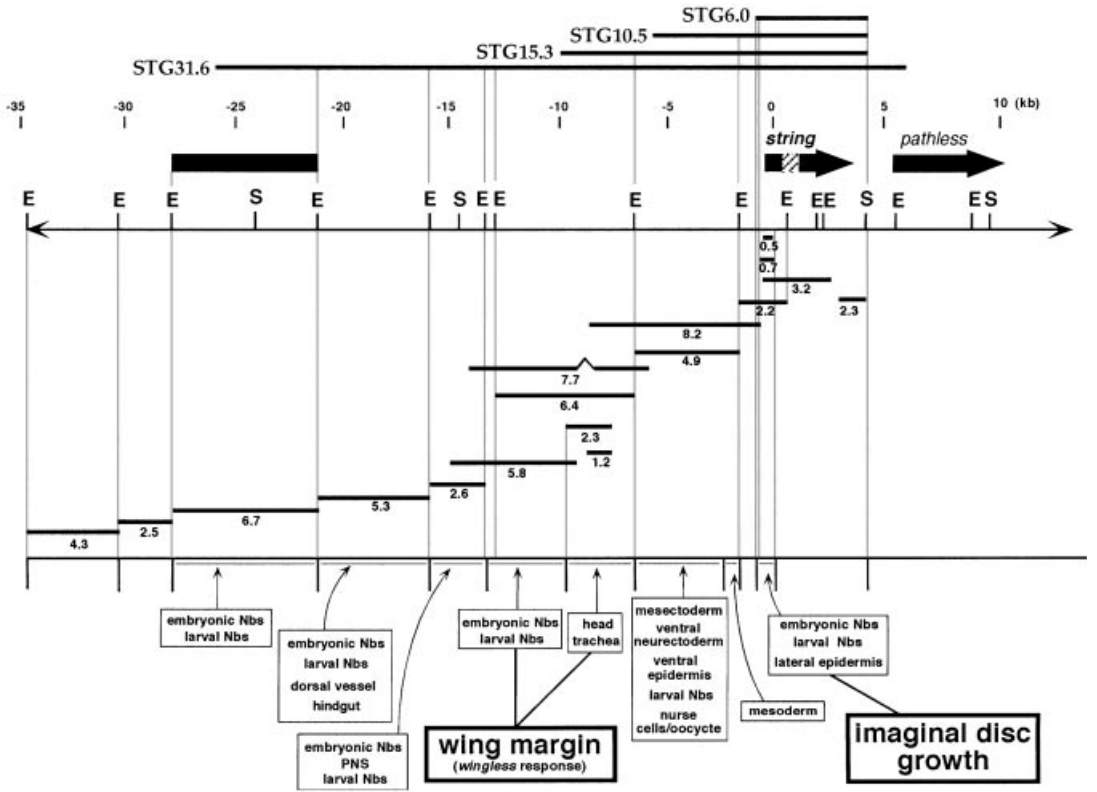

FIG. 1. Map of the *cdc25/stg* locus. The ~50 kb genomic region surrounding *string*. Bold black lines represent transcribed regions. A restriction map indicates EcoR1 (E) and Sal1 (S) sites. Above the restriction map genomic fragments tested for rescue *in vivo* are indicated (STG6.0, STG31.6 etc). Regulatory fragments used to drive *lacZ* reporter gene expression in transgenic animals are shown below the map. Tissues in which expression is driven by these fragments are indicated in boxes at bottom.

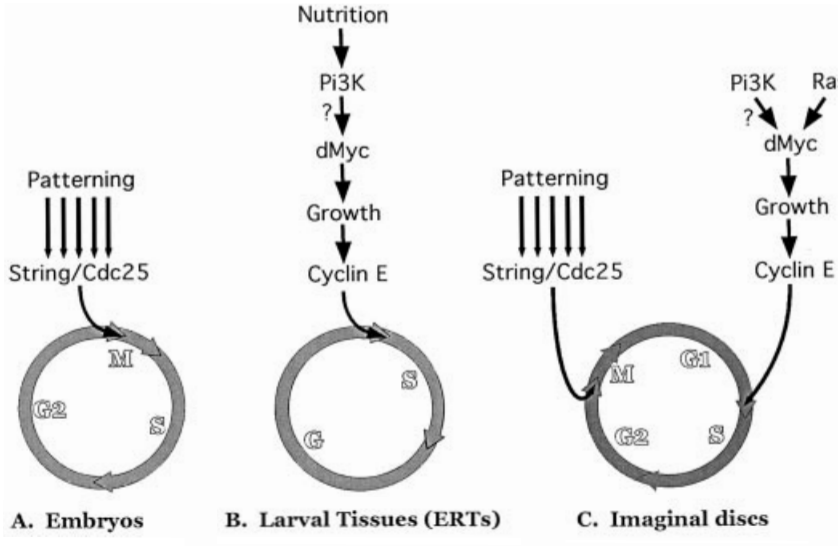

FIG. 2. Three different types of cell cycle.

In summary, transcription of the Cdc25-type phosphatase encoded by *string* limits the progression of many embryonic cell cycles, which are rapid (1–3 h) and require little cell growth. The massive *cis*-regulatory region of *string* acts as a sophisticated 'pattern sensor' that is influenced by a wide variety of cell type-specific transcription factors (Fig. 2A).

Larval endocycles: regulation at G/S by cell growth and nutrition

Drosophila larvae are built for eating and growth. Larvae increase their mass ~200-fold in just four days as they prepare for metamorphosis. Almost all of this growth is due to increases in cell size in differentiated larval-specific tissues that progress through a modified cell cycle — the endocycle — consisting of successive rounds of DNA synthesis without intervening mitoses. Endoreplicative tissues (ERTs) include the larval gut, fat body, salivary glands, malpighian tubules, trachea, muscles and epidermis. The switch from mitotic proliferation to endocycles occurs in late embryogenesis, and appears to be accomplished by loss of String and the mitotic cyclins A and B, whilst continuing periodic expression of the S phase-promoter, cyclin E. Studies of mutants defective in DNA replication and using replication inhibitors show that the increased DNA ploidy resulting from endocycling is absolutely required for larval growth (Royzman et al 1997, B. Edgar, J. Britton, A. de la Cruz, L. Johnston, D. Lehman, C. Martin-Castellanos & D. Prober, unpublished results).

Our studies of the ERT cell cycles show that they are regulated by nutrition (Britton & Edgar 1998). If the newly hatched larva is starved for dietary amino acids, DNA replication in most ERTs is not initiated. Under starvation conditions these tissues express low levels of cyclin E and E2F, the transcription factor which is probably responsible for cyclin E expression. If either E2F or cyclin E is induced in starved larvae, DNA replication in the ERTs is activated, and thus expression of these genes appears to limit the ERT cell cycle. When nutrient-deprived larvae are fed, expression of E2F and cyclin E mRNAs increases approximately sixfold, and DNA replication is initiated in most ERT cells. If the animal is first fed and then starved, the ERT cell cycle is activated and then inactivated quite rapidly. These experiments all indicate that the ERT cell cycle is nutrition-responsive, rather than controlled by a rigid developmental program.

This mode of regulation seems appropriate to the ERTs since their cells are already terminally differentiated, and their primary function is to grow and provide a nutrient rich 'incubator' for the undifferentiated neuroblasts and imaginal cells that eventually produce the reproductive adult. The response of these undifferentiated progenitor cells to food withdrawal is quite unlike that of the ERTs. Larval neuroblasts and imaginal disc cells continue to proliferate for many days after a larva is starved, and seem to complete their normal proliferation programs. In this instance the ERTs lose mass, presumably as they transfer stored nutrients to the developing nervous system and the discs.

These experiments suggest a mechanism in which E2F and cyclin E, which drive G/S transitions in the ERTs, respond directly to nutritional inputs (Fig. 2B). Our working hypothesis is that the link between nutrition and endoreplication in the ERTs is simply cell growth. This is supported by several observations. First, ectopic expression of genes that drive cell growth can promote extra DNA replication in ERTs in fed animals, and can trigger unscheduled DNA replication in the ERTs in starved animals. These genes include the transcription factor d-Myc (Johnston et al 1999), phosphatidylinositol-3-kinase (PI3K) (Weinkove et al 1999) and cyclin D/Cdk4 (unpublished results). Second, we've found that levels of cyclin E protein are post-transcriptionally responsive to increased rates of cellular growth in imaginal discs (see below; Prober & Edgar 2000). Assuming the same mechanism operates in ERTs, we suggest that ERT cells simply grow when nutritional conditions permit, and that one cellular response is increased cyclin E activity and DNA endoreplication.

Imaginal discs: dual control at G1/S and G2/M

Cells in the developing imaginal discs, which form much of the adult body during metamorphosis, exhibit a cell cycle that incorporates both modes of control described above (Fig. 2C). Each imaginal disc has 10–50 cells in the newly

hatched larva, and these proliferate to as many as 100 000 cells before differentiating into an adult structure such as a wing, leg, or eye (Bryant & Simpson 1984). Imaginal cells arrest in G1 during mid-embryogenesis and require the influx of nutrients from larval feeding to reactivate their cell cycle. Their first S phase occurs after a substantial (6×) increase in cell mass (Madhavan & Schneiderman 1977) suggesting that, as in the ERTs, cell growth may be a limiting parameter for progression of their cell cycle. The disc cell cycle averages 8 h and includes extended G1 and G2 periods (Graves & Schubiger 1982, Fain & Stevens 1982). During later disc development *string* is limiting for G2/M progression, whereas cyclin E expression limits G1/S progression (Milán et al 1996a,b, Neufeld et al 1998). Both phase transitions appear to be regulated, since disc cells typically spend several hours in each of G1 and G2, and either phase can be truncated by overexpressing the limiting regulator (Neufeld et al 1998).

We've used clonal activation or inactivation of genes, coupled with fluorescence-activated cell sorting (FACS) and quantitative *in situ* analysis, to probe the mechanisms of cell cycle control in imaginal wing and eye discs. Our initial tests showed that specific cell cycle regulators such as String, cyclin E, E2F, and the E2F co-repressor RBF play little role in regulating growth of the tissue (Neufeld et al 1998). When the cell cycle is either sped up or slowed down by manipulating these genes, cell growth continues at nearly normal rates. Consequently cells with a faster cycle are smaller, and cells with a slower cycle are larger. In contrast, mutations such as *Minutes*, which reduce growth by slowing protein synthesis, effect a slower cell cycle without changing cell size. This implies that cell growth has a dominant, regulatory effect on the cell cycle, and that the cell cycle control apparatus must employ mechanisms for sensing cell growth and adjusting cell division rates to match. We doubt that such mechanisms simply sense cell size, since cell size varies greatly during disc development (Madhavan & Schneiderman 1977, Neufeld et al 1998).

Potentially illuminating findings come from experiments in which we studied the behaviour of disc cell clones overexpressing genes that stimulate cell growth. The d-Myc transcription factor (Johnston et al 1999), the small GTPase Ras1 (Prober & Edgar 2000) and PI3K (Weinkove et al 1999) are all required for normal imaginal cell growth. When overexpressed each gene increases growth, as measured by increased clone sizes. Interestingly, increased growth effected by d-Myc, Ras, or PI3K does not accelerate the disc cell cycle. Instead, increased growth is manifest entirely as increased cell size (Fig. 3). In each case the large, fast growing cells generated have a truncated G1 phase. These observations suggested that increased cell growth is 'sensed' by cell cycle components that control G1/S transitions, but that G2/M controls are insensitive to growth stimulation. To test this notion we coexpressed the G2/M limiter String along with d-Myc (Johnston et al 1999) or activated Ras (Ras^{V12}; Prober & Edgar 2000). In both cases this

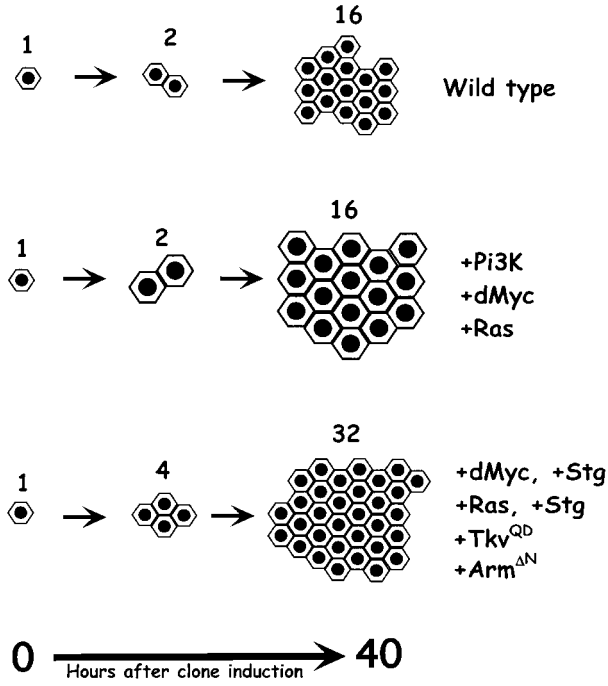

FIG. 3. PI3K, d-Myc and Ras accelerate cell growth, but not cell division. The cartoon depicts the behaviour of cell clones, induced at 0 h, that overexpress d-Myc, PI3K or Ras^{V12}. These cells grow faster and make larger clones, but cell division is not accelerated, and thus the cells are enlarged. Each treatment shortens G1, but appears to have little effect on rates of G2/M progression. Co-expression of the G2/M driver, String, with d-Myc or Ras^{V12} reduces the cell size and accelerates cell division, but does not suppress growth of the clone as a whole. Cell autonomous activation of *dpp* signalling (with Tkv^{QD}, an activated receptor) or *wg* signalling (*with* $Arm^{\Delta N}$, an activated intracellular transducer) also accelerates cell growth and division equally. String expression alone has little affect on growth or proliferative rate (Neufeld et al 1998).

reduced the cell size back to approximately normal, truncated the G2 phase and allowed the cells to divide faster than normal (Fig. 3). Importantly, co-expression of Stg with these 'growth drivers' did not decrease the overall growth rate, as measured in cell clones. This corroborated our hypothesis that the primary effect of increasing d-Myc or Ras activity was stimulation of cell growth, and that truncation of G1 was a secondary response.

How might cellular growth drive G1/S transitions? A mechanism in which an unstable, translationally regulated protein limits G1/S transitions provides one possible explanation. Consistent with this, we found that ectopic Ras^{V12} or d-Myc increased levels of cyclin E, and that this effect was post-transcriptional (Prober &

Edgar 2000). The 5′-untranslated region of the *Drosophila cyclin E* mRNA contains several short open reading frames (uORFs) which could potentially modulate its translation. Polymenis & Schmidt (1997) proposed that a uORF in the yeast G1 cyclin, Cln3, reduces initiation of translation at the downstream Cln3 translation start site. As a result, more ribosomes are needed to achieve efficient translation of Cln3. Since ribosome abundance is coupled to the cellular growth rate, this uORF renders translation of Cln3 growth-sensitive. Our data suggest that a similar mechanism could regulate production of cyclin E in the developing wing. Cyclin E would thus act as a 'growth sensor' to couple cellular growth rates to G1/S progression (Fig. 2B,C).

One intriguing aspect of these findings is that G2/M progression was not affected by d-Myc- or Ras-driven growth. This suggests that the G2/M regulator, String, does not act as a 'growth sensor' in the cycles we studied. Various observations indicate that, in late stage discs, *string* instead becomes subject to dominant transcriptional control by patterning genes, much as described above for embryonic cell cycles. This presumably results from the onset of differential expression of transcription factors that specify cell fates, and also interact with the *string* control region. *string* expression in late stage wing discs is repressed at the dorsoventral compartment border in response to *wingless* (*wg*), which organizes pattern in this region (Johnston & Edgar 1998). Consistently, the *cis*-acting element responsive to *wg* signalling lies several kilobases away from the *string* promoter (fragment 6.4 in Fig. 1). *string* also displays vein/intervein patterning in later wing development, presumably in response to *EGFR*, *Notch* or *decapentaplegic* (*dpp*) signalling.

Interestingly, *string*'s pattern sensing capability appears not to affect the cell cycle significantly during early disc growth. Cells in early discs cycle very rapidly, express *string* RNA constitutively and have a short G2 period (Fain & Stevens 1982, Neufeld et al 1998). This suggests that their cycle is primarily regulated at G1/S transitions and that, as in the ERTs, cell growth may be the limiting parameter. As development progresses however, distinct on/off patterns of *string* expression resolve and disc cells slow their cycle by accumulating in G2. In this way patterned control of G2/M by *string* appears to 'check' cell proliferation (and hence growth), even when growth promoters like PI3K or d-Myc are inappropriately activated. The contrast to the ERT cell cycle, which lacks this fail-safe check and runs wild in response to ectopic d-Myc, is intriguing.

How are G1/S and G2/M progression normally co-ordinated in the disc cell cycles? In closing it is pertinent to note that modulating pattern formation signals directly can indeed drive balanced cell growth. Specifically, cell autonomous activation of either *dpp* or *wg* signalling (two patterning factors required for normal wing growth) gives rise to cells that grow faster than normal, divide faster than normal, and retain a normal cell size (Fig. 3). Thus,

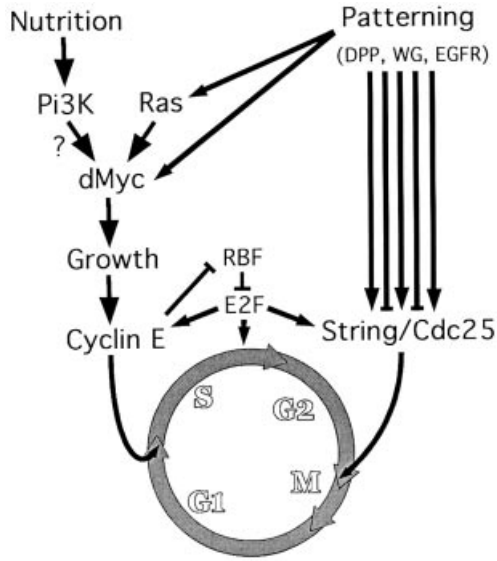

FIG. 4. Summary of imaginal disc cell cycles. Here we emphasize that patterning signals such as DPP and WG can accelerate both cell growth and cell division to the same degree ('balanced growth'). Hence these signals must stimulate both G1/S and G2/M progression as well as cell metabolism. Coupling relationships observed in numerous experiments are shown.

while *dpp* and *wg* signalling stimulate cell growth and G1/S progression, they must also augment the activity of the G2/M limiter *string* (Fig. 4). Determining the specific connections that link these patterning signals to cell growth and the cell cycle promises many interesting surprises.

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DISCUSSION

Bryant: As you showed on the growth curve for the imaginal disc, there is something that happens at the end of its development that stops both growth and proliferation. When you do your cell cycle analysis, is this in discs that have shut down at that point or in discs that are still in the growth phase?

Edgar: We haven't got to the exit phase yet in the wing. Technically this is harder to get at. All our analyses have been done in second and third instar imaginal discs which are still growing but which are starting to slow down.

Bryant: When you study those later phases do you see a change in the cell cycle phase distribution?

Edgar: Yes, this is an interesting point. In FACS profiles from different developmental stages, we see that as the disc grows the cell cycle slows down.

The cell doubling times go from less than 8 h to more than 18 h. Cells are also accumulating in G2. This is coincident with the gradual transition of String from a ubiquitously expressed product to a periodically expressed product. This transition can be largely attributed to regulation of String.

Bryant: Is it true that the only clones that escape this control are those that are the activated thick vein?

Edgar: One thing we don't understand about these discs is that none of the treatments we have tried have yet succeeded in bypassing this slow-down of the cell cycle or the exit of the cells from cycle when they finally differentiate. We can overexpress E2F, String, cyclin E or activated Ras, Myc, or activated thick vein, but in all of these cases the cells still stop cycling and differentiate. I don't know why.

Nasmyth: You overexpress Myc or Ras and this speeds up growth and gives bigger clones, but with bigger cells. Your model is that you are speeding up growth, but whatever it is that is triggering the G2/M transition hasn't been accelerated. That is not the only interpretation of this result. If this is the correct interpretation, one has to think very seriously about what sort of mechanism underlies this. What is it that is completely cell autonomous that can be operating here? If we think about it mechanistically we are beginning to talk about gene-limited synthesis. This brings up a whole other hugely important area for this meeting: what is the relationship between synthesis rate and cell size? Some people would agree that there is a strong relationship between the rate at which a given gene will give rise to a rate of protein synthesis, and generally speaking this is cell-size limited.

Nurse: The relationship between size and growth is an interesting issue that was discussed a lot in the 1960s but has not been discussed much in recent years. But before we address it could I clarify one other fact that confused me. Bruce Edgar, if you overexpress Myc, is the mass doubling time of the tissue unchanged?

Edgar: No, the mass doubling time is decreased. The response of the cell to this is to increase to a larger size and then plateau.

Nurse: This is what I am trying to clarify: cell size increases, but is the mass doubling also increased?

Edgar: Yes. There is more mass in the same time.

Nurse: So when you overexpress Myc, growth in tissue mass is increased and cell size is also increased.

Edgar: Yes.

Nurse: If you overexpress String, for example, are you just reducing the size back to normal under those circumstances, or do you also cause further rate mass increases?

Edgar: We have done this experiment, overexpressing Myc or Ras co-expressed with String. This decreases the cell doubling time and reduces the cell size, but the

clone size is roughly the same as with overexpression of just Myc. However, in both cases it is a little bit higher than normal. One thing you are asking about here may be whether it is necessary to increase the DNA replication rate to increase the growth rate.

Nurse: This is the issue: does advancing mitosis — that is, by elevating String levels — positively feed back on increasing growth rate?

Edgar: Yes. String plus Myc gives more mass than just Myc alone.

Nasmyth: But not much.

Hunt: There may be some confusion here. I suppose a smaller cell with more DNA can take more precursors aboard. And the more DNA it has the faster protein synthesis will be.

Edgar: DNA is definitely limiting for growth.

Nurse: This is the point: does the cell cycle itself feed back on the growth rate of the cell? Ultimately it must do when a cell gets really big, because the genes may not be able to support it.

Nasmyth: The question is, does it do so in the normal physiological range? The answer is largely no.

Leevers: Bruce Edgar's experiments show that division only feeds back on growth when you have already stimulated growth.

Edgar: Another interesting experiment worthy of discussion concerns the difference between what Myc does in a disc cell with a mitotic control apparatus, and in an endoreplicating cell without a mitotic control apparatus. The growth effects in the endoreplicating cell cycle are much more dramatic. Myc will cause a lot more growth in an endoreplication cell cycle than in a mitotic cell cycle. One reason for this may be that the DNA replication cycle is holding it back, basically acting as a developmental checkpoint.

Nurse: So gene concentrations may be limiting in certain circumstances.

Edgar: Definitely.

Nasmyth: There is another interpretation of the Myc result: when you overproduce Myc you actively inhibit String activity. I bet you that is the explanation.

Nurse: There is obviously a further interpretation: you overexpress Myc and increase growth rate, which feeds back on some sort of growth rate-modulated G2/M transition.

Nasmyth: Myc has many effects in the cell. One of the effects — whether it is via growth or independently of it — is that it ends up actively inhibiting String. This would explain why the cells are large.

Nurse: But there is no evidence that this is direct.

Nasmyth: And there is no evidence that it is actually happening. Bruce Edgar's explanation is still a viable one. That is, whatever the mechanism is that makes enough String, it is gene-limited.

Edgar: What do you mean by 'gene-limited'?

Nasmyth: There has to be a mechanism whereby when you speed up growth you fail to speed up the production of String. This is your hypothesis. One explanation for this is that this process is gene-limited. But usually that is not the case.

Nurse: Let's make sure everyone realises what Kim Nasmyth means by 'gene-limited'. This is the concept of gene concentration: the numbers of genes per unit volume of cell is reduced to a point where it becomes limiting. That is a rather crude causality compared with a regulatory linkage, which could be influencing growth rate. The reason I think this is interesting is because all these experiments that were done in the 1960s trying to measure whether genes become limiting might be worth re-addressing here. When does a gene's activity become limiting when cell volume is increased?

Lehner: When comparing cell proliferation in *Drosophila* imaginal discs and in yeast or bacterial cultures, where gene limitation has been studied previously, we should keep in mind that the number of cell population doublings that can be studied in imaginal discs is much lower. Wing imaginal discs progress only through about 10 doublings. Experimentally, imaginal disc cell proliferation is usually studied for a period of only four cell cycles or less. I doubt that a new steady state of cell growth and proliferation is reached after experimental perturbation within this brief period that is analysed. Development is very dynamic. There is essentially never a 'steady state' cell proliferation, where cells progress through the cell cycle during invariant growth conditions. Wing imaginal disc cells grow six times in volume before they begin cell cycle progression. During the imaginal proliferation they shrink. There might be some sort of developmental clock at work that might be just as important or even more important than the coupling of cell growth and progression through the cell cycle, which is presumably quite relaxed. We probably should not think of imaginal disc cells as yeast or bacterial cells where this coupling is tight. Imaginal disc cell proliferation is a brief and dynamic transitional phase; it is not a state.

Nasmyth: If you want a clock to produce that, it has to be completely independent of the growth rate and the cell cycle.

Edgar: The endoreplication cycles might be a better example of growth-limited cycles. Christian Lehner has done experiments in which cyclin E is overexpressed in endoreduplicating cells. They get stuck in G1. We have then expressed Myc in these G1-arrested cells; it doesn't make them grow much. They absolutely require endoreplication for growth.

Raff: Do you know what stops the cell growth itself in the disc towards the end of growth?

Edgar: No. There is one example in a subset of cells in the middle of the wing — the wing margin cells. In this region the wingless activity shuts down Myc transcription. This is a potential mechanism. But this is a subset of cells and it is a transient arrest.

Raff: Would it be correct to say that the link between patterning and stopping cell enlargement is missing?

Edgar: Yes, we don't understand this. The eye disc is reported to undergo lots of growth after cells exit their final cycle.

Bryant: It seems to me that if you accelerate the cell cycle you get smaller cells, and if you slow it down you get bigger cells, but this could also be progressive: with successive cycles the cells should be getting smaller and smaller or bigger and bigger. Can you address this within the number of divisions that are available in the growth of your clones?

Edgar: We haven't addressed this directly. We do know that if we speed up the cell cycle and generate small cells by overexpressing E2F or cyclin E, that there is a lot of cell death. From looking at the FACS traces, it seems that it is the smaller cells that are dying. You can imagine that in every cycle there is a subpopulation of very small cells that are generated which die.

Thomas: When you drive Myc, are you able to follow E2F? E2F should come up, and this should drive String.

Edgar: Possibly. That is an interesting point. We haven't done those experiments. We usually use the RNR2 gene as a reporter.

Thomas: Is your argument that you are driving E2F because you fail to induce cyclin E?

Edgar: The argument is that cyclin E is post-transcriptionally up-regulated by increased growth rate. This is the model that we are currently testing. There is a linkage through E2F, but I don't know whether E2F activity is growth regulated in this system or not. It is certainly required for the cell cycle. E2F can turn on String and cyclin E, and cyclin E can turn on String, probably by E2F.

Hunt: How much of that enormously long promoter is driven by E2F and how much is enhancer-driven by bits upstream?

Edgar: That is another great question that we don't know much about. All I can tell you is about that one experiment I mentioned in which a minigene with just a basal promoter can give low-level ubiquitous string expression and enough cell division to generate a fly.

Schmidt: There is still an assumption here that cell size is telling us about cell growth. If take mammalian Myc null cells, put 6×10^7 cells in a tube of null cells and wild-type cells, the null cells occupy twice the volume. So Myc null mutations in diploid fibroblasts actually gives bigger cells. I have been puzzling about this, saying that this is not what the paradigm is, but if growth is up-stream of S phase, then a slowing of growth should give a commensurate larger slowing of S phase, resulting in bigger cells, at least in one model. I wonder how much of what you are seeing is a decision to enter the cell cycle or not. It is not related to how it is being driven, but is rather a timing decision about whether to enter the cell cycle.

Hunt: You have set a hare running there! What do you mean by 'entry into the cell cycle'?

Schmidt: That in some sense the cells are deciding to just sit and do nothing, not grow. Can you distinguish these two in your assay?

Edgar: Yes. We are taking time points: we are turning a gene on and then tracking the growth of a clone of cells, measuring the area of the clone at different times. These clones get exponentially larger.

Nurse: The classical approach would be to have some marker showing whether the cells are in or out of cycle.

Schmidt: Let me take this back to the original question: how do you know that cell size is tracking cell growth?

Edgar: There is more DNA per unit area, there is more green fluorescent protein per unit area. In endoreplicating cells we have looked for one nucleolar protein, fibrillin, which is massively overexpressed in Myc-overexpressing cells.

Bryant: You have been showing us preparations in which nuclei have been stained. Are you estimating cell size from nuclear size?

Edgar: We measure cell size on the FACS machine by forward scatter and also by dividing number of nuclei over area measured. We get the same result.

Bryant: Do you find that the nuclear size is proportional to cell size in all cells?

Edgar: Generally, yes, regardless of the ploidy.

Newport: When you first presented your results it seemed as though you could split things up into two pathways: one would be Cdc25 and the other would be growth. What happens if the enhancers or the transcription response elements for something like Cdc25 have a narrower dynamic range, and they saturate at lower Myc concentrations? Growth has a much larger dynamic range.

Edgar: That is a good explanation; it makes a lot of sense. It is possible that the normal dynamic range of the growth in the discs is limited, and within that range Cdc25 is capable of responding. But then when you leave this range it can't respond and becomes limiting in an artifactual manner.

Nurse: Why do you think *Drosophila* cells endoduplicate so much?

Edgar: The larva is a fully functional animal made up of terminally differentiated cells, but its purpose is to be an incubator for these imaginal discs that make the fly. It has to bulk up on mass and it transfers this biomass to the discs as they grow. I think it is a really simple way for an organism that is already differentiated to grow. It is a sort of stripped-down cell cycle that can respond to nutrition. There is no need for those cells to proliferate.

Nurse: Is there an advantage in having a 1000 1C salivary gland cell as opposed to 500 2C cells?

Edgar: I don't know, but it is a simpler way to do things.

Lehner: Mitosis is a risky procedure: it involves the disruption of cell–cell contacts, for instance. Broken chromosomes can get lost during mitosis. If it is not worth the effort, why do it?

Leevers: It is unnecessary; most larval (polyploid) cells are going to lyse during metamorphosis.

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Introduction

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Without any reservation I can say that the Novartis Foundation Symposia are amongst the best discussion meetings held throughout the world, and I am sure that this will be yet another excellent meeting. The cell cycle and development is a topical subject covering the border between two research areas that are currently very active. The proposal for holding this meeting, which originally came from Jean Gautier, was an excellent one, and I am pleased to be the chair. Because the meeting straddles two fields, not all the participants will know everyone else, which should be stimulating for the discussion. I should add that the participants have been chosen for their breadth of views and their ability and willingness to discuss.

I will begin by introducing some of the issues that we should consider during this meeting. There are a number of general issues of importance, and whilst chairing the discussion I will try whenever possible to steer us towards these more general issues. We need to address how the cell cycle itself changes during development, how the cell cycle influences development, and how development influences the cell cycle. What are the changes to the cell cycle, how are these brought about, and why do they occur? We want to put these three questions in the contexts of influences on the core cell cycle machinery and interactions with developmental signals. These are two areas about which we already have quite a good body of knowledge, so we can have a sensible dialogue.

What are the sorts of changes in the cell cycle that we ought to be thinking of? The first is the general organization of the cell cycle, in particular the replication and segregation of the hereditary material, DNA. We want to know how progressions through the major cell cycle phases — G1, S, G2 and M phase — are changed during development, and why they are changed. Associated with cell cycle progression are checkpoint controls, which ensure that the events occur in the correct sequence and only proceed if previous events have been properly completed. These checkpoints change during development; for example, in an early embryo, checkpoints are largely lacking. There are also changes in the balance between cell cycle progression and apoptosis at different stages of development. We also need to consider modifications of normal cell cycles. The modification that occurs in all eukaryotic organisms is meiosis. In meiosis, S

phase is followed by two M phases, and there are differences in the cell cycle progression leading to higher levels of recombination and, most importantly, to reduction in chromosome number by a reductional division. These are major changes in the ways in which the cell cycle is organized. A somewhat more specialized change in cell cycle organization is endoreduplication. This occurs when a cell undergoes repeated rounds of S phase, generating cells with large nuclei. What are the mechanisms that underlie this? Is it failed mitosis or reinitiation of DNA replication? Why should this occur and what is its significance for development?

Much of what I have been saying up till now concerns timing — the regulation of the temporal order of events in the cell cycle. There is a whole other area of cell cycle control which is less well understood but which is important in development, namely the spatial organization of the cell. One problem is the symmetry of division. We know many examples where the symmetry of division is altered, but do not know how this is signalled and brought about. Asymmetric segregation of specific factors can also influence subsequent development. The direction of division is important and at different stages of development there are switches in the plane of division. This touches on the core cell cycle machinery: how it is regulated and how it is set in the context of the entire organism.

Finally, there is the whole thorny issue of growth and the cell cycle. How much is the cell cycle coupled to growth and how? How are these integrated together? This is particularly relevant to the effect of the cell cycle on development itself. Here we encounter some fascinating problems that are more developmental than cell cycle-related. These have to do with issues such as what determines the numbers of cell divisions that can occur within an organism or within an organ, which are of relevance to the cell cycle controls and the growth of cells. In some organisms the actual cell cycle itself is likely to have a major effect on the form of that organism, plants being a good example. Where plant meristems are generated with different structures leading to different organs, the contribution of cell division pattern is very important, particularly in an organism where cells don't move very much. The cell divisions and the direction in which they occur have a direct effect on the generation of form within plants. A third issue concerning the interaction of development and the cell cycle is how the cell cycle itself can influence the expression of the subsequent phenotype of a cell. For example, does the process of replication influence subsequent gene expression, this problem has relevance to issues ranging from imprinting to the asymmetric segregation of determinants. This links cell cycle progression with subsequent cell differentiation. These are some of the general issues I think this meeting may find profitable to discuss.

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Imprinted genes and the coordination of fetal and postnatal growth in mammals

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Abstract. A substantial proportion of genes that control fetal growth in placental mammals are imprinted. Imprinted genes can act in fetal tissues to regulate growth by cell proliferation, cell death and the make up of extracellular space. Imprinted growth-promoting genes such as *Igf2* probably act predominantly in an endocrine fashion, thus coordinating organ growth with the growth of the organism. In overgrowth and growth deficiency syndromes, however, imprinted growth factors can act by more local mechanisms, resulting in disproportionate growth. In addition to controlling fetal growth directly and thereby determining the nutritional demand of the fetus, imprinted genes can also apparently limit the nutritional supply to the fetus through the placenta. Imprinted genes may also be involved in postnatal growth up to weaning.

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Growth is essential for life. Being the right size in different circumstances and environments can crucially affect survival and reproductive success. Regulation of growth of an organism in relation to its environment is thus pivotal for evolutionary success and fitness. Related and similarly important is the regulation of the size of individual organs in the body in relation to each other and to the size of the whole organism, so that the body is the right shape and all the components work well together.

Growth at the level of the organism is an increase in cell number, cell mass, extracellular mass, fluid content and other components of the body that result in an increase of body mass. The mammalian body has very different growth phases with different functions. The pre-implantation embryo does not grow, but only divides, fuelled by materials that were deposited in the egg before fertilization. After implantation there is very rapid cell division which is tightly coupled to cell differentiation in the process of gastrulation — the creation of the different lineages

and organ primordia. At this stage the embryo is still minute in comparison with the size of the mother; thus energy and nutrient transfer from the mother is not the critical factor that it becomes soon afterwards when the placenta develops fully, and the fetus becomes completely dependent on food from the mother. Rapid organ growth occurs in this fetal period and the size of the fetus is regulated presumably both by its genetically determined demands for food (e.g. expression levels of growth factors) and the availability of this food to the fetus through the placenta. At this stage there is also the possibility for the mother to even out food supplies between fetuses in mammals that have a number of offspring in a litter. Immediate postnatal growth is similar, in that there is a balance between demand by the offspring for milk (which can be genetically determined; Alexander et al 1999), supply, and competition with other offspring in the litter. During this period there is amazing regulation of growth whereby a pup's current size is compared to its genetic trajectory (the size it should be) and adjustments upwards (catch up) or downwards (catch down) are made. For example, the placental food supply may have limited a pup's growth when its fetal growth factors were set to a higher level of demand; this level of demand may be satisfied after birth by a plentiful milk supply enabling this pup to catch up with its own genetic trajectory (and thereby its littermates).

Finally, growth after weaning is no longer limited by the mother but by other environmental factors many of which are now integrated by functions in the CNS, and growth of the organism is thus largely controlled centrally.

Fetal and immediate postnatal growth are thus similar in that supply of food is controlled by the mother. This results in a genetic peculiarity: conflict between paternal and maternal copies of genes that control growth, with paternal copies programming pups to be greedy, and maternal copies putting the brakes on in the interest of current and future littermates. Genetic conflict of this type — so the theory continues — led to the emergence of imprinted genes in mammals (Moore & Haig 1991).

Here we argue that imprinted genes, those genes that are expressed depending on their parental origin from mother or father, have a central role in the regulation of fetal, and perhaps of early postnatal growth. Imprinted genes can apparently act both in the fetus (increasing for example cell proliferation) and the placenta (influencing placental function and thus food supply). Thus the interplay of imprinted genes is critical for the coordination of whole body growth with nutritional factors, and presumably also for the coordination of organ growth in relation to growth of the organism. The study of imprinted genes, we argue, will lead to fresh insights into old unsolved biological problems such as the control of organ and body growth. This is both of intrinsic biological as well as of medical interest, since fetal growth abnormalities are associated with adult diseases.

Role of imprinted genes in fetal growth

A number of genes affect fetal and early postnatal growth and these fall into different categories (Efstratiadis 1998). We only consider genes as growth controlling (Table 1) if they have a direct effect on fetal size or organ size. We exclude, for example, genes that result in aberrant functioning of the heart leading to poor circulation, which may cause reduced growth. However, in a following section we also consider genes with an indirect effect on growth due to their action in development or function of the placenta, which in turn has an effect on nutrient transfer. More difficult are genes that act in placental differentiation and development before the placenta even begins to function in nutrient transfer. Mutations in these are lethal to the fetus, but more subtle changes might well interfere with nutrient function of the placenta at later stages. The current assessment may therefore require revision in the future.

There is only one major system of interacting growth factors and receptors in the fetus — the insulin (INS) and insulin-like growth factor (IGF) system (Table 1 and Fig. 1). Ignoring various binding proteins that modulate IGF actions in tissues and

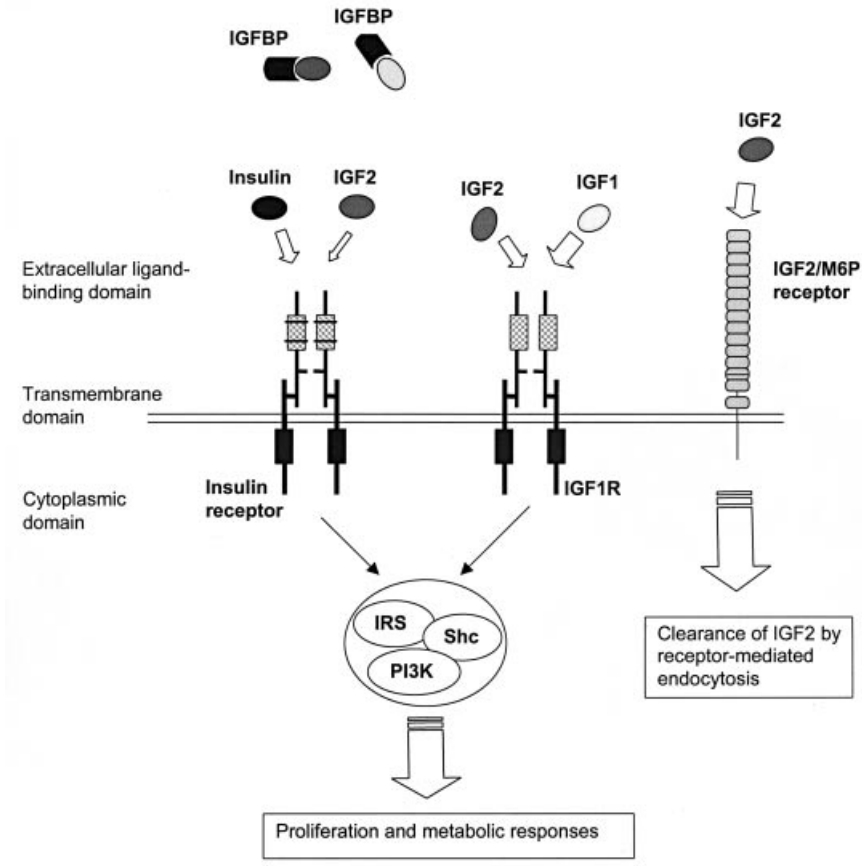
TABLE 1 Major growth control genes in the mouse

Gene members (chromosome)	*Effect*	*Phenotype (loss of function)*
The growth hormone pathway		
Ghrb (2), *Ghrbr* (6), *Gh* (11), *Prop1* (11), *Ghr* (15), *Pit1* (16)	Growth-promoting	Postnatal growth retardation (∼p15)
The insulin (INS) and insulin-like growth factor (IGF) pathways		
Igf1 (10)	Growth-promoting	Fetal and postnatal growth retardation
Igf2 (7)	Growth-promoting	Fetal growth retardation
Ins1/Ins2 (19/7)	Growth promoting	Fetal growth retardation; perinatal death
Igf1r (7)	Growth-promoting	Fetal growth retardation; neonatal lethality
Igf2r (17)	Growth-inhibiting	Fetal overgrowth; perinatal death
Insr (8)	Growth promoting	Fetal growth retardation; death by p5
Irs1/Irs2	Growth promoting	Fetal growth retardation
Grb10? (11)	Growth-inhibiting	Fetal overgrowth (Paternal Disomy)

Imprinted genes are shown in bold; p15, postnatal day 15; p5, postnatal day 5.
(Adapted from Efstratiadis 1998.)

the circulation, the system consists of three ligands (INS, IGF1, IGF2) and four receptors (INSR, IGF1R, IGF2R, receptor X), one of which is a 'scavenging' receptor (IGF2R) and one of which has only been identified genetically so far (receptor X) (Efstratiadis 1998). IGF1 acts mainly on the IGF1R; INS acts mainly on the INSR; IGF2 acts on IGF1R, INSR, IGF2R and receptor X (Fig. 1). IGF1 acts exclusively in the fetus (and postnatally), but the IGF2 system acts both in the fetus and in the placenta. Intriguingly, of these seven components, three are imprinted (*Igf2*, *Ins*, *Igf2r*) and it is thus mainly the IGF2 system that has imprinted components.

On the other hand, of the 45 or so imprinted genes that have been identified so far, seven have been shown by genetic analysis to have an effect on fetal growth as judged by altered birth weights of pups (Table 2).



We propose that additional imprinted genes that affect fetal growth will either turn out to be interacting components of the IGF system (*Grb10* is a strong candidate, for example), or will turn out to be genes that act in the placenta and affect nutrient transfer (perhaps *Mash2* or placental *Igf2*, see below).

Coordination of fetal growth: endocrine versus paracrine

The INS/IGF system is a very ancient system that integrates metabolic signals with growth. Various effects on organism growth have been documented, including the control of cell proliferation, cell death and extracellular fluid accumulation (Gardner et al 1999). Although this has not been studied in detail, it is not likely that in mammals there are major effects on cell growth (i.e. increase in cell mass itself), though curiously in other organisms the same system is involved in cell growth. For example, the *Drosophila* homologue of IRS 1-4, Chico, is involved not only in the control of cell number but also of cell growth (Böhni et al 1999). Some of the signalling pathways for the INS/IGF system have been unravelled but many remain unknown.

Some of the IGFs and receptors are made early after implantation in the embryo, predominantly in mesodermal, endodermal, and extra-embryonic tissues (Efstratiadis 1998). Some IGFs and receptors are also produced in the CNS. Surprisingly, while lack of IGFs can affect brain growth postnatally, growth of the fetal brain is remarkably unaffected by IGFs. Major effects of the IGF system on fetal growth are observed starting from E12 in the mouse (20 days gestation).

FIG. 1. The signalling of both IGF2 and IGF1 is mediated by IGF1R, which is very similar in structure to the insulin receptor (INSR). Both IGF1R and INSR consist of two extracellular α subunits that bind the ligands, and two transmembrane β subunits that anchor the receptor in the membrane and contain tyrosine kinase activity in their cytoplasmic domains (LeRoith et al 1994). IGF1 binds to IGF1R with higher affinity than IGF2 (larger arrow). IGF2 binds to INSR *invitro* with an affinity 10 times lower than that of insulin (smaller arrow). The IGF2/INSR signalling interaction *in vivo* is supported by several gene knockouts (Efstratiadis 1998). An unknown placenta-specific receptor (X), which is distinct from IGF1R and INSR, mediates the IGF2 growth-promoting role in this organ (Efstratiadis 1998). Most of the IGF2 and IGF1 in the circulation and in the extracellular matrix are bound to IGF-binding proteins (IGFBPs). The IGF1R-mediated-pathways overlap extensively with INSR pathways. Ligand binding by the receptors leads to activation of their intrinsic tyrosine domain, resulting in receptor autophosphorylation and phosphorylation of IRS proteins and Shc. These phosphorylations result in the activation of major signalling pathways (e.g. phosphatidylinositol-3-kinase, MAP kinase), leading to the growth-regulating and metabolic effects of IGFs and insulin (reviewed by Blakesley et al 1999). The IGF2R/mannose-6-phosphate (M6P) receptor is a single-chain protein consisting of a large extracellular domain (15 contiguous repeats) and a short cytoplasmic tail that lacks tyrosine kinase activity. This receptor binds IGF2 and numerous ligands tagged with M6P to target them to the lysosomes. In the case of IGF2 binding it does not appear to participate in a signalling pathway, but only leads to turnover of the ligand by receptor-mediated endocytosis.

TABLE 2 Growth effects of imprinted genes

Gene (Chromosome)	*Expression*	*Function*	*Phenotype*	*References*
Igf2 (7)	Paternal	Peptide growth factor	GR first detected at E12; BW 60% N; adulthood 60%N	De Chiara et al 1990
Ins1/*Ins2* (19/7)	Paternal (yolk sac)	Peptide hormone	Fetal GR; phenotypic effect of imprinting undefined	Duvillié et al 1997
Rasgrf1 (9)	Paternal	Ras-associated G protein	Postnatal growth retardation: GR ~weaning 70–75%N; ~adulthood 75–85%N; Decreased levels of growth hormone and IGF1 in serum (up to 50%)	Itier et al 1998
Igf2r (17)	Maternal	Receptor-mediated turnover of IGF2	Fetal OG (140%N); perinatal lethality	Wang et al 1994
Peg3 (7)	Paternal	Unknown (implicated in TNF signalling pathway and in p53-mediated cell death pathway)	GR BW 81%N; ~4 weeks 65% N; reduced postnatal survival rates; impairment of maternal behaviour	Li et al 1999
Mest (6)	Paternal	Hydrolase	GR BW 83% N; ~1 week 65%N; reduced postnatal survival rates; impairment of maternal behaviour	Lefebvre et al 1998
SnrpnIC (7)	Paternal	Imprinting control region	GR BW 77–82% N; early postnatal lethality	Yang et al 1998

E, embryonic day; BW, birthweight; GR, growth-retardation; OG, overgrowth; N, normal; TNF, tumour necrosis factor.
(Adapted from Kelsey et al 1999.)

The most dramatic reduction of fetal weight (30% of normal birth weight) occurs, for example, with a combination of deficiency of *Igf1* and *Igf2*. The most dramatic increase (200% on E18) occurs with loss of imprinting of *Igf2* (due to *H19* mutation) combined with lack of *Igf2r* (Table 3, *Igf2r*/*H19*) (Efstratiadis 1998).

TABLE 3 Mouse mutations and transgenics affecting *Igf2*

Mouse mutation	*Expression and serum levels*	*Phenotype*	*References*
Igf2$^{+/-}$	Expression is absent in both the embryo and placenta	GR first detected at E12; BW 60%N; placenta 65%N; organ growth deficit largely proportionate except the brain	De Chiara et al 1990, Constância 2000
enh$^{+/-}$	Embryonic expression is normal in the heart & skeletal muscle, but reduced in the liver, intestine, kidney and lung Placenta expression is normal	BW 70%N; all organs proportionately small	Leighton et al 1995a
Mnt$^{+/-}$	Embryonic expression restricted to endoderm (liver and intestine)	GR first detected at E12; BW 55%N; placenta also small; most organs proportionately small except brain	Cattanach et al 2000, Davies 2000
DMR1-U2$^{+/-}$	Embryonic expression is normal Placental expression of the P0 transcript is lacking	GR first detected at E14; BW 71%N; placenta 70%N; most organs proportionately small except brain	Constância et al 2000, Constância 2000
H19$^{-/+}$	Embryonic *Igf2* expression increased about 2 fold (maternal activation of *Igf2*) in liver, intestine, heart, skeletal muscle & kidney Serum 114%N at E15	OG first detected at E14; BW 128%N; placenta 140%N; lung, intestine, kidney and heart proportionately large; liver disproportionately large	Leighton et al 1995b, Eggenschwiler et al 1997
Igf2r$^{-/+}$	*Igf2* expression is normal, although it is not cleared, and hence accumulates in tissues and serum levels are 425%N at E15	OG first detected at E14; BW 135%N; perinatal death; placenta 140%N; lung and intestine proportionately large; liver and heart disproportionately large; kidney disproportionately small	Wang et al 1994, Lau et al 1994, Ludwig et al 1996, Eggenschwiler et al 1997
Igf2r|*H19* double mutant	Serum 1167%N at E15	Embryonic lethal by E18, overgrowth (~200%); placenta 230%N; lung and intestine proportionately large; liver and heart disproportionately large; kidney disproportionately small	Eggenschwiler et al 1997
Igf2 transgenics	Serum 270%N at birth	OG first detected E13; BW 160%N; perinatal death; placenta proportionately large; liver and heart disproportionately large	Sun et al 1997, Petrik et al 1999

E, embryonic day; BW, birthweight; GR, growth retardation; OG, overgrowth; N, normal; +/−, paternal transmission of the deletion; −/+, maternal transmission of the deletion.

How are various effects on organ growth coordinated with each other? First, it seems that with the exception of the brain, most other organs are similarly affected by deficiency of IGFs (and thus they show largely proportionate organ growth deficiency), even those organs that do not produce any itself. Thus the main action of IGFs may be endocrine, i.e. secreted by various organs into the circulation and affecting many organs to a similar extent as local concentration rises because serum levels rise. This model is further supported by a knockout of IGF2 production largely limited to fetal liver and intestine, in which growth deficiency is similar in spleen (no IGF2), heart (normal IGF2 production) and liver (no IGF2 production in knockout; Leighton et al 1995a) (Table 3, enh). The same is observed in a mirror image mutation, in which IGF2 production is abolished or down-regulated in muscle, heart, kidney and lung, but not in liver and intestine (Table 3, Mnt mutation; Davies 2000). In this mutant the size of all of these organs is reduced. However, it is important to note that IGF2 is also absent from the placenta, which is likely to limit nutrient transfer to the fetus (see below). The magnitude of the effect of nutrient restriction and growth factor depletion, respectively, are not known.

In situations in which there is severe deficiency or over-expression of IGF2 (see Table 3), however, differential local actions of the growth factor are possible. In these situations there can thus be disproportionate growth deficiency or overgrowth of organs (Sun et al 1997, Eggenschwiler et al 1997, Constância 2000, Davies 2000). It is thus likely that there is differential sensitivity of organs to altered overall levels of the growth factor (this may be brought about for example by different receptor concentrations). In addition, local paracrine mechanisms may also play a role particularly when overall levels are drastically altered, as well as in disease situations in which IGFs are abnormally and locally expressed.

Role of placenta

Mutations of several genes in mice, which are associated with placenta development, result in embryonic lethality or fetal growth retardation (reviewed by Ihle 2000, Cross 2000). In the case of the imprinted *Mash2* gene the lethality is immediate and there is not time for the manifestation of growth retardation. *Mash2* is an essential regulator of trophoblast giant cell differentiation (Guillemot et al 1995). Mice lacking *Mash2* fail to develop a spongiotrophoblast layer and the labyrinthine layer is disorganized. These defects result in placental insufficiency and death of the embryo by mid-gestation (E10) (the visceral yolk sac provides the essential structure for nutrient and waste exchange until E10, when the placenta takes on this function).

Other gene mutations associated with placental development have been described that are not embryonic lethal but result in fetal growth retardation,

e.g. *EsxI*, *PdgfB*, *Wnt2* (Ihle 2000, Efstratiadis 1998), and the imprinted *Igf2* gene.

IGF2 acts both in the placenta and in the fetus (whereas IGF1 action is limited to the fetus). The two effects are difficult to disentangle. Part of the fetal component of IGF2 action has been demonstrated with the knockout that eliminates IGF2 expression exclusively in endodermal tissue (liver, intestine) but not in mesoderm or in the placenta (Leighton et al 1995a). Remarkably, the *Igf2* gene itself possesses a placenta-specific transcript (making the same peptide as everywhere else) which has recently been knocked out without apparently affecting the other transcripts (Constância et al 2000). This knockout results in intrauterine growth retardation (IUGR) to a level of 71% of normal birthweight (Table 3, DMR1-U2). Thus elimination of this *Igf2* transcript exclusively from the placenta has a profoundly growth-retarding effect. Because levels of IGF2 in the fetal circulation are not affected, it is likely that lack of this transcript affects placental function in the supply of nutrients. This model needs to be tested by placental function assays. As before, reduction in organ size — with the exception of the brain — is largely proportionate. Thus both the regulation of demand and the regulation of supply by the IGF system seem to lead to coordinated effects on organ systems. Brain growth is apparently independent of nutritional demand and supply.

Finally, most of the imprinted genes known to date are expressed and imprinted in the placenta. Other imprinted genes are exclusively imprinted in the placenta but not in the fetus, and these may have a role specifically in placental growth or development (Engemann et al 2000).

Regulation of postnatal growth

As pointed out above postnatal growth during the suckling period is under similar genetic laws as is fetal growth. Do imprinted genes have a role in growth up to weaning? First, the major postnatal growth control system based on growth hormone only comes into play shortly before weaning (Table 1). Second, one imprinted gene, *Rasgrf1*, has already been shown to have a role in postnatal growth specifically from birth to weaning (Table 2; Itier et al 1998). Third, the IGF system is likely to operate from birth to weaning as well. This is because *Igf1* and *Igf2* are expressed during this period after which *Igf2* is silenced in rodents, *Igf2* mutants grow at normal rates after birth (INS, IGF1 action?; Efstratiadis 1998), and *Igf1* mutants grow at normal rates to weaning (INS, IGF2 action?; Wang et al 1999). Finally, placental *Igf2* mutants which are born growth retarded (71% of normal) then show accelerated growth to weaning and thus catch up with their normal littermates (INS, IGF1, IGF2 action?; Constância et al 2000). Intriguingly, these mutants express higher levels of IGF2 after birth than their

normal littermates specifically in mesodermal tissues, and have higher serum levels of the growth factor (Constância et al 2000).

Two models can be proposed that involve IGF action in the control of this catch up growth. First, it is possible that sensing of their reduced body weight or size delays the normal silencing of *Igf2* expression postnatally (as witnessed in the mutant) and this contributes to accelerated growth. This should be testable by analysing other models of IUGR followed by catch up growth where IUGR is caused independently of *Igf2*. Second, it is possible that normal postnatal levels of IGFs in a small body lead to increased tissue concentrations of the growth factors, leading to an accelerated growth response. New genetic models by which pre- and postnatal IGF levels can be manipulated more precisely are needed to resolve these questions.

Other interacting factors

The conflict theory proposes that paternally and maternally expressed imprinted genes oppose each others actions on fetal growth. Thus far we have considered how this can happen at the level of the growth factors and their receptors themselves (e.g. paternal IGF2 and maternal IGF2R). However opposing interactions may occur at many different levels. For example most imprinted genes are clustered in the genome and the *Igf2* gene is located in one of the major clusters, being largely surrounded by other maternally expressed genes. This has given rise to the proposal that oppositely imprinted genes in this cluster have opposing actions on fetal growth (Reik & Maher 1997); for *Igf2* and the maternally expressed cell cycle inhibitor p57Kip2 this seems to indeed be the case, at least in some tissues (Lam et al 1999, Caspary et al 1999). This may suggest that in some tissues p57Kip2 has a role in the transduction of the IGF2 signal; in addition they also seem to interact at the level of expression (Grandjean et al 2000).

Adjacent to the *Igf2* gene in the cluster is a gene, *H19*, which is maternally transcribed but does not encode a protein. Some knockout studies have suggested that the *H19* RNA has no function at all (Jones et al 1998, Schmidt et al 1999). Other studies however have shown that *H19* RNA can suppress proliferation in certain assays (Hao et al 1993), that *H19* may have a role in the control of translation (Li et al 1998), and that the *Igf2* and *H19* RNAs share common binding factors (Runge et al 2000). It is remarkable that in an *Igf2* silencer knockout (which has no effect on *H19*) the maternal allele of *Igf2* is transcribed in some tissues without any apparent effect on growth (Constância et al 2000), but in other knockouts which eliminate or down-regulate the *H19* RNA in addition to causing expression of the maternal *Igf2* allele, organ overgrowth

occurs (Leighton et al 1995b, Schmidt et al 1999, Ripoche et al 1997). Thus the final verdict on the role of the *H19* RNA is not established yet.

Finally, a much more remote influence on fetal growth is also worth considering here. Elsewhere we have argued that as far as mechanisms are concerned, the majority of the paternally expressed genes are maternally repressed by methylation originating in the egg. Maternally expressed genes, by contrast, tend to be imprinted indirectly, involving paternally expressed and maternally methylated antisense transcripts (Reik & Walter 2001). The possible reason for this asymmetry in the imprinting mechanism is that there is genome wide and active demethylation of the paternal genome in the zygote, but the maternal genome is protected from this reprogramming event (Oswald et al 2000, Mayer et al 2000). This can be interpreted as an attack on paternal methylation patterns (equalling growth enhancement) by the egg, that is the maternal genome, resulting in reduced growth. This example shows that while we are beginning to understand some of the features of growth control in mammalian fetuses, many other facets remain to be elucidated. It also emphasises how important it is to consider the physiology of growth together with the genetics.

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DISCUSSION

Lehner: What is the paternal defence against the global demethylation immediately after fertilization? Why do the paternal imprints survive this process?

Reik: There are some paternally methylated imprinted genes that survive this demethylation. They have to have evolved a defence against demethylation. We don't know what this is; we can only speculate. One possibility is that active demethylation might have to do with the reorganization of the whole sperm genome, the stripping off of protamines and establishing nucleosomal structure. At some stage we may be dealing with naked DNA, and all the enzymes present in the egg cytoplasm can come in and attack this DNA. If in the sperm genome you could adopt a nucleosomal or slightly heterochromatic structure which doesn't get remodelled, then I think you have a better chance of protecting the DNA. The mouse sperm is 99% protamine; human sperm is 90% protamine and 10% nucleosomal. It would be interesting to look in the human.

Nasmyth: You introduced this demethylation idea as the egg defending itself from the predatory growth genes from the sperm. How do you test this hypothesis? You might be right, but you could also say that the paternal genome is packaged up in protamine and needs to be activated. The null hypothesis is that you are activating the paternal genome. How do you test that sort of hypothesis?

Reik: I don't know. One thing we could do is look phylogenetically to see what the distribution of this phenomenon is versus the distribution of imprinting, for example. The data points are very sparse. In the zebrafish, which doesn't have imprinting, this doesn't happen, even though the zebrafish still needs to activate its paternal embryonic genome at some stage.

McMahon: Presumably the nuclear transfer experiments with somatic nuclei should give you some insight.

Reik: Absolutely; this is what everyone is very keen to do. If you don't get massive demethylation in the somatic nucleus, you might say that it is not necessary for the reprogramming of the nucleus.

Vande Woude: How does this fit with the earlier work where researchers replaced the sperm nucleus with the maternal nucleus and vice versa?

Reik: That just shows what the imprinted genes do collectively.

Vande Woude: Do they undergo this demethylation?

Reik: We don't know, but we would expect them to. The way the androgenetic embryos are made is to take the pronucleus out of the fertilized egg after demethylation.

McMahon: In terms of the model you are presenting that *H19* might have a functional role, what has been done with regard to gene targeting to remove the *H19* transcriptional locus or to ectopically express it in transgenics?

Reik: The general pattern in mice is that in all situations where people have found that overexpression of *Igf2* leads to increased fetal growth, this is accompanied by a reduction or loss of *H19* RNA. Our new knockout does not affect *H19* at all. This is the first clean genetic situation where this has been found. If people overexpress the RNA in transgenics, nothing happens. Although there was some early work that showed there was lethality associated with overexpression of RNA, we don't think that this is generally the case. But it is very difficult to raise the RNA levels in transgenics above a certain point, which is not too far off the actual physiological level.

McMahon: If you leave the *H19* promoter intact and put β-galactosidase in its place, does this generate the phenotype?

Reik: If there is normal *Igf2* imprinting and no *H19* RNA, there is no overgrowth.

Gönczy: Have you done this experiment in the context of your knockout?

Reik: No, but we would love to.

Kubiak: Is the demethylase active specifically in the sperm cell cycle?

Reik: We have no direct evidence for a demethylase of any kind. It is just an activity that we observe here. We know that after this rapid demethylation, there is also passive demethylation, which is replication-dependent. You could ask whether this whole process is a bit of a waste. Why do you want to demethylate your paternal genome at fertilization if you also demethylate the rest of the genome at the blastocyst stage?

Goodwin: What happens with the extraembryonic tissues?

Reik: The experiments that I have described by and large address the direct effect of expressing or not expressing *Igf2* in the fetus. There is another very important phenomenon in mammals, which is an indirect effect. If you do something to the placenta and not the fetus, what happens then? We have specifically knocked out a placenta-specific *Igf2* transcript, which is only expressed in the labyrinthine trophoblast of the placenta. There is no effect on fetal *Igf2* expression or levels of peptide, and these mice are born 70% smaller, so they show intrauterine growth retardation. We don't have any formal proof that the placenta is the only thing that is damaged, but we are hoping to carry out transport assays across the placenta to get to that stage.

Schmidt: What happens to imprinting in the methylase-deficient mice?

Reik: This is the work by Rudolf Jaenisch. All the genes that have been looked at are imprinted wrongly. Those that are methylated and silenced are then bi-allelically expressed and genes such as *Igf2* are not expressed from either chromosome. These mice die at day 9–10.

Thomas: IGF2 is presumably going through the IGF1R receptor, and IGF1 is up-regulated at the time you see this growth effect. They are both working through the same receptor and both doing the same thing. IGF1 is already being developmentally regulated in the knockout when you see the growth effect. What happens under these conditions?

Reik: I don't know; we didn't look.

Thomas: I am trying to understand how excess IGF2 can contribute to the growth, given the fact that these mice are up-regulating IGF1 to carry out that function.

Reik: If you look at ligands and receptors in double knockouts, these actions of IGF1 and IGF2 can be separated; they are additive.

Thomas: This is a function of the time during development when IGF1 and IGF2 are up-regulated. These two events you are talking about happen at the same time.

Raff: Isn't this because IGF1 is limiting? All extracelluar signal proteins seem to be limiting in tissues.

Nurse: Wolf Reik, could you say something about the significance of imprinting on those genes which have a clear involvement in both growth and cell cycle progression? What is the significance of imprinting for these genes?

Hunt: Can I paraphrase that question? There is a twofold difference in the level of *p57* for example, depending on whether it is or isn't imprinted. What difference does this make to cell cycle progression?

Reik: That doesn't happen at the same time. It is not relevant to the animal now that it only has a onefold dosage. The level is not oscillating between twofold and onefold. The animal doesn't use imprinting to regulate levels.

Nurse: But what is the consequence of having onefold versus twofold dosage?

Hunt: To put this another way, why do it this stupid way, using imprinting? Why not turn the genes down?

Reik: But this is not the purpose of imprinting. Its role is to regulate fetal growth in a nutrient-providing environment. This is what creates the selective pressures for these genes to switch one allele off. It has nothing to do with regulating gene expression.

Nurse: Why these genes?

Reik: Because they are very important in fetal growth. The logic goes like this. The first thing that is there is the placenta. There are genes that are not imprinted at this stage, which regulate fetal growth. They do this directly or indirectly by sucking resources out of the mother. This is the selective pressure that leads these genes to become imprinted.

Nasmyth: I think Tim Hunt's question is a sensible one. Normally *p57* is expressed on the maternal chromosome and not on the paternal one, because according to this hypothesis the father's chromosome does not express it because he doesn't want to slow down growth. If this is the case, then if you had an extra copy and introduced it on a bacterial artificial chromosome (BAC) into the maternal genome, what would happen to growth? This is a crucial prediction of the hypothesis.

Reik: To my knowledge this hasn't been done.

Nurse: I was specifically trying to link in whether the particular genes concerned with growth and cell cycle had a special significance.

Reik: So the question is, of the seven genes which are centrally implicated in fetal growth, why are four imprinted and not all seven?

Nurse: There are two questions; that is one, and the second is, are we certain that it is those seven that are crucial for fetal growth and development?

Reik: The first question first: why aren't all seven imprinted? One simple answer is that we are taking an evolutionary snapshot here. The mouse is a certain kind of mouse right now, so it may be on the way to imprinting all seven. Another explanation is that imprinting is costly. One of the major costs is that it results in only one active copy of the gene, so the mutation load is doubled. There may be some genes that can tolerate this and others that can't. Finally, look at all the various mechanisms that imprinting operates by. Some genes may be easier mechanistically to imprint than others.

Nurse: The other question is, are these seven the most important genes?

Reik: If you take all the knockouts in the mouse, give or take one or two it is these seven that always come up in fetal growth control. Therefore the argument holds that a large proportion of important fetal growth genes are imprinted. There is an interesting observation here: if out of 30 imprinted genes, say 15 are involved in fetal growth, then clearly this is more than the seven genes that we currently know to be crucial for the control of fetal growth. Finding imprinted genes and looking at their actions will give you more genes that are involved in fetal growth. This would be my guess.

McMahon: I have a more general question about how the IGF system works. Your explanation for the absence of a tissue-specific effect of modulating IGF levels presupposes that there would be some data that suggest that IGF2 is acting locally. It is certainly expressed broadly throughout the whole embryo. An alternative hypothesis is that there are key places where it has been expressed during embryonic fetal development that are important and some places that are unimportant. What has been done in terms of tissue-specific removal or activation of this pathway?

Reik: Nothing has been published, but people are doing those experiments now. Tissue-specific removal is easier than tissue-specific over-expression. The general point that you are addressing is the endocrine versus paracrine/autocrine mode. There are as many experiments that seem to show that one is more important as there are which show the other is more important.

Raff: You have emphasized fetal growth, but you would think that postnatal growth, at least until weaning, would be equally important for this competition. Are there imprinted genes that affect postnatal growth?

Reik: There is one, *Grf1*, where the knockout has normal birthweight, but there is a growth deficiency effect between birth and weaning. It is an interesting prediction: you would expect the same kind of principle to hold during suckling.

Edgar: I was curious about the indifferent paracrine question, and your comment about the placenta. Do you think that is a direct effect of maternal IGFs on the embryo or is it an indirect effect?

Reik: We think it is an indirect effect. We have measured IGF2 transcription and peptide in the fetal circulation. This is normal. The only thing that is missing is the *Igf2* transcript which is specific to the placenta. The placenta is small from about Day 13. The fetus shows much more protracted growth deficiency: only on Day 17–18 can you see the fetus slow down.

Edgar: So it could be just a general nutrient transfer issue.

Reik: That is what we think, but we have no formal proof.

Raff: Is the placenta normal in size? Is it possible that the effect on fetal growth is indirect via an effect on placental growth?

Reik: You mean via other factors? That is possible.

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General discussion I

Nurse: As we move to a general discussion, I have three questions which I suggest we could focus on, going back to this thorny question of growth and the cell cycle. First, is the cell cycle itself limiting growth? This addresses questions to do with cell size — can you be too big, or too small, and what influence does this have on overall growth of the cell? Second, what actually regulates overall growth of the cell? Third, what do we mean by 'in' and 'out' of cycle? Does the term 'G0' mean anything?

Edgar: I can address the first question concerning the relationship between cell growth and the cell cycle. In all the experiments we have done, we have found that cell cycle progression — especially the DNA replication cycle — is required for growth. This is fairly trivial.

Nurse: You say 'trivial': exactly what do you mean? You are saying that you require DNA replication for growth. What do you mean by that sentence?

Hunt: One can always think of exceptions, such as oocytes which grow very big, or nerve cells which grow very long. Maybe they have to have special ways of doing that.

Edgar: Our experiments have been limited to tissues that are normally growing and cycling. In those situations, the cycling is required for the growth.

Nurse: And you think that is S phase.

Edgar: I think it is how much DNA you have: a certain amount of DNA is needed to support a certain amount of cytoplasm.

Nasmyth: Within what range? You have a cell, and you stop DNA replication. You look after 1, 2 or 3 generations. Within 1 generation, there isn't much effect. If you look after 3 or 4 generations, there is a big effect. It is terribly important to make that distinction. You say that you need DNA replication for growth: the extreme version of that would be that you block DNA replication and the cell immediately stops growing: this is absolutely not true. They go on growing and the rate of growth goes on increasing more-or-less normally, at least for one generation.

Nurse: There is a lack of careful experiments in this area.

Hunt: In the human body, for example, what is the normal range of nuclear to cytoplasmic volume ratios?

Nasmyth: It is vast: look at a neuron.

Nurse: But a neuron isn't growing very fast. There is this ancient idea from Hertwig, called the karyoplasmic ratio of 1903, in which he claimed that the nucleus was limiting for growth. In actual fact, the DNA content per unit mass is not as hugely variable as you might think. It is buffered over quite a range, but you can't go too far without something going wrong.

Nasmyth: Aren't Purkinje cells the largest cells?

Raff: Cardiac myocytes are also very large.

Edgar: Can Purkinje cells proliferate as large cells?

Raff: No. Most neurons grow after withdrawal from the cell cycle. Motor neurons can become even bigger than Purkinje cells, and they too are clearly diploid.

Reik: How significant a phenomenon is cell growth the way you people talk about it in development? If I look at our big mice, their size has little to do with cell enlargement.

Schmidt: Are the cells in these mice bigger, or are there more cells?

Reik: There are more cells, and there is also more water in the extracellular tissue and all sorts of other things. These situations you are talking about where cells grow may not be so important in mammalian development.

Raff: It may be that the IGFs are mainly stimulating cell growth and only secondarily stimulate cell cycle progression. It could be that most of their effects are on cell growth and cell survival.

Reik: When you talk about growth, do you mean proliferation?

Raff: I mean cell enlargement.

Leevers: And that may lead to division.

Nurse: That is the simplest view, that growth leads to cell division. This is why it is intriguing to discover that cell division can feed back on growth in some way. The general point is that embryos can often have very different cell sizes and still cope perfectly well. In this sense individual growth of the cells may not matter.

Gautier: Returning to the nuclear:cytoplasmic ratio, in a situation where you don't block any event in the cell cycle, such as experiments in frogs where a mosaic is made between the diploid and tetraploid tissue, the tetraploid cells are twice as large. There is some regulation here: the amount of DNA is somehow influencing the size of the cell.

Nurse: I'd like to mention a sort of half-relevant observation in coelocanths, the fish that are living fossils. When people looked at pre-Devonian fossils of these fish from 300 million years old, the cell size in sections of the fossilized bone was as much as 10 times smaller than the modern fish. The idea was that the fish have accumulated more DNA and the cells have got bigger, but the form of the fish has remained the same.

Schmidt: Is the question what happens, for example, if you induce p16 (as an arbitrary choice of something that will arrest the cell cycle) and then push on nutrients, how big can you make a cell, and what is it that limits size?

Nurse: The general question I was interested in was close to that: since we generally think that growth initiates the cell cycle, is there any evidence for the cell cycle feeding back on growth? We have only discussed blocking the cycle, which would reduce the gene concentration.

Schmidt: You might be able to get the information by asking what limits growth. For sure, something will limit growth. Then the question becomes a different one: what is it that halts growth at the limit, when the p16 has stopped the cell from dividing any more.

Hunt: There is something called cytolethal distending toxin, which I understand kills millions of infants in the middle east and is produced by almost all enteric bacteria. This produces a beautiful G2 block. The reason why it is called cytolethal distending toxin is that the cells get bigger and bigger, without entering mitosis although they have done replication, and the microtubules never show any sign of adopting their mitotic form. Finally, they get so big they die.

Nasmyth: This is where you have to be incredibly careful. p16 is not just a cell cycle regulator: it is regulating Cdk4, which is regulating not only the cell cycle, but also growth. I think you'll find that p16 is regulating growth directly, quite independently of regulating the cell cycle.

Edgar: With the imaginal disc cells that we have studied we can block the cell cycle pretty specifically with a Cdc25 mutation or cyclin E mutation. The cells continue to grow and eventually will die.

Nurse: In my lab we have looked at the growth of cells with different cell cycle blocks. As Kim Nasmyth has mentioned, they grow exponentially for about one cycle, and then they gradually grow slower. After they get about three or four times bigger, they are linear or very slow.

Hunt: mRNA probably does get a bit limiting.

Newport: In terms of physical limits, we tend to think of DNA, but on the other hand there is RNA degradation and protein turnover. This is how an egg is made. Even though it is only a tetraploid organism, RNA degradation is slowed down significantly, so less DNA is needed. The other physical limitation is the surface area:volume relationship. How many receptors or growth factors can be inserted into a membrane?

Nurse: There is also the question of how big a domain the cell's regulatory system can influence. Can microtubules organize over a huge space, for example?

Hunt: Nerve cells break all these rules.

Nurse: This may only be possible for specialized functions.

Nasmyth: Oocytes are huge compared with other cells.

Hunt: But they import a lot of protein from outside.

Nurse: What about what regulates the overall growth of a cell? What actually determines the limit on growth rate?

Raff: I am not aware of any example where a limit to cell growth has been demonstrated *in vivo*. Generally, if you increase the amount of cell growth factor available, cells will get bigger. Thus the levels of extracellular signals can influence cell size in an animal.

Nurse: I was thinking of growth rate of a cell, rather than the eventual size it reaches.

Raff: But these are related.

McMahon: Perhaps there is one natural example of that — the hypertrophic chondrocyte. The cells exit the cell cycle and then undergo growth, get bigger and bigger, and their eventual fate is apoptosis. One potential reason for this is that they have reached the limits of growth.

Raff: It is a possible reason, but may not be *the* reason. The possibility could be tested.

Nurse: If we are going to ask about the rate of increase of mass per unit of time, what is regulating that in a cell? What determines the rate at which an individual cell grows?

Raff: The difference between macromolecular synthesis and degradation. Synthesis must be greater than degradation for the cell to grow.

Nurse: That is a description: net increase is the balance between those two. What is actually determining the overall rate of those two?

Leevers: Nutrition.

Nurse: Let's do a thought experiment. Let's give them so much nutrition and energy source that it is coming out of their ears, what is then limiting growth?

Leevers: Nutrient uptake.

Nasymth: Growth factors.

Vande Woude: Density, cell volume and stochiometry of product.

Raff: I disagree. I suspect that it is mainly extracellular signals that are limiting, as you can increase growth by increasing the amount of signal. This suggests that growth is not limited by the number of receptors, for example.

Vande Woude: How is receptor different from signal?

Hunt: Signals put more receptors on the surface, don't they?

Raff: Generally, receptors are down-regulated by signals rather than up-regulated.

Vande Woude: Let me modify my answer, then, by saying stoichiometry of products, which includes signalling.

Nasmyth: Martin Raff's question is, if the number of receptors is increased without increasing the signal, does the cell grow faster? He is saying that there are countless situations where the signal is increased without an increase in receptors, and the cells do grow faster. The signalling molecules therefore tend to be rate limiting as opposed to the receptors.

Raff: One needs to look at more cells and signals *in vivo* but, where it has been studied, signals are limiting.

Nasmyth: How many experiments have been done in which the receptor numbers are increased?

Raff: I don't know, but in many cases receptors are present in vast excess.

Nurse: What is limiting the growth rate in simple organisms such as yeast?

Newport: Glucose uptake. How many glucose transporters can the cell put in the membrane?

Schmidt: But they don't have a true stringent response in the way that bacteria do, so that answer is not quite right. It is not really glucose induced.

Nurse: The reason why I am not sure it is as simple as that is if growth rate was determined by a single limiting factor of that type, I would have thought mutationally, if we selected for growth rate, we should rapidly isolate and identify those limiting factors. On the whole, when we select for rapid growth, these factors don't come out.

Hunt: It could be because there are lots of things the cells have to take on board, each of which is limiting in particular circumstances. It is a balance; if the cells only take on one thing then they don't take on the others.

Vande Woude: No one has mentioned mitochondria, but these would almost certainly have a role.

Nurse: When you take growth factors out, and growth rate goes down, what is actually regulating the rate of translation and so on? What actually shuts down?

Thomas: The cells sense the nutrient availability, and then proteins are either degraded or synthesized as a function of available nutrients. This is being sensed by something.

Nurse: Where does this input into the growth machinery?

Thomas: Translation.

Schmidt: More specifically, most people in the translation field would say that it is translation and initiation factors.

Nurse: Do you think that initiation factors are the limiting component for translation?

Hunt: Yes. You only have to look at a cell and initiation shuts down. It is amazingly sensitive.

Thomas: It is not the only place that it is regulated. Elongation is also regulated, but most of the input is at the level of the initiation of translation.

Nasmyth: You can't have any one of these things limiting, because that is a very inefficient way of doing it. I suspect that most steps will be rate limiting because if it is any one of them you are wasting resources.

Nurse: That is consistent with the view that we can't get a faster growth rate even in a simple organism such as yeast.

Thomas: That is not absolutely true. In bacteria, if you change the energy charge you see a direct effect on translation and ribosome biosynthesis. As soon as ATP drops, ribosomal RNA synthesis decreases but not transcription of other genes.

Maller: Neither ribosomes nor mitochondria can explain translational limitations in the oocyte, because there are many more ribosomes and mitochondria than are needed yet protein synthesis is very limited. Every message put in is translated at the expense of some endogenous message. No one knows yet what the limiting factor for translation is in oocytes.

Hunt: I don't think it is simple, either. Lots of things are missing.

Nurse: Is there any meaning in G0?

Thomas: Liver cells are carrying out a differentiated function and reside in G0. Upon damage or hepatectomy the liver cells re-enter the cycle.

Nasmyth: Do you even need terms like 'G1' or 'G0'? These are purely phenomenological terms. One thesis is that we just talk about real things, as opposed to phenomenological things. Do you mean by 'G2' that the cell is a 4C cell? In which case, why not just say that it is a 4C cell. Does it have sister chromatid cohesion or not? Is Cdk1 in some form or another active or non-active? Most of these terms are hangovers from a past when we didn't know anything about the mechanism of the cell cycle. We should just stop using them.

Edgar: We have to use them to communicate. Is G2 going to be different in different cell types?

Nasmyth: Yes, it will be different in different cells. There will be some cells in so-called G2 which happen to have undergone an endomitosis. There are other cells where String hasn't yet been activated. If you want to call this G2 all the time you will make mistakes.

Raff: But at least you know that you are talking about a particular part of the cycle. It would be nice to have terminology that distinguishes permanent withdrawal from the cell cycle, as in a nerve cell, from transient withdrawal, as in a lymphocyte.

Nurse: Just to push what Kim Nasmyth was saying, this problem of G1/G2 becomes very confusing with an endoduplicating cell. What he is saying is that it is best to imagine an assemblage of descriptors which allow us to define a cell preparing for mitosis or S phase. This is probably a useful way to begin thinking about G0: are there a set of descriptors which do make a difference and which might be more meaningful than the term G0? The definition of G0 has always been extremely difficult. The real problem of Kim's point is actually identifying the important descriptors without being distracted by the trivial ones.

Edgar: I could offer one idea of what G0 is. When the cells we study in *Drosophila* are ready to differentiate, they turn off all their cell cycle genes. They are limited for everything: DNA polymerase, Cdc25, cyclin E and E2F. We consider these cells G0 cells.

Hunt: Do all those genes have a sort of 'off' button in their promoters that coordinately regulates them?

Edgar: That would be nice to know.

Nasmyth: The work by Gareth Williams and Ron Laskey with the MCM proteins in cervical cancer is an example of a cell cycle gene that is presumably part of some huge battery of genes that is somehow related to whether the cell is in a proliferative state. It turns out to be rather a good marker for tumour cells. But it is nothing directly to do with proliferation itself.

Nurse: It may be a bit later. It is more likely to be in S phase.

Nasmyth: MCM is not a cell cycle-regulated gene. If this were the case, then cyclins would be good markers, but they are lousy markers.

Gautier: The MCM story tells us that the off button is not the same for all genes. It is not a single mechanism telling everything to go to G0.

Nurse: Another complication is that G0 is often wrapped up with the idea of reduced growth rate, in the sense that cells are not accumulating mass. But you can go into a G0 state in other parts of the cell cycle which are not pre-S phase.

Nasmyth: Is a growing neuron in G0? Is that even an interesting question to ask? It is clearly not cycling, and it clearly is growing.

Raff: It would be useful to compare the cell cycle control proteins in cells that will never divide again with a cell that has transiently withdrawn from the cell cycle. Is the withdrawal from the cell cycle the same in both cells? It would be helpful to know.

Schmidt: The liver is sort of an example, and the practical answer to the question of why it is useful to know is because sometimes we want to reconstitute livers. When you do a two-thirds partial hepatectomy all the cells divide. Suppose you push this to a limit and take out 90% of a liver, the hope is that there is something called a liver stem cell which is at something called G0, which is capable of reconstituting 90% as opposed to 67%. Is this a practical definition of G0?

Nurse: There is a whole set of issues here which have been lost, in my view, under this umbrella term of G0, which is not very useful as it has been applied.

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Regulation of the embryonic cell proliferation by *Drosophila* cyclin D and cyclin E complexes

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Abstract. Cell proliferation during *Drosophila* development occurs in a well known spatial and temporal pattern which can readily be studied *in vivo*. The cells which form the larval epidermis exit from the cell division cycle during embryogenesis after the 16th round of mitosis when they enter for the first time into a G1/0 phase. We are interested in the mechanistic basis of this cell proliferation arrest. We have shown that the arrest requires the down-regulation of cyclin E/Cdk2 activity by inhibition of cyclin E expression and parallel activation of Dacapo/p27 expression. In addition, up-regulation of Fizzy-related is observed and is required for inhibition of Cdk1 activity. Do these processes result from the down-regulation of D-type cyclin/Cdk complexes? Extensive evidence from mammalian cells, and in particular from tumour cells has suggested that these complexes act as master regulators of cell proliferation upstream of cyclin E. Our genetic analyses indicate that *Drosophila* cyclin D/Cdk4, which interacts with the *Drosophila* Rb family member as expected, does not play an essential role in the regulation of cell proliferation.

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Cell proliferation during *Drosophila* embryogenesis generates the roughly 50 000 cells of the larva, which hatches about 24 hours after fertilization. In addition, the embryonic cell proliferation generates about 1000 imaginal cells which proliferate during five days of larval and early pupal development to generate the few million cells of the adult fly. Multiplication of cells during embryogenesis, therefore, is more extensive and considerably faster than after hatching. The prolific embryonic cell proliferation is supported by abundant maternal stores provided to the egg cell during oogenesis. The giant egg cell is progressively partitioned into smaller and smaller cells during embryogenesis. Here, we will focus mainly on our understanding of the regulatory mechanisms that determine cell numbers in the embryo and arrest cell proliferation, in particular in the embryonic epidermis. We will review progress including some of our recent results.

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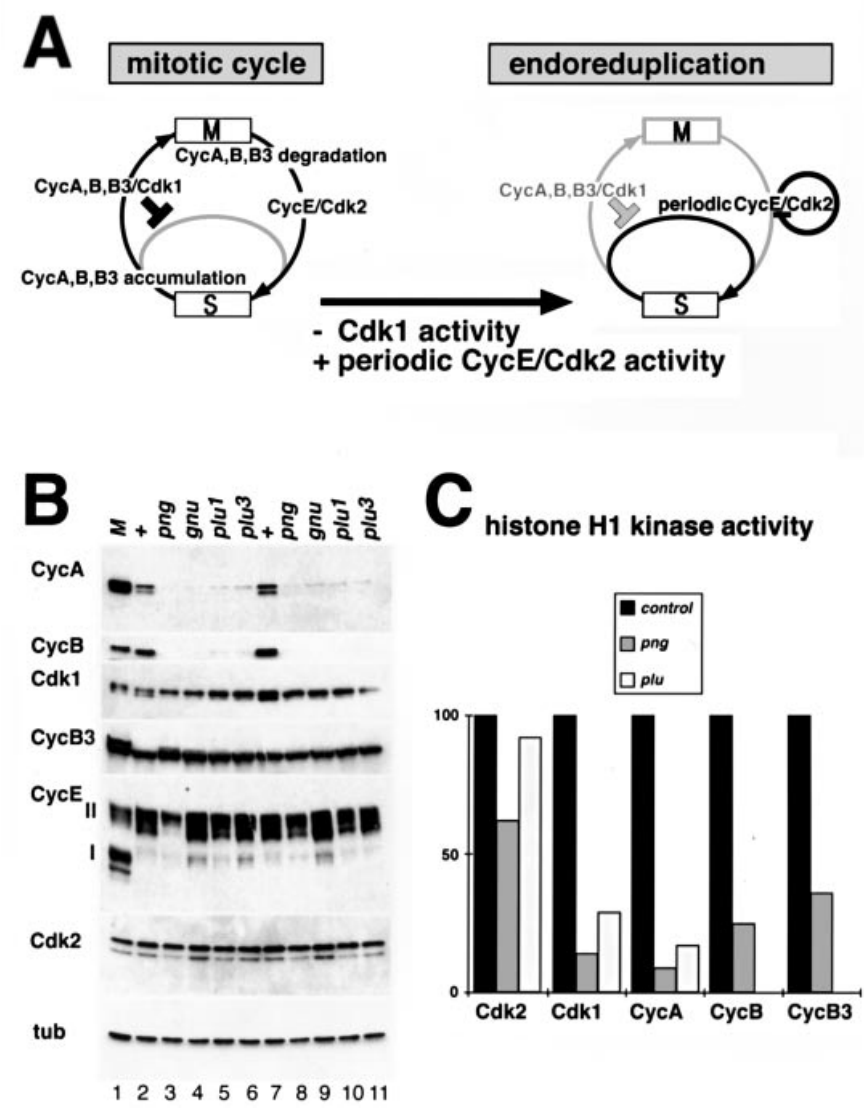
Growth in advance: oogenesis and endoreduplication

The size of egg cells demonstrates that higher eukaryotes have evolved ways to uncouple cell growth and cell cycle progression. The *Drosophila* egg is about 10 000-fold larger than the stem cells in the ovary. Much of the egg content is



synthesized in other organs (fat body) and imported into the egg cell. Moreover, in *Drosophila*, the egg is generated from an egg chamber in which the oocyte is connected to 15 highly polytene nurse cells via ring canals. These nurse cells which produce most of the egg contents progress through about 10 endoreduplication cycles. If the nurse cells were to progress through complete mitotic cycles instead of only endoreduplication, there would be more than 10 000 nurse cells. Making the 10 000-fold larger *Drosophila* egg, therefore, relies largely on uncoupling DNA replication from mitosis.

Oogenesis is not the only example where growth is achieved without a concomitant increase in cell numbers. Larval growth also occurs in the absence of cell divisions and is accompanied by endoreduplication starting in some tissues already late in embryogenesis. While rarely observed in vertebrates, this economic principle of growth by increasing cell size but not cell numbers is widely used in invertebrate and plant species. Cell growth beyond a certain limit, however, is presumably dependent on genome amplification by endoreduplication.

Based on the functional analysis of Cdk1 and Cdk2 complexes, we have proposed a model for the conversion of the mitotic cell cycle into an endoreduplication cycle (Fig. 1A; Edgar & Lehner 1996). Accordingly, this cell cycle conversion is dependent on elimination of Cdk1 activity and periodic activation of cyclin E/Cdk2. Cyclin E is required for endocycles and a pulse of ectopic cyclin E expression is sufficient to trigger endoreduplication. Cdk2

FIG. 1. Conversion of the mitotic cell cycle into an endoreduplication cycle. (A) Cells are switched from mitotic cycles to an endoreduplication cycle by elimination of Cdk1 activity and periodic expression of Cdk2 activity. (B,C) The runaway DNA replication, which is observed instead of regular alteration of S and M phases in eggs derived from females lacking *gnu*, *plu* or *png* function, is paralleled by decreased levels of mitotic cyclins and Cdk1 activity. (B) Extracts were prepared from either embryos synchronised by *Hs-string* expression in prometaphase of mitosis 14 (lane 1, *M*), or from 0–15 minute old eggs collected from females which were either *w* used as controls (lanes 2, 7; +), $png^{13\text{-}1058}$ (lanes 3, 8; *png*), *gnu* (lanes 4, 9; *gnu*), plu^{1} (lanes 5, 10; plu^{1}), or plu^{3} (lanes 6, 11; plu^{3}). These females had been mated to either sterile twe^{HB5} males (lanes 2–6) or fertile *w* males (lanes 7–11). The presence of cyclin A (CycA), cyclin B (CycB), Cdk1 (Cdk1), cyclin B3 (CycB3), cyclin E (CycE), Cdk2 (Cdk2) and β-tubulin (tub) (which was used as a loading control), was monitored by immunoblotting. The different cyclin E forms (I, II) are indicated on the left side. Longer exposures revealed some residual cyclin A and B also in lanes 2–6 and 8–11 (not shown). (C) Extracts were prepared from 0–15 minute old eggs collected from females that were either *w* used as controls (black bars), $png^{13\text{-}1058}$ (grey bars) or plu^{3} (white bars). All these females had been mated to sterile twe^{HB5} males. Antibodies against Cdk2 (Cdk2), Cdk1 (Cdk1), cyclin A (CycA), cyclin B (CycB) and cyclin B3 (CycB3) were used for immunoprecipitation. Histone H1 kinase activity present in the immunoprecipitates was determined and is given in % of the activities precipitated from the *w* control extracts. Results from two (*plu*) and three (*w*, *png*) independent experiments were averaged.

periodicity involves a negative feedback loop whereby cyclin E ultimately inhibits its own expression (Sauer et al 1995). Periodicity of cyclin E expression is important. Maintaining cyclin E expression at a constantly high level, results in inhibition of endoreduplication in salivary glands (Follette et al 1998, Weiss et al 1998). Constant cyclin E overexpression, however, does not only inhibit polytenization in salivary gland cells but also normal growth of the gland. This model for the control of endoreduplication has been supported by a number of highly interesting studies with nurse and follicle cells during oogenesis in *Drosophila* (Lilly & Spradling 1996, Spradling 1999) as well as with mammalian megakaryocytes and trophoblasts (Zhang et al 1996, MacAuley et al 1998, Hattori et al 2000).

From meiosis to mitosis

While the nurse cells endoreduplicate, the oocyte within the egg chamber enters meiosis and arrests in metaphase of the first meiotic division. While recent progress has provided first insights into the role of cell cycle regulators during oocyte choice and has uncovered an interesting meiotic checkpoint co-ordinating this process with embryonic axes predetermination (González-Reyes et al 1997, Ghabrial & Schupbach 1999, Lilly et al 2000), we still know very little about the regulation of the meiotic divisions in *Drosophila*. However, maternal-effect mutations which affect the resumption of mitotic cell cycle progression after release from meiotic arrest, completion of meiosis and fertilization have led to the identification of genes specifically required during the transition from meiosis to mitosis. When females are lacking the function of the genes *giant nuclei* (*gnu*), *pan gu* (*png*) or *plutonium* (*plu*), a runaway replication uncoupled from mitosis results after completion of female meiosis in both their fertilized and unfertilized eggs (Freeman et al 1986, Shamanski & Orr-Weaver 1991). *plu* encodes an ankyrin repeat protein (Axton et al 1994) reminiscent of the mammalian INK inhibitors of Cdk4 and Cdk6. We have failed to find a genetic interaction of *plu* and *Cdk4*. However, we have obtained some hints as to why these mutations might lead to runaway replication instead of regular alteration of S and M phase. According to the model (Fig. 1A), Cdk1 complexes have a crucial role in making S phase dependent on progression through mitosis. Runaway replication in *gnu*, *png* and *plu* progeny might therefore be associated with inhibition of Cdk1 activity. In fact, we find that the levels of cyclins A and B are clearly decreased, and in particular a hypophosphorylated form of cyclin A is absent (Fig. 1B). In case of Cdk1, the fast-migrating form representing the active form is strongly decreased. In the case of cyclin B3, the third *Drosophila* cyclin that associates with Cdk1, we cannot detect the form which is characteristically present in active Cdk1 complexes. Moreover, histone H1 kinase activity in immunoprecipitated Cdk1 complexes is

significantly decreased (Fig. 1C). In contrast, cyclin E/Cdk2 complexes were much less affected (Fig. 1B,C). We conclude, therefore, that mutations in *gnu*, *png* and *plu* affect mitotic cyclins and Cdk1 activity.

Stopping the syncytial cycles

In wild-type eggs, completion of meiosis and fertilisation is followed by progression through 13 extremely rapid cell cycles. Gap phases and cytokinesis are omitted during these syncytial cycles. The omission of cytokinesis is compensated during the process of cellularization which follows after mitosis 13 during interphase of cycle 14 and converts the layer of nuclei that have migrated to the periphery of the egg syncytium into an epithelial layer of cells. Most of the cell multiplication is accomplished during these first two hours of embryogenesis. Why do these very rapid syncytial cycles stop abruptly and precisely after mitosis 13? Recent work has implicated the DNA replication checkpoint which prevents entry into mitosis before DNA replication has been completed (Fig. 2; Sibon et al 1997, 1999). The exponential increase in DNA appears to result in the titration of a still unknown factor required for DNA replication during the late syncytial cycles 10–13. Consequently, the DNA replication checkpoint increasingly delays the activation of Cdk1 and entry into mitosis. The interphase extension facilitates zygotic expression of factors which destabilize the maternal contribution of the Twine and String Cdc25 phosphatases, which are absolutely required for Cdk1 activation (Edgar & Datar 1996). Thus, a positive feedback loop is triggered

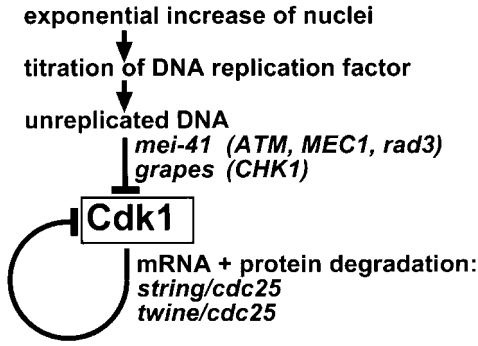


FIG. 2. Stopping the syncytial cycles. The DNA replication checkpoint has an essential function during early embryogenesis. The genes *mei41* and *grapes* which encode *Drosophila* homologues of the universal DNA replication checkpoint components ATM/MEC1/Rad3 and CHK1, respectively, are required to stop the progression through the syncytial cycles after mitosis 13 (Sibon et al 1997, 1999). In addition, the degradation of the maternal String/Cdc25 and Twine/Cdc25 phosphatase contribution is required (Edgar & Datar 1996).

which results in the complete inactivation of Cdk1 after mitosis 13. All subsequent mitoses therefore become dependent on zygotic expression of Cdc25 phosphatase.

Stopping the cell division cycles

Each of the cell divisions after cellularization is triggered by a brief pulse of zygotic *string/cdc25* expression (Edgar & Lehner 1996). Entry into S phase follows after mitosis without an intervening G1 phase. This immediate entry into S phase is most likely explained by the presence of maternal stores. All components required for S phase appear to be present in amounts sufficient to allow all of the embryonic S phases in most of the tissues. Zygotic expression of various S phase genes (like ORC2, MCM, PCNA, E2F/DP, cyclin E, Cdk2, RNR, Chiffon/Dbf4p) has been shown to be dispensable for the generation of normal cell numbers in the epidermis for example.

After completion of S phase, the embryonic cells wait during each G2 phase for a pulse of *string/cdc25* transcription to enter mitosis and another S phase. The complex and dynamic pattern of zygotic *string/cdc25* expression, therefore, controls the embryonic cell proliferation. The intricate developmental programme of *string/cdc25* transcription is controlled by a large array of stage- and tissue-specific enhancers (Lehman et al 1999). Spatially and temporally varying combinations of transcriptional regulators known to specify positional information and developmental fate appear to act directly at these *string/cdc25* enhancers. The programme of embryonic cell divisions is thereby adjusted to the various developmental fates. Cells that share the same developmental fate progress through the embryonic cell division cycles almost synchronously. Moreover, they also terminate cell proliferation with high synchrony. In the epidermis, which we have studied most extensively, the great majority of cells stop proliferating after mitosis 16.

Why is the epidermal cell proliferation arrested at a defined developmental stage? This is no longer simply a result of eliminating String/Cdc25 phosphatase expression and thereby Cdk1 activation, as in the case of the early syncytial cycle arrest described before. Eliminating exclusively the String/Cdc25 phosphatase without concomitant inactivation of the S phase-promoting activity would result in a G2 arrest. However, after the terminal mitosis the cells enter for the first time into G1 and withdraw from the mitotic cycle. The immediate entry into S phase observed after all the previous divisions no longer occurs after the terminal division. Which of the components required for entry into S phase is no longer present or no longer active after the terminal division? The elimination of cyclin E/Cdk2 activity is critical for a timely arrest of the epidermal cell proliferation (Knoblich et al 1994). Epidermal cells fail to arrest in G1/0 when *cyclin E* is overexpressed. *cyclin E* transcripts normally disappear during the final division

cycle. However, some cyclin E protein remains and is still detectable after the terminal mitosis in wild-type embryos. The inhibition of the resulting residual cyclin E/Cdk2 activity requires the expression of *dacapo* (*dap*) which encodes a CIP/KIP-type Cdk inhibitor (de Nooij et al 1996, Lane et al 1996). Expression of this specific cyclin E/Cdk2 inhibitor is not detectable during the embryonic cell division cycles, but starts abruptly in G2 of the final division cycle before the terminal division. In *dap* mutants, the epidermal cells fail to arrest in G1/0 after the terminal mitosis.

While *dap* is clearly essential for a timely cell proliferation arrest in the embryonic epidermis, and while it is likely to be equally important in a number of other tissues, there are also clear cases where *dap* is definitively not involved in stopping cell proliferation (for instance in the zone of non-proliferating cells [ZNC] at the prospective wing margin in third instar imaginal discs or in front of the morphogenetic furrow in the eye imaginal discs). We have not found additional CIP/KIP-type genes in the near complete *Drosophila* genome sequence. However, the conservation of CIP/KIP inhibitors is very limited and they are therefore difficult to identify.

In addition to *dap* mutations, we have also isolated *cdk2* and other mutations in a screen for genetic modifiers of a rough eye phenotype caused by ectopic expression of *cyclin E* in postmitotic cells during eye development (Lane et al 2000). Using these *cdk2* mutations we have addressed whether inhibition of cyclin E/Cdk2 might involve inhibitory phosphorylation of Cdk2 by Wee1/Mik1-like kinases in addition to DAP. However, our experiments indicated that this type of regulation, which is of paramount importance in the case of Cdk1, is not essential for Cdk2 regulation. We found that the lethality resulting from null mutations in *Cdk2* was rescued by expression of mutant *Cdk2* lacking the phosphate acceptor sites (*Cdk2*^{Y18FT19A}). Moreover, this mutant *Cdk2* did not prevent a timely arrest of the epidermal cell proliferation (Lane et al 2000).

Apart from *dap*, we have identified a second gene which is required for a timely arrest of the embryonic cell proliferation. This *fizzy-related* (*fzr*) gene is also transcriptionally up-regulated at the stage when cells become post-mitotic (Sigrist & Lehner 1997). It encodes an activator of the APC/C, a ubiquitin ligase required for the degradation of mitotic cyclins (A, B and B3). The transcription of cyclins A, B and B3 is terminated after the final division. However, residual transcripts endure for some time after the terminal division. FZR brings about the proteolytic degradation of the mitotic cyclins that are translated from these enduring transcripts. Like inhibition of cyclin E/Cdk2, elimination of cyclin A is also required for a timely arrest of cell proliferation. Expression of cyclin A with mutations in the destruction box region, which partially inhibit degradation, phenocopies the loss of *fzr* or *dap* expression. Genetic interactions indicate that FZR activity is negatively regulated by cyclin E/Cdk2, like the yeast FZR

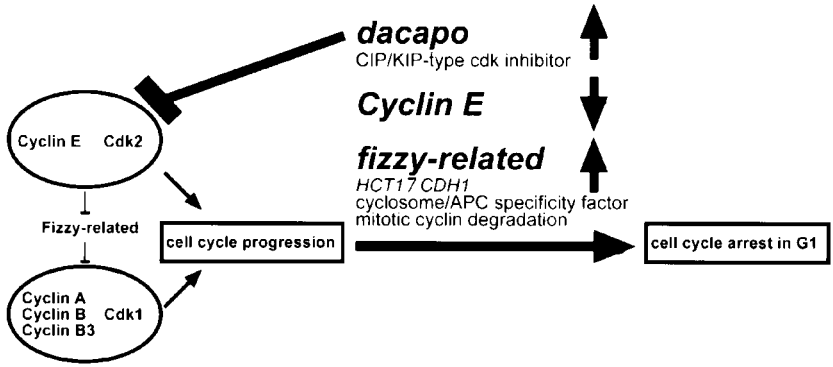


FIG. 3. Stopping the cell division cycles. In *Drosophila* embryos, the timely arrest of cell proliferation in the epidermis requires the transcriptional activation of *dacapo* (Lane et al 1996, de Nooij et al 1996) and *fizzy-related* (Sigrist & Lehner 1997) which occurs during the final division cycle in parallel to down-regulation of *cyclin E* expression.

homologue Cdh1p which is known to be inhibited by Cdk phosphorylation (Zachariae et al 1998). By inhibiting cyclin E/Cdk2 after the terminal division, DAP also appears to maintain FZR activity and thereby contributes to the elimination of cyclin A and other mitotic regulators (Fig. 3).

The activation of *dap* transcription during the final division cycle, therefore, is a very crucial step for the arrest of the epidermal cell proliferation. To analyse this process, we are dissecting the *cis*-regulatory region of the *dap* gene. In addition, we have evaluated whether the activation of *dap* expression is a consequence of the inactivation of D-type cyclin/Cdk complexes. The work in mammalian cells has suggested that D-type cyclin/Cdk complexes play a crucial role in the regulation of cell proliferation (Sherr 1993, Sherr & Roberts 1999). D-type cyclins are induced by mitogens. This results in hyperphosphorylation of the retinoblastoma tumour suppressor protein pRb and thereby activates the transcription of E2F/DP target genes, like cyclin E and ribonucleotide reductase (RNR), which promote entry into S phase and cell proliferation. Inactivation of D-type cyclin/Cdk complexes, therefore, might cause the down-regulation of cyclin E and the parallel up-regulation of *dap* expression in the *Drosophila* embryo.

To address this hypothesis, we overexpressed *Drosophila cyclin D* and its partner kinase *cdk4* using Gal4p-dependent UAS transgenes. In combination with the *prd-GAL4* transgene, we achieved *cyclin D*|*cdk4* (as well as *E2F1*|*DP*) overexpression in seven epidermal stripes (Fig. 4). Analogous overexpression of *cyclin E* is known to abolish a timely G1/0 arrest (Knoblich et al 1994, Sigrist & Lehner 1997). However, *cyclin D*|*cdk4* (as well as *E2F1*|*DP*) overexpression did not prevent epidermal cells from entering into G1/0 after the terminal mitosis. Control

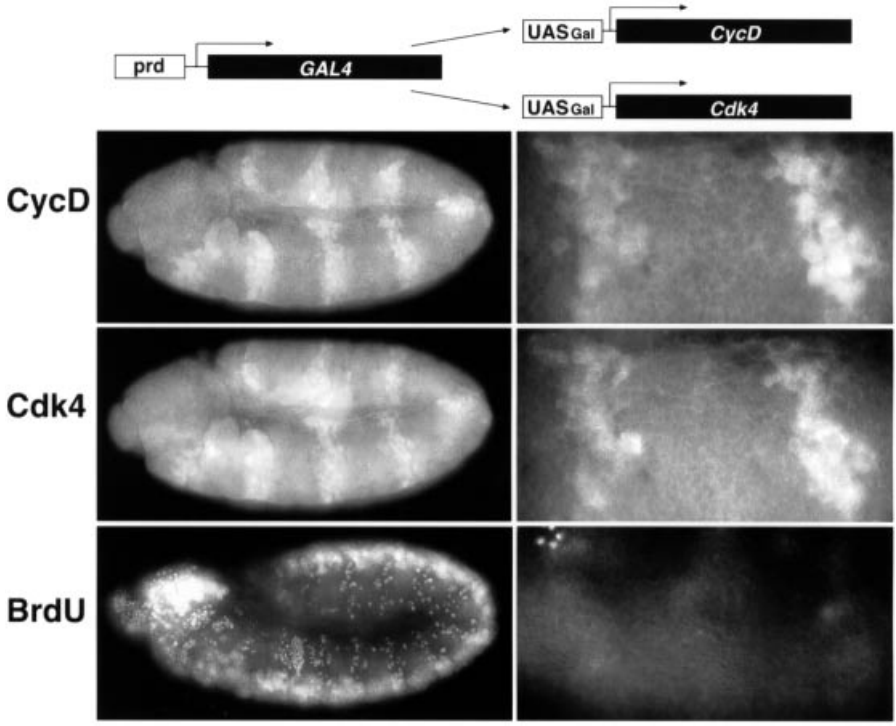


FIG. 4. Overexpression of *cyclin D* and *cdk4* does not prevent the arrest of cell proliferation at the correct developmental stage. *cyclin D* and *cdk4* were overexpressed in *prd-GAL4*|*UAS-cycD*, *UAS-cdk4* embryos in seven epidermal stripes as demonstrated by immunolabelling at the stage of the terminal epidermal cell division with antibodies against cyclin D (CycD) or Cdk4 (Cdk4). BrdU pulse labelling (BrdU) did not reveal incorporation in the epidermis, while incorporation in proliferating cells of the peripheral and central nervous system is readily detectable. High magnification views of epidermal regions from the embryos on the left side are presented on the right side.

experiments clearly demonstrated that the overexpressed complexes are active. *In situ* hybridization demonstrated that the *prd-GAL4* driven *UAS-cyclin D*|*cdk4* (and *UAS-E2F1*|*DP*) expression induced strong expression of the E2F/DP target gene *RNR2*, but *cyclin E* expression was not detectable. Moreover, while *cyclin D*|*cdk4* overexpression did not abolish the initial entry into G1/0, it interfered in a few of the overexpressing cells with the maintenance of this G1/0 arrest. A very similar inability to maintain the G1/0 arrest in some epidermal cells has been described in embryos lacking the function of *RBF*, a *Drosophila* pRb family member (Du & Dyson 1999). Our findings, therefore, indicate that establishment of the cell proliferation arrest in the epidermis is not dependent on inactivation of D-type cyclin/Cdk complexes.

Our genetic characterization of *cdk4* confirmed the notion that D-type cyclin/Cdk complexes might not have a major role in the regulation of cell proliferation throughout *Drosophila* development. *cdk4* is not an absolutely essential gene: *cdk4* null mutations do not prevent development to the adult stage, and even though body size is slightly and fertility greatly reduced, some progeny can be obtained from homozygous mutant parents.

Mammalian D-type cyclins primarily associate with two highly related kinases, Cdk4 and Cdk6. Moreover, in malignant cells binding of cyclin D to Cdk2 is often observed. The mild phenotype of null mutations in *Drosophila cdk4* might therefore be explained by the action of redundant cyclin D-associated kinases. However, a number of observations argue against this explanation. The nearly complete *Drosophila* (and *Caenorhabditis elegans*) genome sequence contains only a single *cdk4/6*-like gene and a single *cyclin D*-like gene. Extensive low stringency screens of *Drosophila* genomic libraries with human *CDK4* and *6* probes have also not revealed a *cdk4/6*-like gene in addition to *cdk4*. In addition, expression of a mutant Cdk4 ($Cdk4^{D175N}$), which fails to rescue the decreased body size in flies lacking the endogenous *cdk4* gene but still binds to cyclin D, has no effect in these flies lacking the endogenous *cdk4* gene, while it results in a significant body size reduction when expressed in wild-type flies. Thus, as expected from analogous mutations in human *CDK1* and *2*, $Cdk4^{D175N}$ appears to act in a dominant-negative fashion by titrating cyclin D. The fact that this $Cdk4^{D175N}$ protein has no effect in *cdk4* mutants argues against the presence of redundant D-type associated kinases.

However, genetic interactions demonstrate that *cdk4* mutants are extremely sensitive to a reduction in cyclin E/Cdk2 function. Cyclin D and cyclin E complexes, therefore, might have partially overlapping functions. Our analysis of the phenotypes resulting from *cdk4* mutations and *cyclin D/cdk4* overexpression in imaginal cells indicate that D-type cyclin/Cdk complexes are primarily important for the regulation of cell growth and not specifically for progression through the G1 phase of the cell cycle.

The activation of *dap* expression is not only independent of inhibition of cyclin D/Cdk4 activity, but also does not require completion of the cell proliferation programme. Mutations in genes encoding mitotic regulators which arrest the embryonic cell proliferation prematurely do not abolish timely *dap* transcription. Preliminary observations suggest that the onset of *dap* expression in the epidermis is already defined during gastrulation by those genes which specify positional information and developmental fate. While the early syncytial proliferation is clearly limited by the number of cell nuclei, therefore, it appears that the genes which specify the fate of the epidermal cells start a clock which eventually activates *dap* expression irrespective of whether the correct cell numbers have been reached. Future analysis will hopefully reveal the details of this D-type cyclin independent control of cell proliferation.

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DISCUSSION

Thomas: How does cyclin E up-regulate E2F? You showed us that it is not through cyclin D, so how is Rb being shut down?

Lehner: In the embryo, cyclin E is a lousy target for the E2F pathway. It might be different in the imaginal disks. There, up-regulation of cyclin E as a result of E2F overexpression has been reported (Neufeld et al 1998).

Edgar: It is still a weak target.

Thomas: Have you any idea of how cyclin E is up-regulated?

Richardson: As with String, cyclin E appears to be regulated by developmental signals in particular tissues (L. Jones, H. Richardson & R. Saint, unpublished results). The only place where it is really responding to E2F is in the embryonic gut and other endoreplicating tissues (Duronio & O'Farrell 1995). In other tissues there might be tissue-specific factors that are controlling its expression.

Harper: There is similar evidence in mammalian cells. There are Rb-independent ways for cyclin E to be expressed.

Edgar: Along similar lines, do either of you know why *cyclin E* transcription is shut off in late-stage embryos?

Lehner: It is technically more difficult to study how genes are switched off, as opposed to switched on. This is why we are presently analysing the switching on of *dacapo* expression when cell proliferation stops. The hope is that once we know how *dacapo* is up-regulated, perhaps it will also shed light on how *cyclin E* is down-regulated. At the moment, we do not know.

Raff: Did you say that all of the regulators are down-regulated at the same time, or is *cyclin E* special?

Lehner: I do not think that *cyclin E* is special. However, for technical reasons it is very difficult to know exactly when this down-regulation starts. I do not know whether all the proliferation genes are turned off at the very same time. It is possible that *cyclin E* is switched off earlier and the mitotic regulators later, because they are still needed for the final mitosis.

Nasmyth: The lack of necessity for this cyclin D/Cdk4 activation of cyclin E is striking. What is the latest in mammalian cells? They have knocked out a number of cyclin Ds; it is a sort of similar story, isn't it? Let's take the growth bit away. It is awfully similar to, say, Cln3 in yeast where it is important but not essential: if you take it away the system still works. Is it not similar in a mammalian cell?

Lehner: In mammalian cells there is more genetic redundancy. We are not sure to what extent the other D-type cyclins compensate when we are looking at cyclin D1 or D2 knockouts. The same is true for Cdk4 knockouts; Cdk6 may compensate. There are many different mutant combinations that have not yet been analysed.

Schmidt: There are some clear answers in different tissues. Mammary glands don't grow in the absence of cyclin D1, for example.

Raff: Do you mean not grow or not proliferate?

Schmidt: There are no lobuloalveolar structures present in cyclin D1 knockout mice.

Raff: Why is that?

Schmidt: That's a harder question. The data are clear; the mouse can make a duct, but it cannot put a lobuloalveolar structure on the outside of this. There is even a third question: is D1 specifying some kind of a cell fate?

Harper: The flip side of the coin is the status of p27 and homologues, and whether or not for cycling in mammalian cells cyclin D is needed to sequester these indirectly. What you are suggesting is that in flies this is not going on.

Lehner: We just can't detect it. Even if we overexpress cyclin D/Cdk4 we cannot detect the association of Dacapo with these complexes. This is negative evidence and should be interpreted cautiously. However, it is clear that the p27 association with mammalian D-type cyclin/Cdk complexes is readily detectable. Thus, there seems to be a difference between flies and mammals.

Nasmyth: Is there only one Rb-like protein?

Lehner: There are two RBF proteins. The genome project has identified a second *RBF* gene.

Nasmyth: You talked about the heterozygote: what is the phenotype of the homozygous mutant?

Lehner: The phenotype has only been described for RBF1. What has been described more carefully is the phenotype resulting from elimination of both the maternal and the zygotic *RBF1* function by generating germline clones (Du &

Dyson 1999). Oogenesis in the absence of *RBF1* appears to be normal as does the embryonic proliferation. In the case of the embryonic epidermis, cells are also known to exit from the cell cycle at the appropriate developmental stage. But some cells have problems maintaining quiescence. They turn on *cyclin E* expression, they progress through another S phase, but they do not seem to complete this extra cell cycle with a division.

Edgar: I have also done some clonal analysis knocking *RBF* out in clones of cells in wing and eye discs. The phenotypes don't phenocopy gain of cyclin D expression, they look more like gain of E2F expression. For example, overexpressed cyclin D/cdk4 can give eye overgrowth, whereas loss of *RBF* gives hypoplasia in the eye. My interpretation of those experiments is that RBF is a very cell cycle-specific molecule and not a growth regulator.

Nasmyth: Whereas Rb is a growth regulator in mammals.

Richardson: What happens with apoptosis in the Rb eyes?

Edgar: They have substantial apoptosis.

Richardson: This could explain why they are much smaller.

Edgar: Yes, the cells are a little smaller, too.

Raff: What about Cdk4 overexpression in clones in the eye?

Edgar: It is neutral by itself, but when it is coexpressed with cyclin D it gives a fast-growing clone.

Lehner: Interestingly, it really also depends on when you do this. As long as cells proliferate, proliferation is effectively inhibited by *RBF1* overexpression. In contrast, *cyclin D/Cdk4* has little effect in proliferating cells. When the cells are postmitotic, the situation is reversed: *RBF1* overexpression has little effect, but there is a very clear stimulation of cell growth resulting from *cyclin D/Cdk4* overexpression.

Raff: How do you explain Rb-independent cell enlargement?

Edgar: An explanation is that cyclin D is hitting targets other than RBF, and those targets are growth stimulators.

Raff: What are these growth regulators?

Lehner: That is an interesting question. We do not know. In endoreduplicating tissues, Myc has very similar effects to cyclin D/Cdk4.

Raff: If this thing that shuts down at the 16th division does not depend on counting cell divisions, what does it depend on?

Lehner: That is a good question. I assume that some sort of clock or timer is being set at the time when the cell fate is specified. In the epidermis there are tracheal pits which eventually form the tracheal tubes. These cells are the first to go through the final division and they are the first to express *dacapo*. There are a number of genes such as *trachealess* encoding transcription factors that specify the fate of these cells. We looked at whether these mutants still express *dacapo* at the correct time. They do. We think therefore that *dacapo* expression is programmed by

the same regulators that also switch on *trachealess* and other fate-specifying genes. We have the impression that the onset of *dacapo* expression is defined at the same time as the developmental fate.

Raff: Which is when?

Lehner: Presumably during gastrulation.

Raff: Can you inhibit DNA synthesis at some before the 16th cycle and still see the same things happen?

Lehner: We haven't done these experiments. But we have looked in *string* mutants where cells arrest in G2 before mitosis 14 and in *cyclin A cyclin B* double mutants where cells arrest in G2 before mitosis 15. The timing of *dacapo* expression in the epidermis appears to be normal in these mutants.

Raff: Is there anything you can do earlier?

Edgar: No, the *string* mutants are the earliest arrest.

Raff: But you could block DNA synthesis.

Edgar: I've done that. It is sort of catastrophic. I don't think the inhibitors give a nice clean arrest.

Simon: Is the exit from mitosis always sharply at cell division 16?

Lehner: In the epidermis it is quite reproducible. After mitosis 13, when the syncytial division cycle is stopped, cellularization occurs followed by a pulse of *string* expression that allows cells to go through mitosis 14. Then they go immediately into S phase and wait in the next G2 phase until the next pulse of *string* comes up, driving mitosis 15. After mitosis 15, they go again immediately into S phase and wait in G2 until *string* comes up, triggering the final division.

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Cell cycle transitions in early *Xenopus* development

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Abstract. *Xenopus* oocytes and embryos undergo two major maternally controlled cell-cycle transitions: oocyte maturation and the mid-blastula transition (MBT). During maturation, the essential order of events in the cell cycle is perturbed in that the M phases of Meiosis I and II occur consecutively without an intervening S phase. Use of U0126, a new potent inhibitor of MAPK kinase (MEK), shows that MAPK activation is essential to inhibit the anaphase-promoting complex and cyclin B degradation at the MI/MII transition. If MAPK is inactivated, cyclin B is degraded, S phase commences and meiotic spindles do not form. These events are restored in U0126-treated oocytes by a constitutively active form of the protein kinase $p90^{Rsk}$. Thus all actions of MAPK during maturation are mediated solely by activation of $p90^{Rsk}$. At the MBT, commencing with the 13th cleavage division, there are profound changes in the cell cycle. MBT events such as maternal cyclin E degradation and sensitivity to apoptosis are regulated by a developmental timer insensitive to inhibition of DNA, RNA or protein synthesis. Other events, such as zygotic transcription and the DNA replication checkpoint, are controlled by the nuclear:cytoplasmic ratio. Lengthening of the cell cycle at the MBT is caused by increased Tyr15 phosphorylation of Cdc2 resulting from degradation of the maternal phosphatase Cdc25A and continued expression of maternal Wee1. Ionizing radiation causes activation of a checkpoint mediating apoptosis when administered before but not after the MBT. Resistance to apoptosis is associated with increased $p27^{Xic1}$, the relative fraction of Bcl-2 or Bax in pro- versus anti-apoptotic complexes, and the activity of the protein kinase Akt.

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It is now generally accepted that major transitions in development are accompanied by major changes in the cell cycle. However, the exact relationship between changes in cell cycle control and developmental transitions is unclear. In some cases changes in the cell cycle may be necessary to initiate developmental transitions, whereas in other cases developmental signals may cause immediate changes in the cell cycle. The two processes could also in some cases be regulated independently of each other. One of the leading model systems for the study of

both cell cycle control and developmental transitions is encompassed by the *Xenopus* oocyte, egg and embryo. In part the prominence of the system derives from the correspondence between release from specific arrested states of the cell cycle and developmental transitions. Other features include the highly synchronous nature of early development, which makes it feasible to monitor developmental progression morphologically, and the ease of microinjection of the cells, which makes it easy to introduce exogenous components that affect the cell cycle and/or development. In more recent times extracts from oocytes and eggs have been developed that can undergo changes in the cell cycle *in vitro* in response to various signals. These extracts make feasible depletion/reconstitution experiments that provide a powerful tool for experimental analysis. In this chapter we outline recent advances in two key processes in *Xenopus* development that exemplify the interface between cell cycle control and development. These processes are meiotic maturation in oocytes and the mid-blastula transition (MBT) in embryos.

Xenopus oocyte maturation

From a cell cycle point of view, oocyte maturation has been an interesting system for many years. In the ovary all post vitellogenic oocytes are physiologically arrested in late diplotene at the G2/prophase border in meiosis I. The meiotic genome of the resting oocyte is transcriptionally active, especially for ribosome synthesis, but growth over several months occurs via uptake of vitellogenin from the blood. The oocyte is one of the clearest cases where cell growth is completely uncoupled from the cell cycle. Biochemically the resting oocyte is known to have relatively high activity of both cAMP-dependent protein kinase and p70 S6 kinase (Maller et al 1979, Schwab et al 1999). p70 promotes the translation of mRNAs containing a 5′ tract of pyrimidines (5′-TOP), and many of the ribosomal proteins being synthesized during vitellogenesis have 5′-TOP sequences (Amaldi & Pierandrei-Amaldi 1997). The cell cycle quiescence of the resting oocyte is broken by the action of progesterone secreted by the surrounding follicle cells in response to pituitary hormones. The ultimate effect of the steroid is to stimulate progression through meiosis I and II (MI and MII) and arrest as an unfertilized egg. Oocyte maturation can thus be considered the first major transition in early development and results in a cell poised to undergo rapid cycles of DNA synthesis and mitosis in the absence of transcription, fundamentally different processes than those occurring in the oocyte. The conversion of an oocyte into an egg is an example of a developmental switch triggered by a hormone that acts to alter the cell cycle.

Biochemical events during progesterone-induced maturation *in vitro*

Reports by Masui (1967) and others in the late 1960s on the ability to obtain complete oocyte maturation *in vitro* by addition of progesterone were a great stimulus to work in the field. Additional stimulation came in 1971 from the work of Masui & Markert (1971) and Smith & Ecker (1971) that described a cytoplasmic activity capable of inducing complete maturation when microinjected into resting oocytes in the absence of progesterone. This activity, known as maturation-promoting factor (MPF), could be detected in all types of M phase cells. It was ultimately purified in this laboratory from *Xenopus* eggs (Lohka et al 1988) and shown to consist of a cyclin B/Cdc2 complex (Gautier et al 1988, Dunphy et al 1988). Combined with genetic work in yeast showing that *cdc2* is a universal mitotic control gene (Lee & Nurse 1987), the field of cell cycle control entered a new, more energetic phase that continues today. The system in which MPF was characterized and purified, the frog oocyte system, has continued as a leading model for cell cycle control from at least three perspectives: mechanism of MPF activation; mechanism of MPF inactivation, and the linkage of consecutive M phases in meiosis (Fig. 1).

MPF activation

Studies in several laboratories showed that dephosphorylation of tyrosine 15/threonine 14 in cyclin B/Cdc2 was the final step in activation of MPF, just prior to progesterone-stimulated germinal vesicle (nuclear envelope) breakdown (GVBD) in MI. Changes in the phosphorylation state of a protein reflect changes in the relative balance between the protein kinase(s) and protein phosphatase(s) acting on the protein. In the case of tyrosine-phosphorylated cyclin B/Cdc2, the phosphatase is Cdc25C and the kinase is Myt1. In this laboratory a major effort has been devoted over the last 10 years to understanding how Cdc25C is activated during maturation. The general approach has been to work backwards up the pathway to elucidate how Cdc25C is activated. The mechanism has importance not only for maturation but also for the DNA replication checkpoint in the somatic cell cycle. Izumi et al (1992) and Kumagai & Dunphy (1992) demonstrated that Cdc25C is activated by phosphorylation on Ser/Thr residues. Although cyclin B/Cdc2 can perform this reaction *in vitro*, forming a positive feedback loop, Cdc25C activation occurs earlier than cyclin B/Cdc2 activation and can be obtained in microcystin-treated extracts in the absence of Cdc2 (Izumi & Maller 1995). The upstream activating kinase was found to be the *Xenopus* homologue of the polo-like kinase Plx1 (Kumagai & Dunphy 1996), and the kinetics of Plx1 activation fit with a role as an upstream activator of Cdc25C (Qian et al 1998a). Two other experiments strongly support Plx1 as an upstream

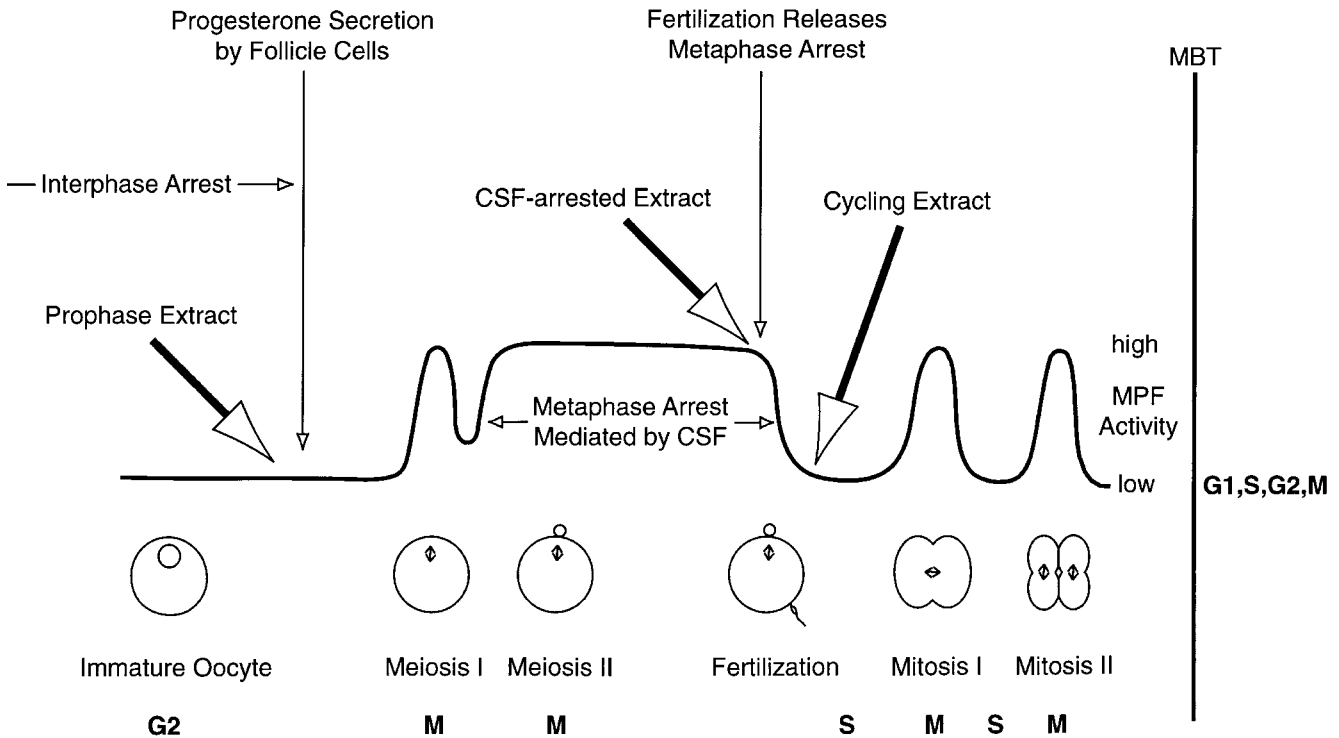

FIG. 1. Schematic diagram of early *Xenopus* development. The figure shows relative levels of MPF (cyclin B/Cdc2) activity from the beginning of oocyte maturation until after fertilization.

activator of MPF. First, constitutively active Plx1 is able to induce oocyte maturation and Cdc2 activation in the absence of progesterone (Qian et al 1998b). Second, extracts from resting oocytes that activate Cdc25C upon addition of the heat-stable inhibitor of PKA (PKI, see below) are unable to do so if Plx1 has been immunodepleted. Plx1 is thus both necessary and sufficient for Cdc25C activation.

Qian et al (1998b) demonstrated that injection of active Cdc25C into resting oocytes leads to Plx1 activation, implying the existence of a positive feedback loop between a component downstream of Cdc25C and Plx1 itself or a component of the upstream pathway regulating Plx1. Inasmuch as inhibition of Cdc2 by $p21^{Cip1}$ blocks Plx1 activation (O. Haccard, personal communication), it has been suggested that Plx1 activation is normally downstream of Cdc2 and not upstream. This possibility appears to be excluded by the kinetics of Plx1 activation during oocyte maturation and the immunodepletion experiments described above. In addition, very early events in maturation, such as c-Mos synthesis, are also blocked by Cip1 injection (Frank-Vaillant et al 1999), making it impossible to evaluate the order of maturation events in Cip1-injected oocytes. These considerations, along with evidence that Plx1 itself is not directly phosphorylated or activated by Cdc2, have focused attention on upstream components regulating Plx1.

Qian et al (1998a) found that Plx1, like its mammalian counterpart, is activated by phosphorylation. A large-scale purification effort for an activating kinase led to purification of a novel protein kinase termed xPlkk1 (*Xenopus* polo-like kinase kinase) (Qian et al 1998a). These results defined the Plx1 activation pathway as a polo-like kinase cascade. Dephosphorylation experiments demonstrated that xPlkk1 itself is also activated by phosphorylation, suggesting that yet another kinase lies upstream of xPlkk1. More recent data show that xPlkk1 can be phosphorylated and activated by itself as well as by Plx1, forming a positive feedback loop between Plx1 and xPlkk1 (E. Erikson & Y.-W. Qian, unpublished results).

Exit from mitosis

The *Xenopus* system has proven instrumental in determining the mechanism controlling exit from mitosis at the metaphase/anaphase transition. Studies in this area have relied heavily on extracts prepared from fully mature oocytes/unfertilized eggs that are arrested at metaphase of the second meiotic division. Upon Ca^{2+} addition, anaphase is initiated and the extract enters the first embryonic cell cycle to replicate DNA. The activity responsible for metaphase arrest was discovered by Masui at the same time as MPF (Masui & Markert 1971), and given the name cytostatic factor (CSF). CSF has never been purified

and therefore its molecular composition is unknown. However, an extensive series of studies show that an active MAPK pathway is necessary for CSF arrest, which is usually monitored by assaying components for cleavage arrest at metaphase in early embryos. Thus CSF arrest can be achieved by expression of Mos, a MAP kinase kinase kinase synthesized during maturation, by constitutively active MAP kinase kinase or by thiophosphorylated, phosphatase-resistant MAP kinase (Sagata 1997 for review). Because activated MAP kinase is localized at kinetochores in mitosis (Zecevic et al 1998) and certain kinetochore-based microtubule motors are altered in CSF-arrested spindles (Duesbery et al 1997), it is generally believed that CSF is located on or acts at the kinetochore. CSF-dependent metaphase arrest is fundamentally different from the spindle assembly/kinetochore attachment checkpoint in the somatic cell cycle, since in the former an intact metaphase spindle is present whereas in the latter microtubules are depolymerized and spindles are absent.

Recently the target of MAP kinase responsible for CSF arrest has been identified as the protein kinase $p90^{Rsk}$. Rsk was originally identified, purified and cloned as the kinase responsible for phosphorylation of ribosomal protein S6 during maturation (Erikson & Maller 1986). Subsequently, it was found to be activated by phosphorylation, and identification in this laboratory of MAP kinase as the upstream activating kinase defined the initial features of the MAP kinase cascade (Sturgill et al 1988). Although Rsk deactivated by protein phosphatases can be reactivated by MAP kinase, recombinant Rsk cannot be activated by MAP kinase *in vitro*. The dilemma was resolved recently by studies that identified PDK1 as an additional kinase that is required for Rsk activation (Jensen et al 1999). With this information and a variety of deletion mutants, Gross et al (1999) generated a constitutively active form of Rsk that requires only PDK1 phosphorylation for activation. This enzyme was found to cause CSF arrest at metaphase when injected into blastomeres (Gross et al 1999), and depletion of Rsk from egg extracts depleted CSF activity, which could be restored by re-addition of Rsk (Bhatt & Ferrell 1999). Importantly, Rsk produced this CSF arrest without activation of feedback loops that activate endogenous MAP kinase or Rsk. These results indicate that Rsk is the only substrate of MAP kinase needed to mediate CSF arrest (Fig. 2).

The MI/MII transition

One of the classical fundamental problems in cell cycle control is 'the alternation problem', i.e. why does S phase always follow M phase, and M phase follow S phase (sometimes separated by intervening gap periods). In considering this problem, it is evident that meiosis is a special situation in that the two consecutive M phases of MI and II occur sequentially without an intervening S phase (Fig. 1). Analysis of

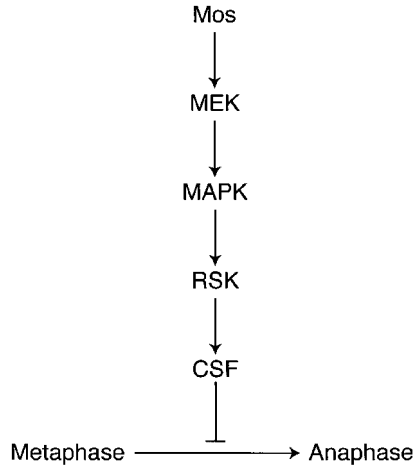

FIG. 2. Pathway of CSF activation. The figure denotes $p90^{Rsk}$ as the target of the MAPK pathway required for generation of CSF activity, which inhibits the metaphase to anaphase transition in MII.

this special situation is expected to elucidate fundamental controls on the alternation problem. The biochemistry of the MI/MII transition has been most extensively studied in *Xenopus* oocytes treated with progesterone. In anaphase I of the MI/MII transition, the level of cyclin B falls transiently to about 50% of the mitotic level, and the H1 kinase activity of Cdc2 falls in parallel (Roy et al 1996). However, chromosomes remain condensed after anaphase I, and MII spindles form 1–2 h later. The mechanism that prevents complete cyclin B degradation involves the activation of the Mos/MAPK pathway, which begins in MI. Inhibition of the MAPK pathway in MI using the MEK inhibitor U0126 causes a delay in the kinetics of the response, but GVBD occurs normally (Gross et al 2000). However, if Mos, a MAP kinase kinase kinase, is ablated by injection of antisense oligonucleotides after GVBD, maturing oocytes exit anaphase into interphase, forming a nuclear envelope and replicating DNA (S phase) (Furuno et al 1994). Under these conditions cyclin B is completely degraded. Similar results occur with cycloheximide treatment after GVBD, which leads to cyclin B degradation and S phase entry even in the presence of active GST–Mos and active MAPK (Roy et al 1996), or on treatment with U0126 (Gross et al 2000). These results have supported a model in which MAPK prevents interphase by promoting accumulation of cyclin B to about 50% of the metaphase level after anaphase I.

The accumulation of cyclin B and the absence of interphase after anaphase I fits well with data in fission yeast that indicate cyclin B 'marks' the G2/M state of the

cell (O'Connell & Nurse 1995). In principle the persistence of cyclin B during anaphase I could be due to increased synthesis of cyclin B or decreased degradation or both. The idea that synthesis of cyclin B was very active during anaphase came initially from studies in which cycloheximide was added after GVBD (Roy et al 1996). Cyclin B was rapidly degraded, indicating the anaphase-promoting complex (APC) was active and that only rapid synthesis of cyclin B prevented complete cyclin B degradation during the MI/II transition. The mechanism responsible for cyclin B synthesis has not been fully elucidated but appears to involve at least two distinct signal transduction pathways. One pathway is MAP kinase inasmuch as premature activation of MAP kinase by GST–Mos injection increases the synthesis of cyclin B1 prior to any detectable activation of Cdc2 (Roy et al 1996). However, in oocytes injected with the Cdk inhibitor $p21^{Cip1}$, cyclin B1 synthesis is stimulated by progesterone in the complete absence of both active Cdc2 and active MAP kinase (Frank-Vaillant et al 1999). These effects on cyclin B synthesis are presumed to be mediated by progesterone-dependent translational activation of maternal mRNA via 3′-untranslated region (UTR) binding proteins (Richter 1999).

The idea that cyclin B degradation might be reduced after anaphase I came originally by extension from studies on the stability of Mos which some investigators (Sagata 1997) but not others (Freeman et al 1992) hypothesized to undergo phosphorylation-dependent stabilization. Recently the question of cyclin B stability has been re-examined because of advances in understanding the anaphase-promoting complex, a complex of eight proteins that functions as an E3 ubiquitin ligase to target proteins for degradation by the 26S proteosome (Fang et al 1999 for review). Abundant evidence indicates that the APC is regulated by phosphorylation and is more active in mitosis than in interphase (Zachariae & Nasmyth 1999 for review). Recently, Gross et al (2000) investigated changes in APC function during maturation as judged by the electrophoretic mobility of Cdc27 in the presence or absence of U0126 to inhibit the MAPK pathway. A shift in Cdc27 that correlates with accumulation of cyclin B during the MI/MII transition failed to occur in the presence of U0126. Correlated with this was entry into S phase as described above. This result suggests that APC function for cyclin B degradation in MII is being regulated by the MAP kinase pathway. To investigate this directly, we injected radiolabelled cyclin B1 into oocytes 4 h after GVBD in the presence and absence of U0126. Cyclin B1 was chosen for study because of previous evidence that its degradation does not require binding to Cdc2 (Stewart et al 1994). The half-life of cyclin B1 in controls was greater than 45 min whereas in U0126-treated oocytes it was less than 15 min. These results provide direct evidence that cyclin B degradation is reduced during the MI/II period due to activation of the MAPK pathway (Gross et al 2000).

Subsequent experiments addressed the possibility that the target of MAPK responsible for these effects is $p90^{Rsk}$. Upon injection of the constitutively active form of Rsk described above, oocytes undergoing the MI/MII transition in the presence of U126 remained in M phase with an elevated cyclin B level, a shift in Cdc27 and metaphase-arrested spindles. These results indicate that these actions of the MAPK pathway on the APC, like those involved in CSF arrest at metaphase, are mediated solely by $p90^{Rsk}$.

The midblastula transition

Once an egg is fertilized, intracellular Ca^{2+} is elevated, cyclin B degradation is stimulated, and CSF activity declines in concert with loss of MAPK and Rsk activity. The first 12 cleavage cycles occur with a cycle time of 26 min and display only M and S phase, except for the first cycle which is longer and has a detectable G2 phase (Fig. 1). Despite these short cycles, the enzymes that control Cdc2 Tyr15 phosphorylation, Wee1 and Cdc25A, are rapidly synthesized after fertilization and new cyclin B/Cdc2 complexes appear to undergo a rapid cycle of Tyr15 phosphorylation/dephosphorylation in every cell cycle. Since no G2 phase is evident in these cell cycles, the function of transient Tyr15 phosphorylation is not clear at this time. The prevailing view is that cell cycle checkpoints do not occur in the first 12 cycles because cleavage is unaffected by inhibition of DNA or RNA synthesis or microtubule depolymerization agents. However, at the MBT, cleavage is blocked by inhibition of DNA synthesis and if DNA is damaged or transcriptionally blocked, a maternally programmed apoptosis response is initiated (Anderson et al 1997). The events of the MBT fall into two classes: those that occur at a fixed time after fertilization and those that require a threshold nuclear:cytoplasmic ratio. Examples of the former include the degradation of maternal cyclin E, and of the latter cell cycle arrest by aphidicolin and zygotic transcription (Hartley et al 1997, Newport & Kirschner 1982). The developmental timing mechanism that controls cyclin E degradation has not been elucidated. In principle, it might have similarities to other fixed timing events in development identified in other organisms (Burton et al 1999). Although the timing of the MBT is independent of DNA, RNA and protein synthesis, the timing of cyclin E degradation and later MBT events is delayed by the Cdk inhibitor $p27^{Xic1}$, which inhibits cyclin E/Cdk2 (Hartley et al 1997). Centrosome duplication, which requires cyclin E, is also inhibited by Xic1 (Hinchcliffe et al 1999), suggesting the possibility the timing mechanism reflects some aspect of centrosome biology.

The DNA replication checkpoint

The study of the biochemistry of the DNA replication checkpoint has been facilitated by the observation that merely adding nuclei to egg extracts at levels where the nuclear:cytoplasmic ratio approaches that of the MBT in the intact embryo makes the cell cycle arrest upon inhibition of DNA synthesis. Available evidence indicates that the block to mitotic entry is a result of failure to dephosphorylate and activate cyclin B/Cdc2. Most efforts have thus focused on the regulation of the phosphatase Cdc25C, which catalyses Tyr15 dephosphorylation and activation of Cdc2. Direct action on Cdc25C has been indicated by checkpoint activation of a protein kinase that phosphorylates Ser287 and creates a docking site for 14-3-3 proteins (Kumagai et al 1998, Peng et al 1997). The 14-3-3 binding is believed to block nuclear translocation and/or interaction with cyclin B/ Cdc2, thus preventing enzyme/substrate interaction. This pathway appears to be highly conserved from the one defined genetically in yeast. Similar events may occur with DNA damage in which a distinct kinase phosphorylates Cdc25 to promote 14-3-3 binding.

Whether the DNA replication checkpoint directly affects the Plx1 activation pathway for Cdc25C has not yet been established. It is possible that the replication checkpoint arrests the cell cycle prior to initiation of the Plx1 kinase cascade. Further characterization of upstream components of the cascade should reveal whether it is directly regulated by replication checkpoint activation. Such a characterization will also have importance for other M phase events, inasmuch as Plx1 also regulates bipolar spindle formation, APC activation and cytokinesis (Qian et al 1998, 1999). These multiple functions of Plx1 are associated with changes in localization of Plx1, and are most likely mediated by protein–protein interaction with the 'polo box' motif in the non-catalytic C-terminal half of Plx1.

The DNA damage checkpoint

Genetic and biochemical evidence in yeast and mammalian cells has indicated that the DNA damage checkpoint has overlapping components with the DNA replication checkpoint, and recent evidence implicates polo-like kinases in the DNA damage checkpoint (Sanchez et al 1999). Moreover, the response to DNA damage (double-stranded DNA breaks) is different in G1 and G2 phase, with the latter response targeted at Cdc25 as described above. To assess the ontogeny of the DNA damage checkpoint, this laboratory embarked several years ago on a study of the effect of ionizing radiation on *Xenopus* development, comparing effects before and after the MBT. In mammalian cells, ionizing radiation in G1 phase activates transcription and/or stabilization of p53, which leads to either cell cycle arrest (allowing time for repair of damage) or apoptosis if damage is extensive or

cannot be repaired (Chen et al 1996). Therefore a related aspect of the work concerned whether this same mechanism becomes operative during early *Xenopus* development, particularly since oocytes and embryos contain a large store of maternal p53 protein (Tchang et al 1993).

Initial studies showed that ionizing radiation given any time before the MBT had no effect on cleavage or cell cycle timing, but irradiated embryos began at the MBT a programme of apoptosis (Anderson et al 1997). Standard features of apoptosis included formation of pyknotic bodies, DNA ladders, cyclin A/Cdk2 activation, and TUNEL labelling. These events could be blocked by prior injection of x-Bcl2 before irradiation, providing further evidence of a genuine apoptotic process. Moreover, the programme only required maternal components inasmuch as α-amanitin and cycloheximide also induced apoptosis when given before the MBT. Although embryos could tolerate a low dose of ionizing radiation and continue post-MBT development after elimination of damaged cells into the blastocoel, no cell cycle arrest option could be observed prior to the MBT. This maternal programme of apoptosis can be induced with irradiation as late as Stage 8.5 (MBT), whereas when ionizing radiation is administered only an hour later at Stage 9 (or later), apoptosis is not induced. With post-MBT ionizing radiation, development does not proceed normally, most likely due to the severe defects in transcription and DNA replication caused by double-stranded DNA breaks, but embryos remain healthy for several hours.

Subsequent analysis has focused on the mechanism of resistance to apoptosis when ionizing radiation is administered after the MBT. Several different mechanisms have become evident. One involves the Bcl2 family of pro- and anti-apoptotic proteins. Studies in several systems demonstrate that these proteins appear to localize in the outer mitochondrial membrane and control the release of cytochrome c, an essential component for activation of the pro-apoptotic caspase cascade. Bcl2 family members include anti-apoptotic agents like Bcl2 and Bclxl, and pro-apoptotic agents like Bad and Bax. In part the decision to undergo apoptosis or not depends on the relative abundance of pro- versus anti-apoptotic complexes. To investigate this in the context of the MBT, x-Bcl2 was re-cloned and x-Bax was cloned by PCR using degenerate primers based on mammalian Bax. mRNA encoding various combinations of FLAG-Bcl2, $(Myc)_7$Bcl2, FLAG-Bax, and $(Myc)_7$Bax were injected into embryos shortly after fertilization, and ionizing radiation was administered either before or after the MBT. It was observed that with ionizing radiation after the MBT relatively little Bcl2 was present in pro-apoptotic Bax immunoprecipitates. However, with administration of ionizing radiation before the MBT, a large fraction of Bcl2 was present in a pro-apoptotic Bcl2/Bax heterodimer. This suggests that one mechanism of resistance to apoptosis that develops at the MBT is a decrease in the relative proportion of pro-apoptotic Bax complexes.

Another key mechanism implicated in apoptosis in mammalian cells is activation of the protein kinase Akt, also known as protein kinase B. Akt can phosphorylate the pro-apoptotic protein Bad and impair its interaction with Bcl2. Activation of Akt occurs via a phosphoinositide 3-kinase pathway involving activation of phosphatidylinositol-dependent protein kinase 1 (PDK1) and phosphorylation at two specific residues in Akt (Vanhaesebroeck & Alessi 2000 for review). These sites have been conserved in *Xenopus* Akt, and phosphospecific antibodies to the phosphorylation sites, denoting active enzyme, react readily with x-Akt. Examining embryos irradiated before and after the MBT revealed that Akt activation occurred with ionizing radiation after the MBT, but not before, with no change in the total Akt level before and after the MBT. These results suggest that at least part of the resistance to ionizing radiation-induced apoptosis after the MBT is due to Akt activation. To assess the relative importance of this activation, newly fertilized embryos were injected with mRNA encoding constitutively active Akt and irradiated prior to the MBT. When assayed 5 h post-MBT, the fraction of embryos exhibiting apoptosis was reduced from over 90% to 36%. This indicates one important mechanism for prevention of apoptosis is Akt activation.

The MBT beginning with the 13th cleavage cycle marks the end of maternal cycles with a period of 26 min. Fluorescence-activated cell sorting (FACS) analysis of isolated nuclei indicates that cycles 14 and 15 have progressively longer S phases and G1 phase first appears at cycle 15, corresponding to Stage 12. At this time the $p27^{Xic1}$ cdk inhibitor is also up-regulated, as is the expression of xRb. The increased expression of Xic1 suggests that it could provide the basis for a 'cell cycle arrest' option to ionizing radiation after the MBT. To assess this directly, extracts from embryos irradiated before or after the MBT were immunoblotted for Xic1. It was observed that pre-MBT Xic1 expression was unchanged by IR, but in embryos irradiated post-MBT the expression of Xic was up-regulated much earlier. Whether this reflects transcriptional or post-transcriptional regulation remains to be determined. FACS analysis of nuclei from such post-MBT embryos revealed a larger fraction in G1 phase than in non-ionizing-radiation-treated controls. When specific cdk complexes were examined, the increased Xic1 was bound to both cyclin D1/Cdk4 complexes and cyclin E/Cdk2 complexes, both consistent with a G1 arrest. In addition, the cyclin E/Cdk2 complexes were also phosphorylated on Tyr15. These results suggest that part of the resistance to ionizing radiation-induced apoptosis after the MBT is due to cell cycle arrest afforded by increased expression of Xic1.

These results indicate that multiple mechanisms account for the resistance of post-MBT embryos to ionizing radiation (Fig. 3). The relative importance of these mechanisms for resistance is not yet fully clear. However, over-expression of x-Bcl2 in pre-MBT embryos is sufficient to completely block apoptosis, and as indicated above, over-expression of constitutively active Akt reduces or delays the

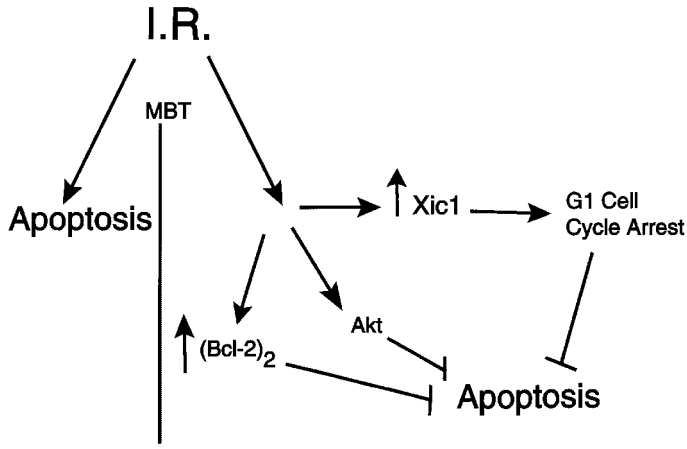

FIG. 3. Apoptosis regulation at the MBT. The figure denotes the relative resistance of the embryo after the MBT to apoptosis induced by ionizing radiation (IR, X-rays) and the multiple mechanisms implicated in this resistance.

onset of apoptosis and reduces the percentage of embryos that are apoptotic at any given time. Xic1 injection into pre-MBT embryos delays the onset of the MBT and increases cell cycle length (Hartley et al 1997), consistent with the G1 delay correlated with its up-regulation after ionizing radiation. In contrast to Xic1, no change in the abundance of x-Bcl2 or Akt is evident before or after the MBT. This suggests these elements needed for an anti-apoptotic response are not missing in pre-MBT embryos but rather in pre-MBT embryos either the signal from DNA damage is not sent until the MBT or is sent but not heard until the MBT. The mechanism that links these damage signals to these anti-apoptotic responses, after but not before the MBT remains to be determined. As mentioned above, oocytes and embryos contain a large store of maternal p53 protein (Tchang et al 1993). The protein is functional as judged by interaction with XMDM2, the suppression of tumours in embryos expressing mutant human p53, and degradation in the nucleus by the papilloma virus E6 ubiquitin ligase (Wallingford et al 1997, C. Finkielstein & J. Maller, unpublished data). However, to date injection of E6 into embryos has been insufficient to degrade all p53, precluding an evaluation of its importance in the response of embryos to ionizing radiation.

Summary

Xenopus oocytes and embryos present a model system for understanding the interface between cell cycle controls and developmental decisions. Maturation of

an oocyte into an egg is a developmental switch triggered by a hormone that encompasses precise controls on M phase entry and M phase exit. Pre- and post-MBT embryos exemplify a switch from maternal to zygotic control of the cell cycle. MBT embryos provide a developmental system to study the stepwise establishment of cell cycle checkpoints and the connection of signalling pathways to regulation of cell cycle progression. Further work in the *Xenopus* system should continue to provide fundamental insights into the interface between cell cycle control and developmental decisions.

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DISCUSSION

Raff: In pre- and post-MBT, was p53 involved in any of those responses?

Maller: That is a question I have been wanting to address. The apoptosis response can be driven wholly by a maternal programme that doesn't require transcription. If it is p53-dependent, it would be a non-transcriptional effect of p53. We haven't been able to completely get rid of p53. The most direct approach we have tried is to inject the papilloma virus E6 protein into embryos. This is an E3 ubiquitin ligase that degrades p53. There is something like 10 ng of p53 in every oocyte, so we can only get rid of half of this. Thus we haven't been able to answer the question of whether this response is p53 dependent or not.

Edgar: I'm mystified by this developmental timer that is independent of protein synthesis. How do you think this works?

Maller: I have some speculations. The other thing that goes on in an embryo that is independent of protein synthesis is centrosome replication. We showed last year that if cyclin E/Cdk activity is blocked, so is centrosome replication. One possibility is that the timer is actually monitoring centrosomes, as a sort of autonomous thing that replicates. The two things that are replicated in cells are the genome and centrosomes; very little is known about centrosome replication.

Raff: What is the time scale of these events?

Maller: About 5 h at room temperature.

Hunt: Do you have any idea what actually degrades cyclin E or Cdc25A?

Maller: We are assuming that it is a ubiquitin-mediated degradation. The only thing we have done directly has been with cyclin E. You may have noticed that when the degradation of cyclin E was delayed by injection of $p27^{Xic1}$, the cyclin E was shifted down. It is now clear that for the mammalian G1 cyclins, phosphorylation of a particular threonine targets them to the F box for degradation. This threonine is conserved in *Xenopus* cyclin E and its phosphorylation causes the gel shift *in vitro*.

Hunt: But if you mutate it it doesn't make any difference.

Maller: We have not directly compared them yet.

Hunt: We have looked at this and there is a lot of phosphorylation, but most of it is in the N-terminus and not the C-terminus. There are a lot of sites there.

Maller: Mutating that conserved threonine site does change the electrophoretic mobility of cyclin E. What is interesting here is that in the Xic-injected embryos, cyclin E is completely inhibited. It still gets degraded. It is not as though you start dribbling away the cyclin E in a gradual way. It is still a developmental degradation that occurs in a short period; it is just delayed by injection of Xic1.

Newport: Is Xic1 still present at the point where degradation occurs? Is this somehow being destroyed?

Maller: We haven't done that experiment, but cyclin E/Cdk2 is still inhibited when cyclin E is degraded. We have made a mutation in a particular threonine in Xic1 that is analogous to a threonine in mammalian p27 that targets p27 for degradation. If we make this mutant we get these effects with about 5% of the normal amount of Xic1, presumably reflecting a longer half-life. This would probably be the reagent to use for future experiments.

Newport: In the MBT if you trigger something that degrades the p27, it could just take longer for it to happen in Xic1 injected embryos.

Maller: There is no decrease in p27 at the MBT. Comparing wild-type and mutant Xic1 degradation would be a reasonable experiment to do. But p27 actually goes up dramatically during gastrulation.

Edgar: John Newport, I recall that you did some work on this timer. Do you have any speculations on how it might work?

Newport: I think there is a set of timers. We have actually characterized a couple of them, but we haven't published much on this. For instance, if you look at *cdc2* mRNA, this decays right at the MBT. This is completely autonomous of replication. It is autonomous of centrosomes, as well.

Edgar: Is it autonomous of transcription?

Newport: Yes, and our speculation is that at the time of fertilization a degradative pathway is activated which is starting to turn over proteins that prevent mRNA degradation from occurring. This protein, which is an inhibitor of RNA degradation, eventually runs out at around the MBT, thus allowing turnover of message. At the MBT, zygotic transcription is turned on and you want to remove the maternal programme.

Maller: Murakami and Vande Woude published a nice paper a couple of years ago where they took a Mos-arrested blastomere and re-started the cell cycle with Ca^{2+}, getting a new cycle of tyrosine phosphorylation equivalent to cycle one, even though the embryo was actually entering cycle two or three. This would suggest that MAP kinase sets the timer back to zero. Of course, MAP kinase is very high in the unfertilized egg, the cell in which the timer is activated.

Newport: We actually got some Mos from George at one point. That was the only thing we could find that would stop the timer once the eggs had been fertilized.

Nasmyth: Do you know whether Rsk is required to maintain the CSF? Can you deplete it?

Maller: This was done in Jim Ferrell's lab. They made a CSF extract that has Rsk activity. If Rsk is depleted, CSF activity is lost, and when Rsk is added back some activity is restored.

Nasmyth: Have you tried adding Rsk to the APC ubiquitin ligase assays directly?

Maller: As you know from Jan-Michael Peter's work, these are difficult assays to do. What we have done is to try to phosphorylate Cdc27 *in vitro*, both as a purified protein and as an IP from interphase extracts. The results are not terribly encouraging: we get some labelling, but it is not enough for me to believe there is a direct regulation of Cdc27 by Rsk.

Hunt: In the experiments you showed us, it is generally accepted that when Cdc27 is in its upper-phosphoryated state it is in its most active form, but your results were exactly the other way round.

Maller: No, it is more complicated than that.

Nasmyth: What you have is an arrest in an APC/Cdh1 active state.

Hunt: Except there is no Cdh1 there.

Maller: That is why we did the *in vivo* injection of labelled cyclin B, because I think that is the best way to ask about the activity of the APC, rather than trying to do IPs. IPs may wash off things that are crucial here. I think that our results argue that during meiosis it is the less shifted form of Cdc27 that reflects active APC, and that in CSF Cdc27 is shifted even higher than usual, correlated now with even lower APC activity.

Hunt: There is another complication here: it turns out that the cyclin A in CSF-arrested frog eggs is extremely unstable. The APC is certainly active in that situation, and the checkpoint that prevents the degradation of B-type cyclins can somehow recognize and spare the cyclin B, but has no effect on cyclin A.

Maller: Of course, cyclin B is being degraded at a certain rate: it is not zero.

Hunt: It is pretty slow.

Maller: The half-life is 30–45 min. If you consider that an egg can survive in the oviducts and in the water for 4 h during stable metaphase arrest, that is a couple of

half-life's worth of cyclin B. So there must be synthesis occurring. It is not as if APC is completely shut off.

Hunt: I think it is very mysterious. The other very mysterious thing is since we know that MAPK is required for entry into meiosis I, how come there is this round of degradation of what is presumably the Pds1 equivalent and the B-type cyclins between meiosis I and meiosis II?

Nasmyth: Why not?

Hunt: Because MAPK is active, so this should stabilize cyclin B.

Vande Woude: It is homologous in meiosis versus sister chromatids that CSF works on.

Lehner: Can you find CSF activity before meiosis I?

Vande Woude: That is defined by MAP kinase.

Hunt: The MAP kinase is actually necessary but not sufficient for CSF arrest and, by implication, something else is made in that interval. Jim Maller published some data (I don't think I'd agree with them) that cyclin E/Cdk2 may be part of the contribution. There certainly is a cohort of proteins that are made in that interval, one of which may be the vital additional component for CSF. But no one knows what it is.

Maller: The bulk of the evidence is against a role for cyclin E/Cdk2. Whatever the additional component made during meiosis II is must persist into the embryo, because when the MAPK pathway on its own is put into the embryo, the arrest occurs, but when it is put into meiosis I, the arrest doesn't occur.

Vande Woude: It is only in those cleavage embryos that we see this.

Hunt: But even that is more complicated: many people have reported that high MAPK levels in the first cell cycle will cause a G2 arrest.

Vande Woude: That is not true for somatic cells at all.

Maller: Our hypothesis is that CSF is at the kinetochore. George Vande Woude published some data showing that the kinetochore protein CENP-E has certain masked epitopes only in a CSF-arrested spindle and not in a mitotic spindle.

Vande Woude: That was true for both mouse and *Xenopus*. Even though the protein can be detected by western blot, it is occluded from recognition by antibodies at metaphase II arrest. We speculated that this was somehow uncoupling the interaction with microtubules, which activates some kind of checkpoint. This is the only place that this is seen. If you look at CENPE in somatic cells during M phase, it can always be detected.

Maller: The question is, does CSF affect the APC, or does it affect the spindle? I don't think we currently have the answer, but our data favour the APC as the target.

Thomas: What happens in the Rsk-deficient mice?

Vande Woude: We haven't looked. One would predict that this knockout is carcinogenic.

Maller: Null Rsk knockout mice are alive. The Coffin–Lowry syndrome is only found in *rsk2*-deficient patients.

Thomas: This is contentious. The *rsk2*-deficient mouse has no phenotype, apparently.

Maller: The mutations we made in *rsk1* to make it constitutively active don't work if you make them in *rsk2*. They are not totally interchangeable.

Kubiak: We found in mouse oocytes undergoing GVBD that Rsk is phosphorylated by two successive steps, one of which is dependent on Cdc2 and the following one on MAP kinase. There is also evidence of phosphorylation of Rsk by another kinase, which is Pdk1.

Maller: For the last 15 years since Rsk was discovered no one could activate it *in vitro* from a recombinant source. This turns out to be because in addition to MAPK, Pdk1 is required. There is no phosphorylation of Rsk by Cdc2. What we essentially made with our constitutively active construct is an enzyme solely dependent on Pdk1.

Kubiak: I show results in my paper (Kubiak & Ciemerych 2001, this volume) suggesting that double phosphorylation could regulate Rsk providing Rsk is indeed the CSF.

Nasmyth: Some Rsk researcher must have knocked it out in *C. elegans*.

Maller: I'm not aware of this. I know Gerry Rubin looked hard for Rsk mutations in the Sevenless pathway in *Drosophila*, without success.

Vande Woude: It is also involved in activation of MPF.

Maller: That's right. Nebreda reported that Myt1, a kinase that phosphorylates Cdc2 on both Thr14 and Tyr15, is phosphorylated by Rsk during MI and is inactivated. Moreover, there is a binding site on the C-terminus of Myt1 that binds to Rsk, so they form a kind of complex. All our experiments in resting oocytes where we have tried to modulate this system by playing with the kinases have not yielded very large effects. I don't know whether this is because the kinases are hard to work with, or just because Cdc25 is the key factor that is being regulated during meiosis.

Vande Woude: There again, Rsk is doing it in *Xenopus* but not in the mouse.

Raff: Do either you or John Newport have an explanation for why apoptosis occurs at the MBT, and not before?

Maller: Either the signal is not sent, or it is sent but not heard. You can irradiate at any time from the two-cell stage right up to the MBT (4000 cells), and get the same readout. This suggests that DNA content is not monitored. I would speculate that the system doesn't sense damage until the MBT.

Newport: Our feeling was that if you irradiate before the MBT, you haven't accumulated enough DNA, so when zygotic transcription begins you start clearing out internal messages and don't have enough zygotic transcription to replace them.

Raff: So a maternal inhibitor goes away?

Newport: Certain proteins are degraded and they are not being replaced; the cell interprets this as a time to go into apoptosis.

Maller: Our original model was based on data that J. Tata published showing that *bcl2* was one of the first genes transcribed by the embryo. This was a sort of default model where unless you produced zygotic Bcl2 you would apoptose. But when we made antibodies to Bcl2 we found that there was plenty of it present in the oocyte.

Gautier: The idea we have is that there is a maternal inhibitor present. Is it something that is very general that is regulating the degradation of many maternal RNAs, or is it something specific for apoptosis? This is not clear.

Maller: What is clear is that we can take an egg extract, add cytochrome C and induce apoptosis quickly. If the inhibitor is there it is not very dominant.

Reik: Is anything known about what happens during nuclear cloning? If a somatic nucleus is put back into the egg, are these cell cycle controls disrupted?

Maller: I don't know of any data on that directly, but the egg cytoplasm instructs the nucleus. John Gurdon has shown 30 years ago that if you put a somatic nucleus that is making RNA into an egg, it will cease making RNA and start making DNA like the egg is doing with its own nucleus.

Reference

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Cell cycle regulation in early mouse embryos

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Abstract. For a long time it has been thought that the cell cycles of the early mouse embryo do not differ from the somatic cell cycle. They are long and are composed of classical G1, S, G2 and M phases and have functional checkpoint controls. However, a few characteristics observed during the earliest mitotic cleavage divisions suggest that the embryonic cell cycle could differ significantly from the somatic ones. Understanding these differences could have an important impact on our understanding of both general cell cycle mechanisms as well as the developmental programme of the early mouse embryo. Over the last few years our laboratories have undertaken a project focused on describing the differences in the first two cell cycles of the mouse embryo. We discuss here the results concerning (1) the way mouse oocytes switch from the meiotic to the mitotic cell cycle upon activation of development (inactivation of the cytostatic factor, CSF); (2) how the entry into the first and the second mitotic M phase is regulated (nucleus-independent activation of M phase-promoting factor, MPF); and (3) how the duration of the early embryonic mitoses is regulated. These data show that developmentally regulated phenomena are superimposed on and highly coordinated with the cell cycle machinery.

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Special control of the early embryonic cell cycles in the early mouse embryo

The first cell cycle of the mouse embryo differs in many respects from the second and the following cell cycles. It is characterized by a long G1 phase that starts after the penetration of the spermatozoon or artificial activation of the oocyte. During this period the chromatin of the oocyte completes the second meiotic division and forms the female pronucleus. At the same time, in the fertilized egg, the highly condensed chromatin of the sperm nucleus decondenses and sperm-specific proteins, protamines, are replaced by histones. After the initial sperm chromatin

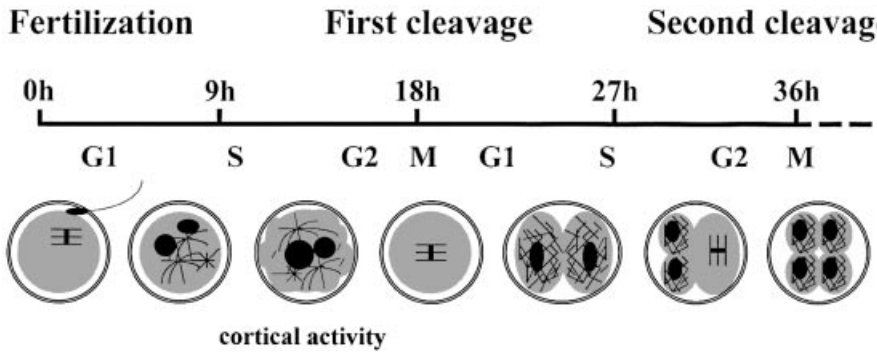

FIG. 1. Timing and morphology of mouse embryos during the first two cleavages. The cortical activity of the one-cell embryo begins during late G2 phase shortly before the entry into the mitotic M phase. Scheme represents: shapes of embryos and morphology of their chromatin and microtubule cytoskeleton.

decondensation, it recondenses (Borsuk & Mańka 1988) and the interphase male pronucleus is formed. The two pronuclei are assembled along two different routes and remain separated in the egg cytoplasm during the S and G2 phases until they enter first mitosis and their chromosomes align at the common metaphase plate forming a single metaphase spindle. The first mitotic division is usually completed 15–18 h after oocyte activation. The second cell cycle is as long as the first one, however the G1 phase is very short and the S phase starts soon after the first mitotic division is completed (Bolton et al 1984). The progression of the first cell cycle is under maternal control since the major activation of the embryonic genome takes place at the two-cell stage (Flach et al 1982) and it is independent of nuclear control (Waksmundzka et al 1984, Ciemerych 1995; Fig. 1). All these one-cell embryo-specific phenomena require a special control and coordination between the cell cycle machinery and the developmental programme triggered by oocyte activation. We do not know much about how this coordination is achieved and only start to understand mechanisms governing this period of exceptional importance for further embryonic development.

Switch from the meiotic to the mitotic cell cycle

The metaphase arrest in vertebrate oocytes is maintained by cytostatic factor activity (CSF; Masui & Markert 1971). CSF was first demonstrated in amphibian oocytes by transferring cytoplasm from metaphase II (M II)-arrested oocytes into one blastomere of a two-cell embryo. The injected blastomere was arrested in M phase while the non-injected blastomere continues to cleave (Masui & Markert

1971). We have demonstrated the presence of an equivalent activity in mouse M II-arrested oocytes using a cell fusion technique (Kubiak et al 1993). CSF was defined as an activity that stabilizes the M phase-promoting factor (MPF; Masui & Markert 1971). The proto-oncogene c-*mos* gene product (Sagata et al 1989) was the first molecule implicated in the CSF activity. Further studies have shown that Mos is the MAP kinase kinase kinase that activates the MAP kinase pathway during meiotic maturation (Posada et al 1993, Nebreda & Hunt 1993). Activation of MAP kinase involves its phosphorylation on threonine and tyrosine residues (Posada & Cooper 1992) which correlates in mouse oocytes with a shift in the electrophoretic mobility of the two isoforms of MAP kinase, ERK1 and ERK2 (for extracellular regulated kinase; Verlhac et al 1993, 1994). After activation of M II oocytes, MAP kinase activity drops progressively during 3–4 h post-activation (Verlhac et al 1994). Mos is destroyed after MPF inactivation when MAP kinase activity drops (Weber et al 1991).

MPF is heterodimer composed of a catalytic subunit, the $p34^{cdc2}$ kinase, homologue of the fission yeast *cdc2* gene product, and a regulatory subunit, cyclin B. Since inactivation of MPF requires the proteolytic degradation of cyclin B, CSF may act by preventing cyclin B degradation. In mouse oocytes, both cyclin B degradation and synthesis were detected during the M II arrest (Kubiak et al 1993, Winston 1997) suggesting that CSF only partially inhibits the cyclin B degradation machinery in this species. The inactivation of CSF upon oocyte activation would facilitate proteolysis of cyclin B (Kubiak et al 1993, Winston 1997). However, CSF inactivation upon oocyte activation has never been observed directly and was only anticipated from the behaviour of MPF and the activity of the cyclin B degradation machinery. Fertilization or parthenogenetic activation of oocytes triggers a transient increase in the cytoplasmic free Ca^{2+} (Miyazaki 1988) and leads to the release of the meiotic arrest by inactivation of MPF (Lorca et al 1993) and CSF (Watanabe et al 1989). However, the detailed analysis of the dynamics of MPF and CSF inactivation in *Xenopus* oocyte has led to an apparent paradox: CSF activity was still detectable after MPF inactivation (Watanabe et al 1991). Does CSF inactivation follow the inactivation of MPF? Such a sequence of events contradicts, however the role of CSF in stabilization of MPF. Degradation of Mos and inactivation of MAP kinases and their substrates (e.g. $p90^{rsk}$) also follow inactivation of MPF in mouse oocytes (Weber et al 1991, Verlhac et al 1994, Kalab et al 1996). Is therefore CSF inactivation parallel to inactivation of the MAP kinase pathway? To find the answer to this question we investigated the dynamics of CSF activity after mouse oocyte activation using a biological assay based on a cell fusion method.

Parthenogenetic one-cell embryos were fused very soon after an activating treatment with parthenogenetic one-cell embryos entering first mitosis. We expected that hybrids obtained in such experiments would either arrest in M-

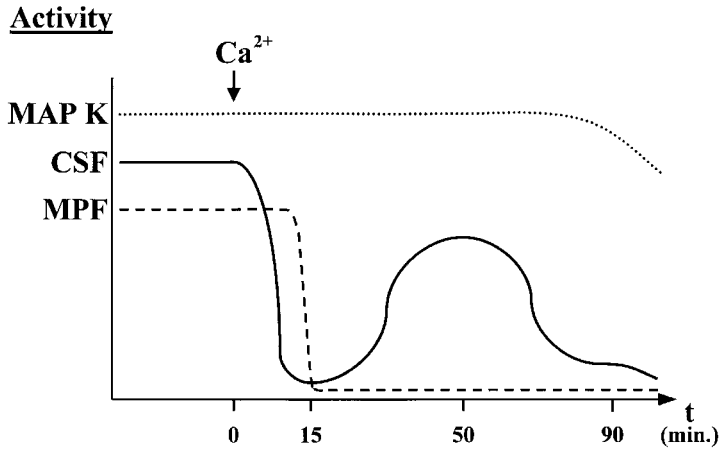

FIG. 2. Relationship between activities of CSF, MPF and MAP kinase during the first 90 min after fertilization of the mouse oocyte. Data from Ciemerych & Kubiak (1999), Szöllösi et al (1993), Verlhac et al (1994).

phase or cleave and enter interphase of the second embryonic cell cycle. If freshly activated oocytes had CSF activity they should stabilize the MPF present in mitotic embryos. If these oocytes did not contain CSF activity, the hybrids should complete mitosis and enter interphase within 1–2 h, as control mitotic embryos. Analysis of cell cycle arrest or cell cycle progression in the hybrids demonstrated that CSF activity decreased dramatically within 20 min after oocyte activation. Then it increased (attaining 60% of the activity observed in M II-arrested oocytes 50–60 min after activation) and finally decreased steadily (Fig. 2). Moreover, we found that MAP kinases ERK1 and ERK2 remained active in M phase-arrested hybrids and were inactive in those that completed cleavage division and entered interphase of the second cell cycle (Ciemerych & Kubiak 1999).

The results show that CSF activity fluctuates after oocyte activation. Inactivation of CSF proceeds in two steps: first, CSF is transiently down-regulated by a mechanism independent from Mos degradation and MAP kinase inactivation to allow exit from the M II arrest. Second, the disappearance of CSF activity after the transition to the first embryonic cell cycle requires inactivation of the MAP kinase pathway.

The relationship between MAP kinase activity and/or its direct substrates (e.g. $p90^{rsk}$) and CSF inactivation is unclear. Abrieu and colleagues (1996) found that in cell-free *Xenopus* eggs extracts MAP kinase remains active when MPF is inactivated. Thus, despite the fact that MAP kinase remains continuously active and phosphorylated, the CSF activity seems to be inactivated. A similar pattern

of uninterrupted MAP kinase activity also takes place in mouse oocytes upon activation (Verlhac et al 1994, Kalab et al 1996). Low CSF activity, when MAP kinase remains fully active, suggests that CSF activity at the time of activation is regulated by a mechanism independent from MAP kinases. Transient inactivation of CSF upon oocyte activation could therefore imply a down-regulation of the Mos/.../MAP kinase pathway downstream from the MAP kinases. Since Ca^{2+} transients trigger mouse oocyte activation (Cuthbertson et al 1981) it is likely that a Ca^{2+}-dependent mechanism is involved in the modulation of CSF upon oocyte activation. This is in agreement with the role postulated by Abrieu et al (1996) for Ca^{2+}/calmodulin-dependent protein kinase II in CSF and MPF inactivation in *Xenopus* egg extracts. A transient activation of the Ca^{2+}/calmodulin-dependent protein kinase II has also been observed in mouse oocyte at the time of activation (Winston & Maro 1995).

The very beginning of the first mitotic cell cycle of the mouse embryo seems to be controlled by the mechanisms characteristic for both meiotic and mitotic cell cycles. Active MAP kinase, its substrate $p90^{rsk}$ and the CSF activity itself could influence the cellular processes within the one-cell embryo. Indeed, we have observed that despite the entry into the interphase (as judged by the low activity of MPF) some proteins are actively phosphorylated as during the meiotic M phase (e.g. 35 kDa complex; Howlett et al 1986, Szöllösi et al 1993), the nuclei and the microtubule interphase network start to form only 1.5 hours after activation (Szöllösi et al 1993). This delay in the phenomena characteristic for the interphase could be linked to the mixed meiotic/mitotic character of this early period. This delay probably allows the correct transformation of the sperm nucleus into the male pronucleus. In species like *Xenopus* or *Drosophila* the transitional period between the meiotic and the mitotic cell cycle control is probably much shorter since it is proportional to duration of the short first cell cycle of these rapidly cleaving embryos. Mammalian embryos are perhaps the most suitable to study this transition because of the exceptionally long first embryonic cell cycle.

Regulation of the entry into the early mitotic M phases

The entry into the first mitotic M phase at the end of the first embryonic cell cycle requires activation of MPF. In the mouse one-cell embryo this activation is fully autonomous from the nucleus (Ciemerych 1995, Ciemerych et al 1998). It proceeds within the cytoplasts obtained either by enucleation or by bisection of the embryo. Other autonomous phenomena are the cortical activity, or the deformation of the one-cell embryo, directly preceding the entry into first mitosis (Waksmundzka et al 1984) and the cyclic activity of K^+ ion channels (Day et al 1998). The role of the cortical activity remains unknown; however, the fact that it directly precedes the entry into the first mitotic M phase suggests that it could be linked to the activation

of MPF. The presence of autonomous activities expressed only during the first embryonic cell cycle suggests that the mechanisms controlling this particular cell cycle would be modified in comparison to the next cell cycles. Chromosomes participating in the first embryonic division come from two separate pronuclei in contrast to chromosomes originating from a single nucleus in blastomeres. The autonomy of the cytoplasm is typical for the oocyte, suggesting that its presence in the zygote could be the remainder of the meiotic cell cycle.

We studied the autonomous activities in bisected one-cell embryos and two-cell blastomeres obtained at G2 of the respective cell cycles. MPF activity rises in anucleate one-cell embryo halves during the cortical activity period and achieves maximum while the embryo halves become smooth (Fig. 3A). The level of MPF activity (as measured by histone H1 kinase activity) in the anucleate one-cell embryo halves reached 35% in halves undergoing the cortical activity and rose to 46% in the halves which rounded up, as compared to the activity observed in the nucleate halves obtained by cutting the mitotic one-cell embryos (100%). Surprisingly, during the second cell cycle the anucleate halves also activate the MPF, albeit to a much lower extent than during the first cell cycle (maximum 27%; Fig. 3B). Neither two-cell blastomeres nor their halves undergo any cortical deformations. The mitotic depolymerization of microtubules took place in one-cell anuclear halves which attained 46% of histone H1 kinase activity, while it was never observed in the two-cell blastomere anucleate halves activating only 27% of the histone H1 kinase, as well as in cortically active anuclear halves activating 35 % of histone H1 kinase activity. This enabled us to quantify the window in the degree of the MPF activity necessary to induce mitotic phenotype of microtubules (Ciemerych et al 1998).

These data show that the autonomous activation of MPF proceeds in the one-cell and the two-cell embryos. However, this phenomenon is less intense during the second cell cycle. It suggests that the autonomy of the embryo cytoplasm diminishes progressively beginning from the activation of the developmental programme. This could have a link to unsuccessful remodelling of foreign nuclei introduced into to zygote cytoplasm (McGrath & Solter 1984), in contrast to much more efficient remodelling within the oocyte cytoplasm (Wakayama et al 1998) in cloning experiments.

Regulation of the exit from the early mitotic M phases

The duration of the M phase is largely determined by the time necessary for the formation of a functional metaphase spindle and the correct alignment of all chromosomes in the metaphase plate. The spindle assembly checkpoint prevents the exit from the M phase before the proper alignment of all chromosomes into a metaphase plate in many cell types. This kind of control is already operational

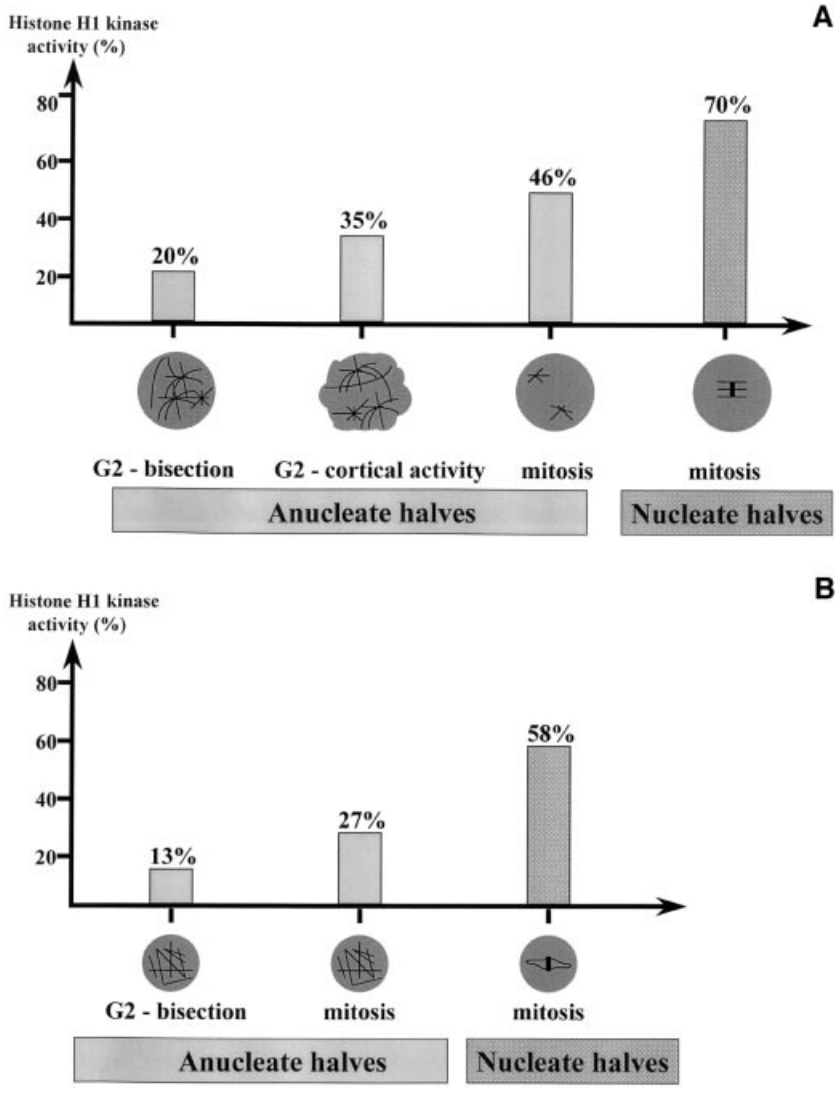

FIG. 3. Histone H1 kinase activity and schematic representation of the morphology of one-cell mouse embryos (3A) and two-cell stage blastomeres (3B) bisected at the respective G2 phases. Note that histone H1 kinase activity rises autonomously in anucleate halves of both embryos and blastomeres. However, the degree of the autonomous activation is lower than in their nucleate counterparts. Activity detected in nucleate halves obtained during respective M phases was taken as 100%. Note that the nucleate halves obtained at their respective G2 stages do not activate histone H1 kinase to the levels observed in the halves obtained in the M phase, and that the mitotic disassembly of microtubules was observed only when the level of histone H1 kinase was between 35% and 46% in anucleate halves.

during the first embryonic cell cycle in the mouse. The first mitotic M phase of the mouse embryo lasts about 119 min, while the second embryonic M phase lasts only about 70 min and the third one 60 min (Ciemerych et al 1999). Therefore, we focused our attention on the regulation of the exit from the first and the second mitosis asking what mechanism(s) delays the exit specifically during the first mitotic M phase?

MPF is activated rapidly during the nuclear envelope breakdown in both the first and second mitosis. Its maximum, however, is followed by a plateau only during the first mitosis. Relatively higher stability of MPF is observed in the cytoplasts obtained from mouse embryos during the first embryonic M phase than during the second one. This stability is largely increased by the presence of the mitotic apparatus in both M phases (karyoplasts). The mitotic spindle assembly during the first and the second mitoses differs. The first metaphase spindle is stabilized during the period of the maximum of the MPF activity.

Interestingly, prolonged duration of the first embryonic M phase is also observed in other mammalian and non-mammalian embryos. It was found in rabbit embryos (X. Yang & M. Deng, personal communication) and in sea urchin embryos (J. Z. Kubiak & P. Cormier, unpublished observation). Further studies will show whether it is a rule during animal development.

To understand the molecular background of the prolongation of the first embryonic M phase we studied cyclin A2 behaviour in the embryos. Cyclins A are known to be the first cyclins degraded before the exit from the mitotic M-phase in the somatic cells. Two A-type cyclins (A1 and A2) were identified in the mouse embryo. Cyclin A1 is not essential for the embryonic development (Liu et al 1998) while cyclin A2 seems to be essential since the embryos deprived of cyclin A2 gene products die after a few cleavages (Murphy et al 1997). We found that during the first embryonic M phase, cyclin A2 is stable and only partially degraded upon cytokinesis. It remains stable and abundant during the second mitotic metaphase as well. However, it is totally destroyed during the second cytokinesis. The stability of cyclin A2 during the first two embryonic mitoses might be linked to the unique mechanism operating in early mouse embryos in contrast to somatic cells. We postulate that a cyclin A2-dependent mechanism stabilises MPF and delays metaphase to anaphase transition. This mechanism enables the metaphase state to be maintained and therefore operates downstream from the spindle assembly checkpoint (Fig. 4).

Do later embryonic cell cycles differ from the somatic ones?

Cyclin A2 knockout mice are not viable (Murphy et al 1997). However, a few early embryonic divisions proceed despite the absence of any cyclin A2 gene product in homozygous −/− embryos obtained by crossing +/− heterozygotes (Winston et al

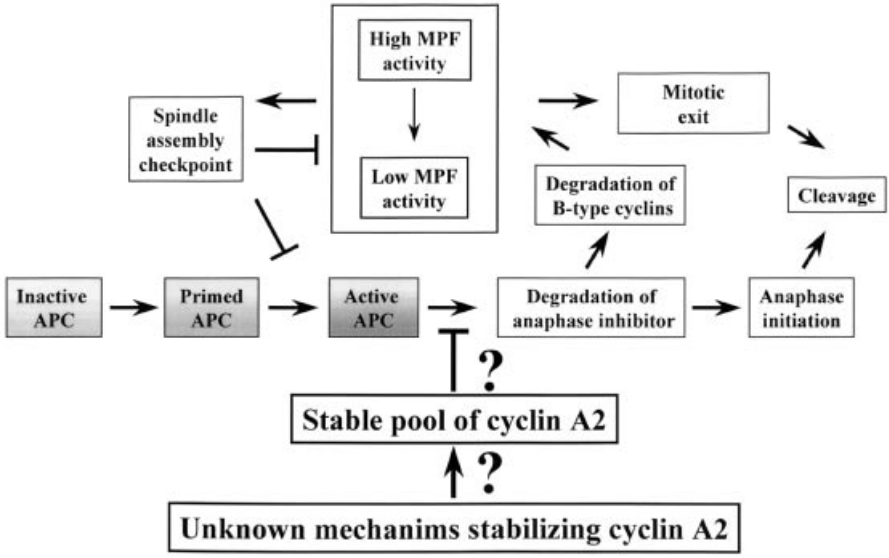

FIG. 4. Hypothetical action of the cyclin A2-dependent mechanism retarding exit from the first embryonic M phase in the mouse.

2000). This suggests that cyclin A2 is not essential for the early embryonic cell cycles. Also D-type cyclins seem to be dispensable for the early mouse embryo cell cycle progression since embryonic stem (ES) cells do not express them at all before differentiation (Savatier et al 1996). We do not know, however, whether the D-type cyclins are also absent in the early embryo. These observations suggest that not only could the first cell cycles of the mouse embryo have specific modifications, but also further embryonic cell cycles are specifically modified as well. Mammalian embryonic cell cycles are probably modified often during development. Such studies could allow us to determine a profile of a minimal cell cycle in mammals which must, however, be much more complex than a simple S:M phase embryonic cell cycle of amphibians or insects.

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DISCUSSION

Gönczy: In the normally fertilized egg there is a centrosome. However, you are saying that the spindle is assembled using a non-centrosomal pathway in the one-cell stage mouse embryo. This seems surprising to me in light of Rebecca Heald's work, which shows that the centrosomal pathway of spindle assembly is dominant over the non-centrosomal one (Heald et al 1997).

Kubiak: There is a puzzling phenomenon observed in mammalian eggs. The spermatozoon brings a centriole that is disorganized and does not participate in

division spindle formation, at least in the mouse embryo, until the fourth or fifth cleavage division. The first cleavages are acentriolar. Entirely maternal centrosomal material is sufficient for normal formation of the spindle. Parthenogenetic mouse embryos cleave normally and develop perfect centrioles and centrosomes.

Gönczy: What makes the centrosome mature and competent again?

Kubiak: We can only speculate. In different mammals the centrioles appear at different times. We do not know whether pericentriolar material (PCM) foci duplicate like mature centrosomes. Do they preserve any structure? We don't know, although we find them amorphous at the electron microscope level.

Vande Woude: I thought the mouse was different from other mammals. Many of the others use the sperm aster to bring the pronuclei together.

Nasmyth: I was interested by the long mitotic arrest you showed. Perhaps you should get together with Peter Sorger, who has knocked out *Mad2* in mouse (Dobles et al 2000). One of the early phenotypes he saw was a failure to arrest when nocadazole was added.

Kubiak: Indeed, but given that it is a checkpoint which is activated at that time, there must be a reason to activate this checkpoint delay in the first mitosis and not in the second one. Whatever the mechanism is, it is maternally determined and it is specific for this particular mitosis. This is the only mitosis that depends entirely on maternal genes in the mouse embryos, so we must look for a mechanism triggering a checkpoint delay.

Hunt: I thought that the maternal mRNA was degraded even before the first mitosis. Is that not the case?

Kubiak: No, the majority of maternal mRNA is only degraded before the second mitosis. There are some mRNAs, however, that persist until the blastocyst stage.

Maller: So there was a transient inactivation of CSF. Have you looked to see if MAPK or Rsk are transiently deactivated?

Kubiak: We didn't see any change in the MAPK activity at that moment. Marcel Dorée has looked carefully in *Xenopus* and concluded that MAPK was still active at the moment of inactivation of MPF.

Maller: What might change that story are the phospho-specific antibodies for Rsk and MAPK, because they can pick up changes of just a few percent. The CSF on kinetochores may require only a tiny pool of those enzymes for regulation.

Reik: Can you comment on the spatial segregation of chromosomes during the first mitosis that you mention? The Mayer et al (2000) study shows this very clearly. We have also carried out the same kind of immunofluorescence studies and we don't see that. This may need some reassessment with other markers. The methyl C antibody may not be the best thing to use.

Kubiak: Recognition of paternal and maternal chromosomes was a nice explanation for the prolongation of the first mitosis in the mouse, but

surprisingly we found the first mitosis is also longer in invertebrates such as sea urchins. Pierre Gönczy told me that the first mitosis might be slightly longer in *Caenorhabditis elegans*, too. In these cases the imprinting hypothesis didn't really fit. What we would like to do now is to find a suitable model in which the first mitosis is exceptional, and to identify mutants. Why not in *C. elegans*? Then we would look back to the mouse.

Vande Woude: Certainly it is that way in *Xenopus*. Monica Murakami has published two couple of papers showing that the MAPK activity, prolonged after the p34 kinase destruction, is key to lengthening the first cycle to 60 min instead of 30 min.

Hunt: I don't think she would claim what Jacek Kubiak is claiming, that the first mitosis is a particularly long one. It is a prolongation of the interphase, not the mitosis.

Kubiak: I don't have any data about the differential regulation of duration of mitoses during development except for the 14th and 15th mitosis in *Drosophila*, where there is a difference (Foe & Alberts 1983).

Edgar: The first meiosis in *Drosophila* is also very long.

Nasmyth: The chromosomes are all in a twist. Have you attempted to look at Cdc20? Is this taking a while to accumulate?

Kubiak: No, we didn't look at that.

Maller: Why do you say that the first spindle poles are made of PCM? Is there some reason to think that PCM is needed without a centrosome? In *Xenopus*, I have never heard anyone argue that there is PCM organizing the meiotic spindles. If you look at the γ-tubulin staining pattern, it is totally different in a meiotic spindle than in a mitotic spindle.

Kubiak: In *Xenopus* it was recently shown by Peter Kalab that there is also the possibility of forming the bipolar structures without chromosomes (Kalab et al 1999).

Maller: I don't argue with that, but why do you say 'PCM'? You are saying that one part of the centrosome is still operating in the first cycle, but not the centriole part, which comes from the sperm.

Kubiak: I don't know what happens to the centriole material that is brought by the sperm. What we detect at the spindle pole is a material that is reactive to γ-tubulin or MPM2 antibodies. We call it PCM because the same material accompanies centrioles in other cells. All that we know is that there are plenty of these small spots in mouse oocytes also out of the spindle and that they can be gathered to opposite places with respect to the chromosomes. Moreover, as I mentioned, we have shown recently that this also happens in the absence of chromosomes (Brunet et al 1998).

Vande Woude: You see lots of these forming in meiosis I in the mouse.

Kubiak: It varies from experiment to experiment.

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General discussion II

Regulation of *Drosophila* imaginal disc growth by the insulin/IGF signalling pathway

Leevers: I would like to give a brief summary of what is known about the role of signalling by the insulin/insulin-like growth factor (IGF) pathway during the growth of *Drosophila* imaginal discs, and then contrast this with what we have heard in the previous papers in this book. There is a pathway in *Drosophila* that is highly homologous to the insulin/IGF signalling pathway in mammals (Fig. 1 *[Leevers]*). A number of different labs have studied various genes on this pathway (Edgar 1999, Leevers 1999, Lehner 1999). The pathway is activated by an insulin/IGF receptor homologue, Inr, which autophosphorylates and phosphorylates an insulin receptor substrate protein, Chico (Chen et al 1996, Bohni et al 1999). This results in the activation of a class IA phosphatidylinositol-3-kinase (PI3K) made up of a catalytic subunit (Dp110) and adaptor subunit (p60) (Leevers et al 1996, Weinkove et al 1999). In addition, at least two downstream components have been identified and studied: they are the serine/threonine kinases, DAkt1 and DS6K, and the *Drosophila* p70S6 kinase (Verdu et al 1999, Montagne et al 1999). Akt has multiple targets in mammalian systems, though we don't know which are the relevant ones for its role in growth regulation in *Drosophila*. p70S6 kinase has a specific role and increases translation of a specific family of mRNAs, many of which encode ribosomal proteins.

We have done a number of experiments using an approach similar to that taken by Bruce Edgar to investigate the function of this pathway, by manipulating the activity of the PI3K, Dp110/p60. For example, we have made clones of cells overexpressing active or dominant negative transgenes and looked to see what happens to clonal growth (mass increase) and cell size in the imaginal disc as it is developing. These experiments have shown that if you increase signalling via this pathway, you increase growth rate. They have also shown that increasing signalling via this pathway increases cell size (Weinkove et al 1999). If the activity of this pathway is manipulated in a region of or throughout the imaginal discs, the final size of that imaginal disc is altered as is the size of the adult organ that it gives rise to (Leevers et al 1996).

To summarize, modulating signalling by this pathway alters three parameters: growth rate, cell size and organ size. What is unclear is the nature of the relationship

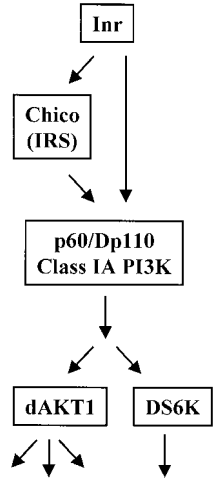

FIG. 1. (*Leevers*) The insulin/IGF signalling pathway in *Drosophila*.

between these parameters. Simplistically, these results suggest that when growth rates are altered, cell division is unaffected, so the altered growth rate alters cell size. Likewise, if you alter growth and the developmental programme and patterning machinery are unaffected, then the altered growth rate alters organ size. However, there are several examples of the way in which other molecules modulate growth, which suggest that this explanation of the results is too simplistic.

Firstly, Bruce Edgar has shown that if Myc or Ras activity is increased in the imaginal discs, then like the insulin/IGF pathway, this increases growth rate and cell size (Prober & Edgar 2000, Johnston et al 1999). In contrast to the insulin/IGF pathway, when Ras and Myc activity is increased, organ size is not altered. Somehow the imaginal discs seem to accommodate the changes in growth induced by Ras and Myc. Presumably compensatory apoptosis or effects on cell division may ensure that correct final organ size is achieved. Secondly, Bruce Edgar and Christian Lehner have spoken about thickveins and cyclin D/Cdk4. Overexpressing these molecules promotes growth (i.e. increases clonal growth) but does not affect cell size. Overexpressing these molecules also increases final organ size. Thirdly, if growth rate is altered by slowing global protein synthesis, cell size and organ or organism size are not necessarily affected. This observation is based on studies of a class of mutations in *Drosophila* called Minutes. Minute genes, when mutated, dominantly slow growth. Not all of them have been cloned, but those that have are ribosomal genes. Heterozygous Minute organisms grow slowly and are developmentally delayed, but in most cases the cells in the imaginal discs of these organisms do reach appropriate sizes. Likewise, Minute organisms usually

reach the right final size, so there seems to be a coordinated slowing of growth and development.

Hunt: How much longer to they take to get there?

Leevers: A two or three day extension of the larval period (when disc growth occurs) is typical. The larval period is normally four days.

Thomas: They are usually hypomorphs.

Nasmyth: Have you or anyone else looked at the epistasis of these effects? PI3 kinase affects all three parameters: growth rate, cell size and organism size. It could be that it independently goes for all three things — this would be the simplest null hypothesis. The prediction of that would be that if you over-produce PI3K in a Minute background or Cdk4 background, you should still get this dramatic effect on cell size and organ size.

Leevers: If you did that experiment you would probably find that PI3K still increases growth rate. I wouldn't predict that a Minute mutation would kill the effect that PI3K has on growth.

Nasmyth: You would predict, though, that it would still produce a big fly, even though it took longer to get there.

Edgar: I would agree with that.

Bryant: Minutes have small bristles. This would be an affect on cell size.

Leevers: That is true. Most of what we are looking at here in the discs is growth in mitotic cells. Bristle size may mainly reflect post-mitotic growth.

Edgar: Some of the Minutes have been reported to give small cell phenotypes in adults. We have looked at a number in discs before the cells differentiate, and we have not seen any effects here.

Hunt: What happens if you just put a low dose of cyclohexamide in their food?

Leevers: I expect you would get a Minute phenotype. That is what happens if you put rapamycin, which inhibits p70S6 kinase activation, into their food.

Thomas: We have identified a mutation in the *Drosophila* S6 kinase (DS6K) in which we obtain a few adults. These flies have smaller cells and the animal is about 50% smaller. Initially we were quite happy with this phenotype because we knew from work that we had done in mammalian systems that this kinase was involved in regulating the expression of ribosomal protein (Rp) messages, which would have a direct effect on translation. We then read Bruce Edgar's paper describing cell cycle mutants and realized that Minute cells are not smaller. Therefore one can distinguish two events. In the DS6K mutants the rate of growth is slowed down quite dramatically. The flies that emerge are quite sick, and only a few survive. They are also quite lethargic. Thus these flies are delayed due to a decreased rate of growth. The second event we need to explain is why the cells of these mutants pass through the cell cycle at a smaller cell size. We thought initially that this had something to with translation rather than the kinase regulating a cell cycle checkpoint for cell size. Indeed, a more simple explanation

might be that unlike the Minute, in which only the expression of an Rp message is suppressed, which is then rate-limiting for ribosome biogenesis, in the S6 kinase mutants all Rp mRNAs are being suppressed. The Rp mRNAs are represented by a small gene family, but they can make up 20–30% of the cell's total mRNA. One must also consider that mRNA is in excess over ribosomes. So these Rp mRNAs essentially monopolize the translation machinery. The Rp mRNAs are characterized by this polypyrimidine tract at their transcriptional start site. This tract acts as a repressor, not an enhancer, in the sense that if you change the first C to an A the Rp mRNAs are mobilized into polysomes. What started to emerge for us was the idea that if you suppress the translation of these mRNAs, ribosome biogenesis is decreased, which will account for the slow growth rate. But how does the smaller cell size arise? What we think is happening is that by taking these Rp mRNAs away, you favour the translation of mRNAs which don't have a tract. If these mRNAs encode a cell cycle regulator, which in general have long 5′-untranslated regions and very short half-lives, their expression would be favoured. This would then lead to a cell cycle regulator reaching a critical concentration at a smaller cell size. Interestingly enough, in development, at least in the frog and the fly, ribosome biogenesisis is dramatically up-regulated during oogenesis. In the fly, approximately 50 billion ribosomes are made in a few hours at stage 10 of oogenesis. As soon as embryogenesis ensues, they move off polysomes and are stored in mRNP particles, not degraded, until the completion of embryogenesis. During embryogenesis, ribosome biogenesis is suppressed, i.e. when the cell is passing through multiple rounds of cell division with no growth. The explanation by default was rationalized from an experiment performed by Jim Maller's group. They showed that if you treat *Xenopus* oocytes with a rapamycin, it induces precocious maturation in the presence of progesterone. They saw that this correlated very nicely with the induction of Mos, and they showed that they could block this effect by microinjecting a rapamycin-resistant allele of the S6 kinase. They then examined mitotic cell cycles, showing that addition of rapamycin caused a more rapid and robust rise of Cdc25. We think then growth is antagonistic to proliferation, and maintains its dominance through monopolizing the translational machinery. Thus ribosome biogenesis is initially acting as an antagonist of proliferation, because Rp mRNAs are simply monopolizing the translational machinery when you stimulate cells to grow. Indeed, in a proliferating cell, about 80% of its energy is being used for ribosome biogenesis. As the cell begins to accumulate ribosomes, the pathway begins to desensitze, Rp mRNAs are diluted, and the cell can begin to translate other messages. I wonder whether this might be an explanation for the fact that in the experiments described by Bruce Edgar, Myc drives expression of cyclin E protein without increasing *cyclin E* mRNA. What may happen is that by driving Myc you are driving ribosome biogenesis as the pool of ribosomes increases, you begin to

translate mRNAs such as cyclin E, which are generally not well recognized by the translational apparatus.

Raff: Among other things, this raises the question of whether cells sense size at all, or sense something else. What is the evidence that an animal cell monitors its size?

Edgar: There is rather little. In the disc cells that we have studied, we initially assumed that they coupled their cell cycle to their growth rate, but on closer examination we found that they have a variable size. They start out large and they get smaller with each division as they go through development.

Raff: Many of these experiments that suggest that cells have a size threshold are done in culture, with saturating amounts of extracellular signals that stimulate cell growth and cell cycle progression. In these conditions, cells will tend to be the same size, but these signals are not saturating *in vivo*.

Nasmyth: There is a classic experiment to address whether there is size control. That is to transiently delay the cell cycle so that you produce abnormally large cells. Then you look at the cell division time of subsequent cycles. If these experiments are done with bacteria or yeast, the subsequent cycles are greatly accelerated. These experiments could be done by Bruce Edgar and his imaginal discs. They are technically harder, but they could be done.

Edgar: This would show that there is an equilibrium of some sort at that normal cell size, but does this mean that cells are measuring their size?

Hunt: Martin Raff, you say that cells are variable in size, but my impression is that if you look at a bit of liver or skin, for example, cells are not that variable.

Raff: I think they are actually surprisingly variable. What would you consider to be the most definitive experiment showing that yeast cells care about their size?

Nasmyth: Wee1 was discovered as a size mutant.

Raff: But what is this set point, if not the level of some cell cycle regulator?

Nasmyth: In the case of the wee1 system, not very much is known about how fission yeast is doing it. In budding yeast, it is the level of Cln3.

Raff: So is that size?

Nasmyth: The level of Cln3 is a pretty good metric for cell size.

Edgar: It is not measuring cell size.

Nasmyth: Well of course it's not getting a ruler out and measuring it!

Raff: That is my point: you talk about cell size when you mean Cln3.

Leevers: Perhaps cells measure their growth rate, not their size.

Nasmyth: Cln3 is a transcriptional activator of the Cln1 and Cln2 cyclins, which are somewhat similar to cyclin E. It is a very unstable protein whose rate of synthesis is proportional to cell size. Its concentration is going to be directly and immediately proportional to the rate of synthesis. This stuff goes into the nucleus, which is roughly constant in size.

Raff: But simply by altering the concentrations of extracellular growth factors and/or mitogens, you can change cell size.

Nasmyth: Right, because this regulates things like Cln3.

Raff: So the cell doesn't care about size; it cares about Cln3.

Nasmyth: But why does it care about Cln3? Because it cares about cell size! Now we are getting into a teleological argument. The hypothesis is that the cells do care about cell size, and they have to have a mechanism for measuring it. They do this by measuring the concentration of unstable proteins in the nucleus.

Raff: This has been the dogma, but it is based on little experimental evidence.

Nasmyth: It is not testable: how do you test what is the purpose of something in biology?

Bryant: How is it that the concentration of those proteins you are describing is proportional to size?

Nasmyth: This relates to something I was trying to say earlier on. Generally speaking, probably the bigger the cell, given one copy of that gene, the rate of synthesis of that product will be proportional to cell size. This probably acts at every level of a synthetic and degradative pathway. Everybody grinds up their cells, measures the amount of RNA (which is a measure of ribosomal RNA), and then they do a Northern with GPDH. It is always the same. In yeast these sorts of things have a fairly short half-life, so the level is in a sense a measure of the rate of synthesis. It is always constant relative to the number of ribosomes in a cell. This is what those Northerns tell you, otherwise you wouldn't have nice normalized controls. If you have a small cell or a big cell, this doesn't matter; it always looks the same level. The conclusion is that the rate of synthesis of that mRNA must be proportional to the number of ribosomes, which is a measure of cell size. This is just anecdotal.

Leevers: I think it is more likely that there is a link between protein synthesis, growth rate and the production of a specific cell cycle regulator. This is a great mechanism to ensure a minimum cell size, making sure that cells don't divide until they are growing at a certain rate and producing everything they will need to divide at a reasonable level.

Edgar: I wanted to add a general comment about the PI3K/insulin pathway. All the analysis that has been done in flies so far suggests that it is not a pathway that is used to regulate differential growth in terms of forming and shaping organs, but rather is a nutrition-sensing pathway. Other signalling pathways such as bone morphogenetic protein (BMP), Wnt and extracellular growth factor receptor (EGFR) seem to be the ones stimulating differential growth.

Raff: That is probably also true in mammals in the sense that IGFs tend to stimulate proportional growth. They don't seem to be involved in patterning.

Edgar: The question is, how do those other signals (BMP, Wnt, EGFR) feed into protein synthesis, or whatever regulates growth?

Thomas: In the tissue culture model following insulin stimulation, such signalling elements as protein kinase B, PI3K and MAP kinase are all activated in the absence of amino acids or glucose, but the activation of S6K is blocked. If nutrients are returned to the media, S6K is activated. Indeed, raising nutrient concentrations in the media will activate S6K, without addition of a ligand. This is all being mediated by TOR (target of rapamycin), an effector for nutrient sensors.

Raff: What about TOR in mammals?

Thomas: If you challenge animal cells with rapamycin, you block TOR function. If you put in rapamycin resistant alleles you can protect against the antibiotic.

Hunt: There is another side to this coin, which is what happens during starvation. There is a hierarchy of what tissues go first.

Raff: If you starve hydra, they just shrink. Cell proliferation carries on almost normally, but cell death (apoptosis) greatly increases. The animals survive by eating their own apoptotic cells.

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Timing cell-cycle exit and differentiation in oligodendrocyte development

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Abstract. During animal development many cells permanently stop dividing and terminally differentiate. For the most part, the mechanisms that control when the cells exit the cell cycle and differentiate are not known. We have been studying the mechanisms in the oligodendrocyte cell lineage. Studies of oligodendrocyte precursor cells (OPCs) in culture suggest that each OPC has a built-in timing mechanism that helps determine when the cell stops dividing and differentiates. This intrinsic timer consists of at least two components — a timing component, which measures elapsed time, and an effector component, which stops cell division and initiates differentiation at the appropriate time. The timer seems to involve both transcriptional and post-transcriptional mechanisms, with some proteins progressively increasing and others progressively decreasing over time.

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Most mammalian cell types develop from precursor cells that divide a limited number of times before they stop and terminally differentiate. In no case do we understand why the cells stop dividing when they do. The stopping mechanisms are important because they determine how many differentiated cells are produced and when differentiation begins. We have been studying the stopping mechanism in oligodendrocyte precursor cells (OPCs) isolated from the developing optic nerve of rats and mice.

The optic nerve contains the axons of retinal ganglion cells and two major classes of macroglial cells — astrocytes and oligodendrocytes. The oligodendrocytes myelinate the axons, while the astrocytes provide structure and help control the environment in the nerve. The astrocytes develop from the neuroepithelial cells of the optic stalk, the primordium of the optic nerve, whereas the oligodendrocytes develop from OPCs that migrate into the developing optic nerve from the brain just before birth (Ono et al 1997, Small et al 1987). The

OPCs divide a limited number of times in the nerve and then terminally differentiate into postmitotic oligodendrocytes. The first OPCs stop dividing and differentiate on the day of birth (Miller et al 1985), and new ones do so over the next six weeks (Barres et al 1992). We have studied the mechanisms that control when the OPCs stop dividing and differentiate in culture and find that both cell-intrinsic programmes and extracellular signals are involved.

A cell-intrinsic timer

The timing of OPC differentiation can be reconstituted in cultures of dissociated embryonic optic nerve cells, as long as there is sufficient mitogen to drive OPC proliferation (Raff et al 1985, 1988). As we discuss below, the main mitogen for these cells is platelet-derived growth factor (PDGF). The reason that OPCs stop dividing and differentiate in these cultures is not because PDGF becomes limiting or that PDGF receptors disappear or can no longer be activated. The addition of excessive PDGF does not alter the timing of oligodendrocyte differentiation (Raff et al 1988), and newly formed oligodendrocytes still express large numbers of PDGF receptors (Hart et al 1989a), which can induce both an increase in cytosolic Ca^{2+} (Hart et al 1989b) and immediate-early gene expression (Hart et al 1992) when stimulated with PDGF.

The timing of oligodendrocyte differentiation can even be reconstituted in cultures of purified embryonic OPCs (Gao et al 1998), suggesting that the timing mechanism is built either into the OPC population or into each individual cell. Clonal analyses in culture suggest that at least part of the timing mechanism is built into each individual cell (Barres et al 1994, Temple & Raff 1986). Such analyses show that the OPCs in the P7 rat optic nerve are heterogeneous in the number of times they divide in response to PDGF stimulation. The cells divide from 0–8 times, but the progeny of an individual OPC tend to stop dividing and differentiate at approximately the same time (Barres et al 1994, Temple & Raff 1986). Moreover, sister cells transferred to different microwells behave similarly (Temple & Raff 1986), suggesting that an intrinsic mechanism operates in each OPC to limit its proliferation to a certain period of time or a certain number of cell divisions. The finding that precursors cultured at 33 °C divide more slowly but stop dividing and differentiate earlier, after fewer divisions, than when they are cultured at 37 °C, suggests that the mechanism does not operate by counting cell divisions but instead measures time in some other way (Gao et al 1997). We therefore refer to the mechanism as an intrinsic timer.

Control by extracellular signals

Although the timer is cell-intrinsic, it requires signals from other cells to operate normally. It depends on PDGF, for example, the main mitogen for OPCs. The

PDGF is probably provided by astrocytes and is the only mitogen that on its own can stimulate purified OPCs in culture to proliferate (Ibarrola et al 1996). Moreover, in mice in which the *PDGFA* gene has been inactivated, OPCs do not proliferate and very few oligodendrocytes develop (Fruttiger et al 1999). When OPCs are cultured on their own with survival signals but without PDGF, they prematurely stop dividing and differentiate (Barres et al 1994, Temple & Raff 1985).

The timer is also regulated by thyroid hormone (TH), which has been known for many years to influence oligodendrocyte development. In developing animals that are hypothyroid, for example, myelination is greatly delayed (Dussault & Ruel 1987, Rodriguez-Pena et al 1993), whereas in developing animals that are hyperthyroid, myelination is accelerated (Walters & Morell 1981). The evidence that TH influences the intrinsic timer in OPCs comes from experiments that compare the behaviour of purified OPCs cultured in PDGF in the presence or absence of TH (Barres et al 1994). When OPCs purified from the optic nerve of postnatal day 8 (P8) rats are grown at clonal density in PDGF and TH, the cells divide a maximum of eight times before they stop and differentiate; in the absence of TH, by contrast, most of the cells tend to keep dividing and do not differentiate. If the OPCs are cultured at clonal density in PDGF in the absence of TH for 8 days, and then TH is added, most of the cells stop dividing and differentiate within 4 days, suggesting that the cells can measure time in the absence of TH. It seems that the timer consists of at least two components — a counting component that measures time independently of TH and an effector component that is regulated by TH and stops the cell cycle and initiates differentiation when time is up. TH also seems to be required for normal oligodendrocyte development in the optic nerve: hypothyroid rats (Ibarrola et al 1996) and mice (Ahlgren et al 1997) have many fewer oligodendrocytes in their optic nerves at P7. As TH influences the differentiation of many types of vertebrate precursor cells, it may help to co-ordinate the timing of cell differentiation in tissues throughout the body, just as it co-ordinates the events of amphibian metamorphosis.

Role of p27/Kip1

It is uncertain whether the intrinsic timer in OPCs primarily controls the onset of differentiation, the cessation of proliferation, or both. It seems likely, however, that the timer at some point interacts with the cell-cycle control system that regulates progress through the cell cycle. As the cells stop dividing and differentiate in G1, it is the part of the control system that operates in G1 that is most likely to be relevant. In principle, the components of the cell-cycle control system could be components of the timer. The timer, for example, could depend on a decrease in one or more positive intracellular regulators, such as a cyclin or a Cdk

that promotes progression through G1, an increase in one or more negative intracellular regulators, such as Cdk inhibitors that block such progression, or on both types of changes. It would be advantageous to have the timer involve changes in multiple components, as this would make it more robust: if one component were defective, the timer would still work, although inaccurately.

Several lines of evidence suggest that an increase in the Cdk inhibitor p27/Kip1 (p27) is part of the timer. First, p27 is high in all oligodendrocytes, whereas it is variable in OPCs (Durand et al 1997). Second, p27 progressively increases as precursor cells proliferate in culture in the presence of PDGF and the absence of TH, even though most of the cells do not stop dividing and differentiate in these conditions (Durand et al 1997). p27 reaches a plateau value at around the time that most of the cells would have stopped dividing and differentiated were TH present, consistent with the possibility that the progressive rise in p27 is part of the timing mechanism. Since the cells continue to divide with this high level of p27, however, it is clear that the rise in p27 is not enough on its own to stop the cell cycle. Third, p27 levels rise faster at 33 °C than at 37 °C (Gao et al 1997), which may be one reason why the timer runs faster at the lower temperature. Fourth, artificially increasing p27 levels by transfection speeds up the timer (J. Apperly, unpublished work). Fifth, many OPCs isolated from the optic nerves of p27-deficient mice go through one or two more divisions in clonal culture in the presence of PDGF and TH before they differentiate than do any OPCs from wild-type mice of the same age (Durand et al 1998).

The p27-deficient mice are larger than normal and have more cells in all organs that have been examined (Fero et al 1996, Kiyokawa et al 1996, Nakayama et al 1996), suggesting that p27 may be required in many cell lineages for normal exit from the cell cycle. Genetic studies indicate that p27-like proteins are also present in *Caenorhabditis elegans* (Hong et al 1998) and *Drosophila* (de Nooij et al 1996, Lane et al 1996). If the genes encoding these proteins are inactivated by mutation, developing cells in multiple lineages go through one or more extra divisions before withdrawing from the cell cycle (de Nooij et al 1996, Lane et al 1996). It seems that Cdk inhibitors are involved in stopping the cell cycle at the appropriate time during development in all animals. On the other hand, precursor cells still stop dividing and differentiate in the mutant mice, worms and flies, suggesting that p27 and its relatives are only part of the stopping mechanism. What are the other components of the stopping mechanism? The phenotype of mice deficient in another Cdk inhibitor, p18/Ink4c (p18), is very similar to that of p27-deficient mice, suggesting that p18 may be another component in mice (Franklin et al 1998). In mice deficient in both p27 and p18 some organs are even larger than in mice deficient in either protein alone (Franklin et al 1998), but even in these doubly-deficient mice precursor cells still stop dividing and differentiate during

development, suggesting that there are additional components that stop the cell cycle in these mice.

How are the levels of p27 controlled such that they progressively increase in dividing OPCs? RT-PCR studies indicate that mRNA levels do not change appreciably as the protein levels increase, suggesting that the controls on p27 protein levels in these cells are all post-transcriptional (Y. Tokumoto, unpublished work). Transcriptional controls, however, may also play a part in the timer. This is suggested by studies of the inhibitory helix-loop-helix protein Id4, which decreases progressively as OPCs proliferate *in vitro* and *in vivo* with a time course expected if Id4 is part of the timer (Kondo & Raff 2000a). Enforced expression of *Id4* stimulates OPC proliferation and inhibits OPC differentiation, suggesting that the normal progressive fall in Id4 protein may help determine when OPCs withdraw from the cell cycle and differentiate (Kondo & Raff 2000a). Id4 protein and mRNA decrease in parallel as OPCs proliferate *in vitro* and *in vivo*, suggesting that the control of *Id4* expression in these cells is transcriptional (Kondo & Raff 2000a).

Thus the intrinsic timer in OPCs is complex. Some proteins increase over time and others decrease, and both transcriptional and post-transcriptional mechanisms seem to be involved.

An intrinsic maturation programme

OPCs are present in the rat optic nerve throughout postnatal life. When they are visualized at different developmental times, they are seen to become progressively more complex with increasing age (Fulton et al 1992). Moreover, when embryonic day 18 (E18) and P7 OPCs are purified from optic nerve and studied in culture, the embryonic OPCs are found to be different from the postnatal OPCs. The embryonic cells have a simpler morphology, migrate and divide faster, and divide more times before they differentiate in the presence of PDGF and TH (Gao & Raff 1997). If the purified E18 OPCs, however, are cultured for 10 days in PDGF and the absence of TH, so that they become equivalent in age to the P7 OPCs, they acquire the properties of the P7 cells (Gao & Raff 1997). These findings suggest that OPCs have an intrinsic maturation programme that plays out over time, even when the cells are maintained in a relatively constant environment and in the virtual absence of other cell types. It seems likely, therefore, that the intrinsic timer that controls when an OPC stops dividing and differentiates in the presence of PDGF and TH is only one part of a more complex maturation programme that changes almost all aspects of the cell's behaviour over time.

The maturation programme may continue to change OPCs over months, or even years. When purified P7 OPCs are cultured in PDGF without TH, many of the cells continue to proliferate indefinitely and continue to change, acquiring

some of the properties of OPCs that can be isolated from the adult optic nerve (Tang et al 2000). It remains a mystery how such a maturation programme is controlled so that it changes the cells in a predictable way over such a long period of time.

Surprisingly, purified P7 OPCs can be induced by extracellular signals to revert to a more multipotential state resembling that of CNS neural stem cells. When treated with fetal calf serum for a few days, purified P7 OPCs become responsive to the mitogenic effects of basic fibroblast growth factor (FGF)2. They then seem to be able to proliferate indefinitely in FGF2 and give rise to neurons, astrocytes and oligodendrocytes (Kondo & Raff 2000b). It seems, therefore, that OPCs are not irreversibly committed to forming glial cells. These findings emphasize the difficulties in arriving at a satisfactory definition of a stem cell.

Conclusion

As far as we are aware, OPCs are the only normal mammalian cells, other than eggs and blastomeres, that have been shown to survive, proliferate and differentiate in serum- and extract-free culture in the absence of other cell types. Indeed, a single OPC can survive and proliferate in these conditions in the absence of any other cells, suggesting that diffusible autocrine factors are not required (Y. Tokumoto, unpublished work). These properties make OPCs especially attractive for studying how intracellular programmes and extracellular signals can combine to control when the cells exit the cell cycle and differentiate. It seems likely that similar mechanisms operate in other cell lineages.

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DISCUSSION

Nurse: Martin Raff has set up two general questions that I think we should try to address in this discussion. One concerns the nature of this timer (and we could add to this, why is the timer needed). The second is this relationship between stem cells and progenitors, and what we mean by this. But I'll start with a specific question. If it is only 6–7% of the cells that undergo one or two more extra divisions, is that sufficient to account for the $p27^{-/-}$ mutant phenotype with respect to cell number?

Raff: In the population of postnatal day 7 (P7) precursor cells, there are some that will only go through one division before they stop, and others that go through up to eight cell divisions. The ones that will go through up to eight divisions are in a minority. In the p27-deficient population, the whole curve is shifted to the right, but the ones at the extreme right hand side of the curve, which we suspect are the least mature, are easiest to detect as abnormal because they go through more divisions than any normal P7 precursor cell. In normal mice, the cardiac myocytes have all dropped out of division by P1 or P2. In the p27 knockout, there are still cells dividing at P4 and you end up with many more cells, but the cells are much smaller.

Reik: Is it true that the phenotype of that knockout is not present at birth, so at birth they are normal size?

Raff: They are normal size if you weigh the mice. But if you look carefully at cell number, there is a phenotype at birth.

Leevers: What happens to the p27 levels when you shift the temperature?

Raff: They go up faster at 33 °C than at 37 °C.

Leevers: In the p27 knockout mice, is the expression of the other proteins you showed us affected?

Raff: We don't know.

Maller: What sort of process could be going faster at a lower temperature?

Raff: It could simply be that the degradation of p27 is decreased more by low temperature than p27 synthesis.

Maller: Have you used these new phospho-specific antibodies to Cdc2 to examine the cycles of tyrosine phosphorylation and dephosphorylation?

Raff: No, it would be interesting to do that.

Maller: In *Xenopus*, raising p27 also raises tyrosine phosphorylation of Cdc2, possibly through indirect effects on Wee1.

Nasmyth: The key underlying question is what determines how big an organ or organism gets, for which this model system might be relevant. There are two hypotheses that come to mind. One is that the bigger you get, the more of something you make and you create a concentration that is somehow related to the mass of the tissue. The other is the timer. It seems to me that one of the predictions of the timer hypothesis is that in the absence of any cell death, it is not clear how the timer can be regulated. Is that correct?

Raff: There are many things that can regulate the timer. TH is one example. If you take TH away, most of the cells just keep dividing. If you add FGF2 as well as PDGF, to stimulate proliferation, the cells also just keep proliferating and don't differentiate. As it happens, at least half the oligodendrocytes produced in the optic nerve undergo programmed cell death, in a process that adjusts their numbers to the number and length of axons.

Nasmyth: In this particular case I was getting the impression that timers would be one way of determining final organ size. In this particular case you clearly get regulation through death.

Raff: It would surprise me if any system depended exclusively on such timers to get the final cell numbers right.

Nasmyth: Can you use it at all?

Raff: The phenotype of the p27 knockout mice suggests such timers do play a part in size control. The simplest explanation for why you get more cells in these mice is that p27 normally plays a role in taking cells out of division at the right time. Cdk inhibitor mutations seem to play similar roles in flies and worms. p27 is clearly not the only component of the stopping mechanism. If you take it out of action, the cells still stop, just not at the right time.

Nurse: I wonder whether the word 'timer' is perhaps the most useful one. You probably came up with the term 'timer' to contrast it with counting cell divisions. It is a timer that is influenced enormously by external factors, and *in vivo* it will be

very complex, whereas generally when we think of timers we tend to think of something that is measuring time. What you really mean is that it is something that is cell autonomous which in a defined set of conditions measures time rather than cell divisions, but it is a timer that is so influenced by external factors that it is not like a little clock ticking in the cell.

Raff: What is being affected by these manipulations may not be the timing component. p27 goes up and plateaus at the same time even if you remove thyroid hormone or add FGF to the PDGF-containing culture medium. The appropriate signals are required, however, for the timer to stop the cell cycle and initiate division at the right time. Thus, there is an intrinsic timing mechanism that operates without thyroid hormone or in the presence of FGF; it is just that you can't see that it is working unless you measure something like p27 levels.

Nurse: Perhaps it would be more helpful to think of this in a negative sense, in that whatever it is doing it is not counting cell divisions.

Raff: Yes, it is very unlikely that it is counting divisions. I don't think that cell-intrinsic timers are responsible for all of timing in development. It is quite clear that they are not. But cells do change as they develop, and some of the changes seem to reflect intrinsic programmes operating within the cells.

Reik: If you consider an organ that is already fully differentiated at birth, and then it simply grows, is the timer off in that situation?

Raff: It depends what timer you are talking about. I have been talking about only part of the oligodendrocyte development process, where the precursor cell withdraws from the cell cycle and expresses one differentiation antigen. But the oligodendrocytes then continue to differentiate over many days, turning on myelin-specific genes and wrapping axons. This is a very complex process and I have no idea how these events are controlled.

Hunt: I want to ask about PDGF. It seems a little unlikely that these are really responding to PDGF in the context of the optic nerve.

Raff: The evidence is very strong that PDGF is a major mitogen for these precursor cells *in vivo*. PDGF is made by astrocytes in the optic nerve. If the *PDGFα* gene is inactivated in mice, oligodendrocyte precursors don't proliferate and very few oligodendrocytes develop.

Hunt: It seems to me that this is a system par excellence where cell–cell interactions are very important. These things are ultimately designed to wrap neurons. There have to be enough of them so that they will totally insulate the neurons. When they put them into these pure cultures, I wonder how that is replicating the real world situation.

Raff: The remarkable thing is that in the presence of the right combination of signals, the precursors seem to stop dividing and differentiate on much the same schedule as they do *in vivo*. Axons are essential, however, for newly formed oligodendrocytes to live *in vivo*.

Hunt: Do you know what the axon normally gives them?

Raff: We know that one of the signals is a neuregulin, glial growth factor; there are almost certainly others.

Nurse: You see, naïve frog people think that PDGF only comes from platelets!

Edgar: I have two simple questions about the timer. First, what starts it ticking and, second, does it have any influence on cell growth?

Raff: I don't know the answer to the second question. I suspect that the timer starts ticking when the precursors first arise from multipotential CNS stem cells, but we don't know.

Edgar: Is that determined by a cell–cell interaction?

Raff: The ventralizing signals such as Sonic hedgehog that are required for motor neuron development are also required for oligodendrocyte precursors to develop from CNS stem cells, at least in the spinal cord.

Schmidt: What happens to the six cortical divisions in the p27-deficient mice? Likewise, has anyone ever done the heroic thing of taking the basal layer that gives rise to the six cortical divisions and transplanting it down one day to see whether it still has five divisions left?

Raff: I'm not aware that anyone has done it in that way, but it is an important question. Cortical neuron precursors in the mouse, for example, go through many fewer divisions than do cortical precursors in primates, which is why mice have a smaller cortex than primates. The question is why is the behaviour of the two types of precursors so different? Is it because intrinsic timers are set differently? Is it because the mitogens and growth factors that drive proliferation are around for longer in primates? These are questions that could be addressed without a need for new technology.

Schmidt: There is an even simpler one, which is what about hypothyroidism, which exists in all species and can be induced?

Raff: You end up with pretty normal cell numbers in hypothyroid animals, although it takes longer to get there. Thyroid hormone seems to play a coordinating role in development.

Schmidt: Do the p27-deficient mice get more cortical divisions?

Raff: I assume so, as there seem to be more cells in the cortex.

Kozma: Are you referring to a problem intrinsic to all cells, or something specific to these oligodenrocytes? Because in the development of an organism, the size of an organ is also controlled by the time when puberty is reached: hormones are stopping or inducing organ growth. Another type of growth regulation is tied to the organ pattern. In the case of the oligodenrocytes you are dealing with an isolated cell. Thus, is this representative of all cell types?

Raff: I think the timing mechanism may be similar in many cell lineages where precursors divide a limited number of times and then stop and terminally differentiate. The best evidence for this is that if you inactivate p27 there are more

cells in every organ. I think it is unlikely that these timers play much of a role in the fine adjustments in cell proliferation that determine patterning and organ shape.

One thing that is interesting about the p27 knockout mouse is that cell death does not bring cell numbers down to normal levels. Why not? It may be because there are more cells in multiple lineages in each organ, and they support the survival of one another. If only one cell type is increased within an organ, then cell death would presumably bring the number back down to normal.

Edgar: Has that been done with p27 to make a mosaic?

Raff: Not as far as I know.

Nasmyth: It would be nice to make a $p27^{-}$ heart. If one wants to analyse the molecular nature of this phenomenon, one of the things one would like to know is what is the variability? How well does it keep time? What is the molecular mechanism behind this? How do clocks work in the absence of a mechanical system? At the moment we use atomic decay. The same sort of principle could be occurring here. What you have is cells that started off at various epigenetic states. Genes are on or genes are off. There is a huge literature showing that these epigenetic states are unstable. They decay at a certain frequency. What we may be looking at here is the stochastic decay of epigenetic states. There are ways of making this less variable. If just one gene was involved there would be a huge standard deviation, but if a combination of genes were involved this may produce quite an accurate clock.

Nurse: This is possible, but it would depend on what was the rate-limiting step. You would only get such a state if they were all decaying with rather similar dynamics.

Nasmyth: We know that when these states do decay, it is completely gene autonomous. This is the key thing.

Nurse: Even if it was one gene you could set up the system so that it would have limited variability. It would depend on the numbers of molecules involved in that circuit. You can devise limited variability in different ways. One is by having many different elements in the way that you have described, but you can also do it by having lots of molecules involved in a single regulatory circuit, which would reduce variability.

Nasmyth: Then it is not stochastic.

Nurse: The amount of variability will be determined by the number of components in that system. If you are saying it is stochastic, if it is really stochastic you need some factor in there which is limiting and decaying. This you would have to produce by having one component that is rate limiting and stochastic.

Nasmyth: My understanding is that the gene itself, whether it is on or off, is completely stochastic.

Nurse: The regulation of it is not stochastic.

Nasmyth: No, it is epigenetically controlled; it is not affected by the environment — that is the whole point.

Nurse: It will be on or off, but the dynamics of it are not stochastic.

Nasmyth: No, that is what we know about these epigenetic states: they are either on or they are off.

Nurse: That is a description of the final state: it is not a description of how you achieve that final state.

Reik: There is another way of doing it. This can be determined quite precisely. For example, in *Schizosaccharomyces pombe* the grand parental DNA strands are marked epigenetically to affect mating type switching.

Nasmyth: This doesn't give you the clock.

Reik: It can, because if you switch one system on like that, you have something two cell divisions later.

Nasmyth: The point is, this doesn't depend on cell divisions.

Raff: Remember that there is an accurate timer in *Xenopus* embryos that doesn't depend on DNA, RNA, or protein synthesis. It is still a mystery how it works.

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Splitting the chromosome: cutting the ties that bind sister chromatids

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Abstract. In eukaryotic cells, replicated DNA molecules remain physically connected from their synthesis in S phase until they are separated during anaphase. This phenomenon, called sister chromatid cohesion, is essential for the temporal separation of DNA replication and mitosis and for the equal separation of the duplicated genome. Recent work has identified a number of chromosomal proteins required for cohesion. In this review we discuss how these proteins may connect sister chromatids and how they are removed from chromosomes to allow sister chromatid separation at the onset of anaphase.

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Back to basics: chromosome mechanics

Instructions for the behaviour of every cell in the bodies of worms, flies and humans will soon reside in public databases for all to read. A complete set of these instructions, packaged as chromosomes, is inherited by most cells in our body. Because of this, many if not most somatic nuclei in mammals are totipotent; that is, they are capable of programming all of mammalian development when injected into enucleated eggs (Wilmut et al 1997). Dolly's creation had dramatic practical consequences but its feasibility was never improbable on theoretical grounds. How cells inherit two complete packages of the genome at each cell division is one of the most fundamental questions in biology (Fig. 1a).

Recent studies have concentrated on control mechanisms, such as the crucial part played by cyclin-dependent protein kinases in triggering chromosome duplication and segregation (Nurse 1990) and surveillance mechanisms (checkpoints) that monitor the fidelity of these two processes (Hartwell & Weinert 1989). This focus on 'control' is however a recent phenomenon. Earlier studies, largely

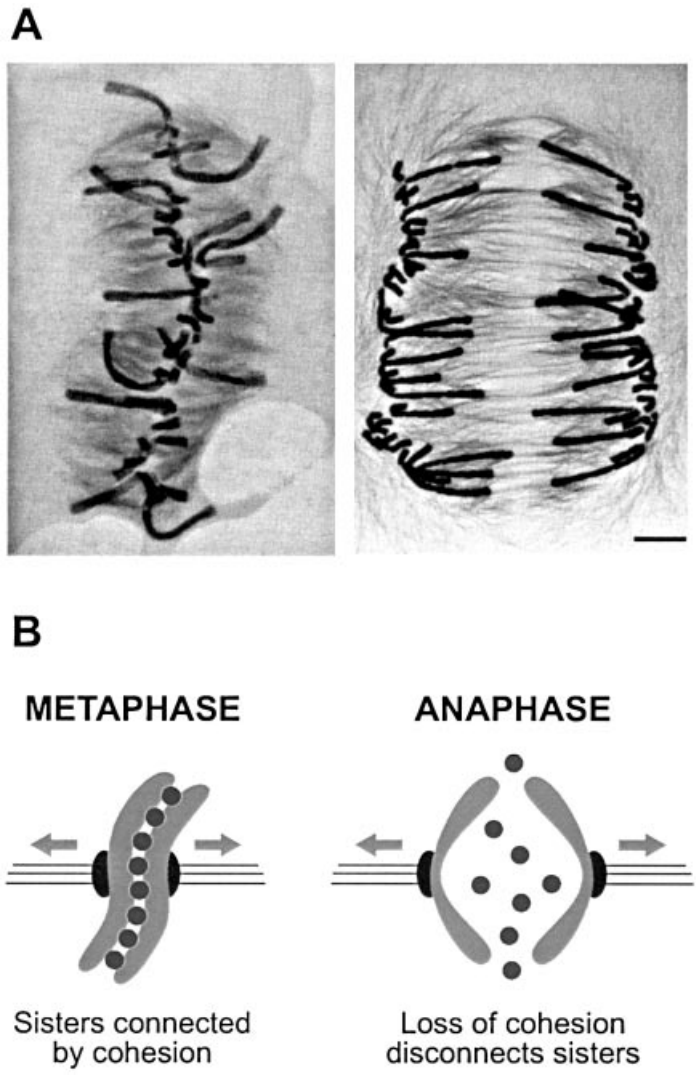

FIG. 1. The metaphase to anaphase transition. (A), Light micrographs of mitotic figures in endosperm of the African blood lily *Haemanthus katherinae* Bak. Microtubules are stained in light grey and chromosomes in darker, thicker lines. In metaphase (left panel) centromere regions are aligned on the spindle equator, whereas in anaphase (right panel) the arms of separated sister chromatids trail behind centromere regions which move poleward. Size bar, 10 μm. Reprinted, with permission from Khodjakov et al (1996). (B), A model depicting how cohesion structures (dots) physically connect sister chromatids during metaphase. Cohesion antagonizes the pulling forces exerted by spindle microtubules (horizontal lines) on kinetochores (attached to the sister chromatids). During anaphase, loss of cohesion liberates sister chromatids for poleward movement.

cytological in nature, concentrated on the mechanics of chromosome segregation (Flemming 1879, Wilson 1925, Schrader 1944, Mazia 1961). What, for example, was 'the nature of the initial act of doubling of the spireme thread (chromosome)' (Wilson 1925) and how were the sister threads moved to opposite poles of the cell during mitosis?

The elucidation of DNA's structure largely answered the first of these questions (Watson & Crick 1953) and work on cytoskeletal proteins like tubulin and the spindle fibres assembled from it, has gone a long way towards solving the mystery of chromosome movement. In contrast, until recently the mechanisms by which sister chromatids are tied together after chromosome duplication and then separated at the metaphase to anaphase transition was largely neglected, despite being equally crucial for the mitotic process (Maguire 1990, Miyazaki & Orr-Weaver 1994).

Importance of sister cohesion

The ability of eukaryotic cells to delay segregation of chromosomes until long after their duplication distinguishes their cell cycle from that of bacteria, in which chromosome segregation starts soon after the initiation of DNA replication (Lin & Grossman 1998). This temporal separation forms the basis for the partition of the cell cycle into four phases, G1, S, G2 and M, and it has played a central role in the evolution of eukaryotic organisms. Meiosis, during which two rounds of chromosome segregation follow a single round of duplication, requires separable S and M phases. Furthermore, mitotic chromosome condensation, without which large genomes cannot be partitioned between daughter cells at cell division, would not be possible if chromosome segregation coincided with DNA replication. A gap between S and M phases therefore made possible the evolution of large genomes. It is sister chromatid cohesion that permits chromosome segregation to take place long after duplication. Cohesion provides a memory of a duplication process that may have occurred long ago (up to 50 years in the case of human oocytes); a memory that defines which chromatids within a nucleus are to be parted from each other at cell division. Were chromatids to drift apart before building a mitotic spindle, there would be no way cells could determine whether chromatids were sisters (to be segregated to opposite poles) as opposed to being merely homologous chromosomes, a distinction that is crucial for all diploid organisms.

The structures holding sister chromatids together are responsible for generating bilaterally symmetrical chromosomes during mitotic divisions. The bilateral symmetry of chromosomes underlies the symmetry of the spindle apparatus and hence forms the basis for the exact and symmetrical partition of chromosomes and the roughly equal partition of most other cell constituents at cell division. In addition, tying sister chromatids together generates a centromere geometry that

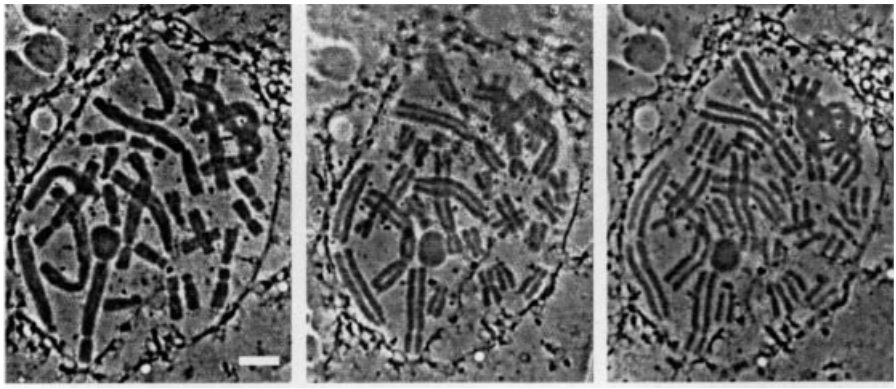

FIG. 2. Sister chromatid separation does not depend on the mitotic spindle. Light micrographs of mitosis in living flattened endosperm from *Haemanthus katherinae* BAK. treated with colchicine (c-mitosis). The micrographs were taken at 10 min intervals. Size bar, 10 μm. Reprinted with permission from Mole-Bajer (1958).

favours the attachment of sister kinetochores to spindles that extend to opposite poles. Only those kinetochore–spindle connections that result in tension are stabilized, which enables the chromosome alignment process to be proof-read (Nicklas & Ward 1994). Despite its importance, the mechanism by which sister chromatids are tied together is still poorly understood.

Chromatid separation independent of the spindle apparatus

The chromatid separation process has also remained mysterious. It is an autonomous process that does not directly depend on the mitotic spindle (Wilson 1925, Mazia 1961). This is most vividly seen in cells whose spindles have been destroyed by spindle poisons such as colchicine. In many organisms, in particular in plant cells, the cell cycle delay induced by colchicine is only transient and chromatids eventually split apart in the complete absence of a mitotic spindle (Mole-Bajer 1958, Rieder & Palazzo 1992) (Fig. 2). Mitosis in the presence of colchicine or colcemid (known as c-mitosis) leads to the production of daughter cells with twice the normal complement of chromosomes. This process is routinely used for manipulating plant genomes and may contribute to the therapeutic effects of taxol in treating breast cancer.

A tense period in the cell cycle

Changes in the interaction between sister chromatids, as opposed to changes in the activity of spindle fibres, is thought to trigger the sudden movement of chromatids

to the poles at the metaphase to anaphase transition. Destroying the spindle fibre that connects a chromosome to one pole using UV or laser microbeams (McNeill & Berns 1981) causes the entire chromosome (i.e. both chromatids) to move rapidly to the opposite pole. The implication is that sister chromatid pairs on the metaphase plate are under tension. Sisters are being pulled away from each other by spindles attached to oppositely-oriented sister kinetochores. The apparatus that will move chromatids to the poles during anaphase is therefore already engaged during metaphase. Metaphase is therefore viewed as a state of equilibrium in which traction exerted on kinetochores by spindle fibres is opposed by cohesion between sister chromatids (Fig. 1b) (Mazia 1961).

Loss of sister chromatid cohesion would therefore be sufficient for the sudden movement of chromatids to opposite poles at the metaphase to anaphase transition. According to this hypothesis, a specific apparatus binds chromatids together during replication, holds them in an orientation that facilitates the attachment of sister kinetochores to spindles extending to opposite poles, and resists the splitting force that results from this bipolar attachment to the spindle. Destruction of this specialized cohesive structure triggers movement of chromatids to opposite poles at the onset of anaphase.

In the absence of molecular details, this notion has remained a working hypothesis only. Indeed, until recently there has been little direct evidence that chromosome separation is due to the loss of cohesion as opposed to the onset of chromatid repulsion (Mazia 1961, Darlington 1939). An affinity between sister chromatids might be sufficient to resist their tendency to be split by spindle forces up to and during metaphase. Anaphase could be triggered by a repulsive force that overcomes the sister's 'natural' affinity. The notion that the midzone of anaphase spindles (or Belar's Stemmkörper [Belar 1929]) might exert this repulsion is now discredited, but more mysterious forces may yet lurk in the crevices between sisters.

Ties that bind chromatids together

In many organisms, the regions around centromeres have a special role in holding sister chromatids together during metaphase. Fluorescence *in situ* hybridization (FISH) shows that most sister DNA sequences separate from each other (at least a short distance) soon after DNA replication (Selig et al 1992). Nevertheless, sister chromatids usually do not acquire morphologically separate axes until pro-metaphase, well after the onset of chromosome condensation. Human chromosomes, for example, appear as undivided 'sausages' during prophase even though they are already highly condensed (Sumner 1991) (Fig. 3A). When sister chromatid arms eventually emerge as separate entities during pro-metaphase, sister centromeric sequences still hug each other in a compact embrace known as the

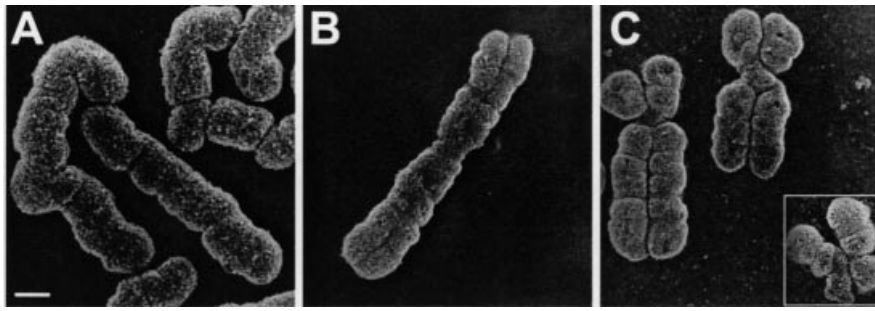

FIG. 3. Chromosome arms begin to separate in prometaphase. Scanning electron micrographs of human chromosomes isolated from cells in prophase (A), prometaphase (B), metaphase (C) and early anaphase (insert in C). Size bar, 1 μm. Reprinted with permission from Sumner (1991).

central constriction (Fig. 3B, C). When late mitotic events are inhibited by treatment with spindle poisons, separation of arm sequences continues while that of centromeres is blocked (Rieder & Cole 1999). The consequence is sister chromatid pairs connected only at centromeres, which though an artefact of drug treatment is a classic image of mitotic chromosomes.

The robust cohesion at centromeres may be due more to their heterochromatic nature than to their ability to form attachments to the mitotic spindle. Other heterochromatic chromosome domains, like the entire Y chromosome in flies, also remain tightly stuck together during mitotic arrest (Sigrist et al 1995). A relationship between heterochromatin and stickiness is also seen during normal mitoses. Human chromatid pairs move to the poles during anaphase with different kinetics and the laggards are invariably chromosomes with the greatest amount of centromeric heterochromatin (Vig 1981).

Despite the extra stickiness of centromeres, it is often this region which splits first at the onset of a normal anaphase. Traction exerted at centromeres peels sisters apart, with distal regions of chromosome arms being the last to separate (Wilson 1925, Rieder & Cole 1999). In several organisms, including budding yeast (T. Tanaka & K. Nasmyth, personal communication, Goshima & Yanagida 2000), diatoms (Tippet et al 1980) and the crustacean *Ulophysema oeresundense* (Melander 1950), sister centromeres are pulled most of the way to the poles even during metaphase, long before arm sequences separate. In these organisms, it appears that loss of cohesion along chromosome arms and not at centromeres is what triggers anaphase (Rieder & Cole 1999).

Despite these valuable insights, over a century of cytological observation has shed little light on the identity of the sister chromatid cohesion apparatus. In the absence of a biochemical approach, one way forward was inspired guesswork. Once it appeared likely that chromosomes contained one double stranded DNA

molecule, it was proposed that the central constriction might be due to the late replication of centromeric DNA. However, pulse labelling experiments suggest that little or no DNA is replicated during mitosis (Comings 1966). Another ingenious idea is that sister chromatids are held together by the intertwining (catenation) of sister DNA molecules that arises when two replication forks converge (Murray & Szostak 1985). According to this notion, increased topoisomerase II (Topo II) activity triggers the final decatenation of sister DNA molecules at the onset of anaphase. Though Topo II is clearly essential to disentangle chromatids (Dinardo et al 1984), there is evidence for an independent cohesion apparatus. First, mini-chromosomes in yeast are clearly held together in nocodazole-treated cells without any intertwining of sister circles (Koshland & Hartwell 1987). Second, centromeres (though not entire chromosomes) disengage from each other and move to the poles in the absence of any detectable Topo II activity in fission yeast (Funabiki et al 1993). Third, addition of Topo II inhibitors to mammalian cells in metaphase fails to block separation of sister centromeres at the onset of anaphase (Downes et al 1991, Gorbsky 1994).

Cohesin and its friends

Genetics is the method of last resort when other approaches reach their limits. The identification of mutants such as desynaptic in maize (Maguire et al 1991) and MeiS332 in *Drosophila* (Davis 1971, Goldstein 1980), in which sister chromatids dissociate prematurely during meiosis, provided the first inkling that sister chromatid cohesion might be mediated by special proteins (Kerrebrock et al 1995). Despite its important role during meiosis, MeiS332 is dispensable for mitotic divisions and is therefore unlikely to be a universal component of the cohesion apparatus.

Genetic studies in yeast have meanwhile uncovered a multi-subunit complex called cohesin that is essential for sister chromatid cohesion not only in yeast (Tóth et al 1999) but also in vertebrates (Losada et al 1998). An important breakthrough in cohesin's identification was the discovery that proteolysis (Holloway et al 1993), mediated by a ubiquitin protein ligase responsible for destroying mitotic cyclins (Irniger et al 1995, King et al 1995), is needed for sister chromatid separation. This ligase, known as the anaphase-promoting complex (APC) or cyclosome (Zachariae & Nasmyth 1999), was initially thought to mediate proteolysis of cohesion proteins. Its role in sister separation turns out to be less direct; it in fact mediates destruction of an inhibitor of the sister-separating apparatus (Yamamoto et al 1996a, Cohen-Fix et al 1996, Funabiki et al 1996a, Ciosk et al 1998). Nevertheless, the premise that the APC destroyed cohesion proteins provided a new impetus to the search for proteinaceous bridges connecting sister chromatids. Screens for mutations that permitted separation of sister chromatids in

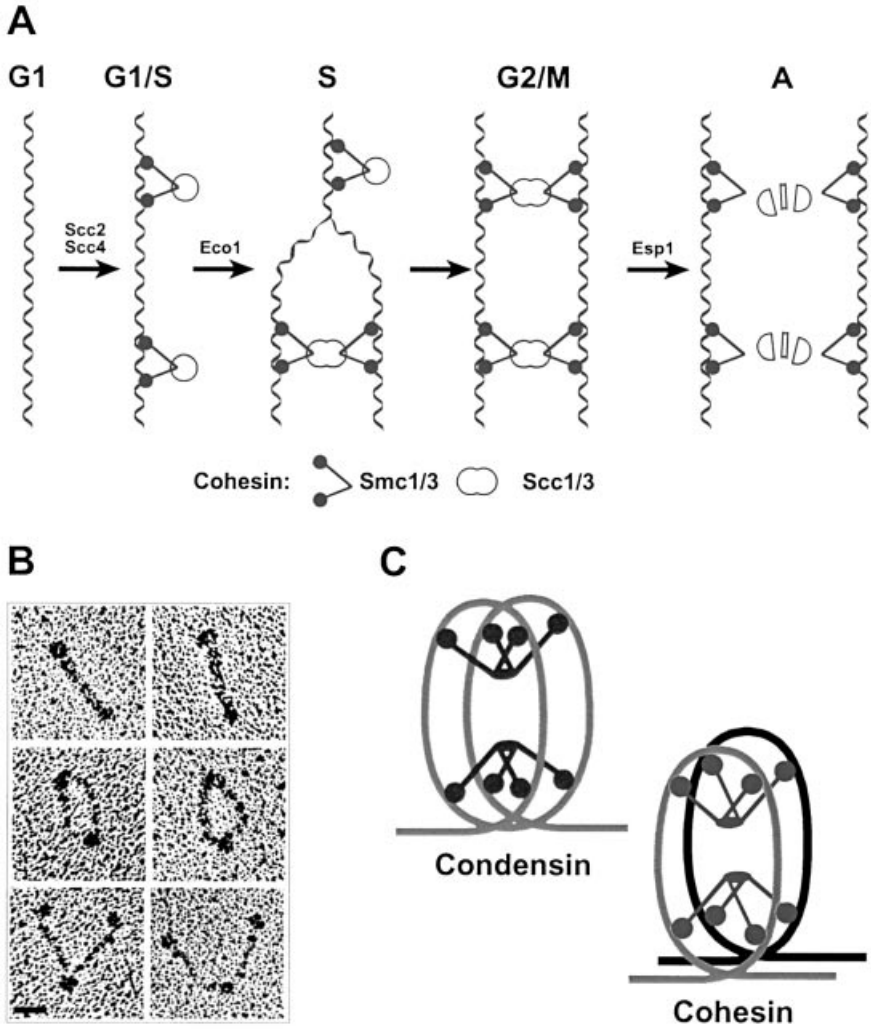

FIG. 4. Cohesin and friends. (A), a model illustrating how yeast proteins required for cohesion connect sister chromatids during DNA replication and maintain this association until the onset of anaphase. (B), electron micrographs of homodimers of the *Bacillus subtilis* SMC protein. Three commonly observed conformations are shown. Size bar, 20 nm. Reprinted with permission from Melby et al (1998). (C) A speculative model for how cohesin might join sister chromatids together, which is based on the premise that cohesin forms large supercoiled loops analogous to those proposed for condensin (shown alongside).

cells lacking APC activity have now identified at least eight proteins essential for sister chromatid cohesion (Tóth et al 1999, Michaelis et al 1997, Guacci et al 1997, Furuya et al 1998, Skibbens et al 1999). Remarkably, the function of all these proteins seem to be intimately connected (Fig. 4A).

Four of these proteins, Smc1, Smc3, Scc1 (also called Mcd1 and Rad21) and Scc3 form a multi-subunit complex called cohesin (Tóth et al 1999, Losada et al 1998). Indeed, Mcd1/Scc1 was independently isolated as a dosage suppressor of an Smc1 mutation (Guacci et al 1997). All four cohesin subunits are required both for establishing cohesion during S phase and (at least in yeast) for maintaining it until the onset of anaphase. Two other proteins, Scc2 (Mis4) and Scc4, form a separate complex that is required for the association of cohesin with chromosomes (Ciosk et al 2000). Cohesin binds to specific chromosomal loci (including centromeres) for much of interphase (Tanaka et al 1999, Blat & Kleckner 1999, Megee et al 1999) but it can only establish cohesion between sister chromatids during DNA replication, possibly when sister DNA molecules emerge from replication forks (Uhlmann & Nasmyth 1998). Establishment of sister cohesion is therefore an integral part of S phase.

Another protein, Spo76 is required for orderly sister chromatid cohesion in *Sordaria* (van Heemst et al 1999). Spo76 has homologues in many organisms, and is called Pds5 in budding yeast (V. Guacci & D. Koshland, personal communication) and BimD in *Aspergillus nidulans* (Denison et al 1993). In budding yeast a protein called Eco1 or Ctf7 is essential for establishing cohesion during S phase but not for maintaining it during G2 or M phases (Tóth et al 1999, Skibbens et al 1999). Its fission yeast homologue Eso1 is also required for establishing sister chromatid cohesion (Tanaka et al 2000). Of all known cohesion proteins the cohesin complex may lie at the heart of the cohesion process because cleavage of one of its subunits is essential for the separation of sister chromatids, at least in yeast (Uhlmann et al 1999). *Xenopus* cohesin is also needed for proper sister chromatid cohesion (Losada et al 1998). Nevertheless, it is still uncertain how, or indeed if, cohesin holds sisters together during metaphase in animal cells, as most of it dissociates from chromosomes by pro-metaphase (Losada et al 1998). It is therefore possible that other important players remain to be identified.

Two cohesin subunits, Smc1 and Smc3, are members of a large family of related proteins whose evolution predates the split between eukaryotes and bacteria (Hirano 1999). All Smc proteins have related globular domains at their N- and C-termini, joined by two long stretches of α-helical coiled-coil, which are linked by a central flexible hinge. Bacterial Smc proteins form anti-parallel homodimers whose terminal globular domains are proposed to form an active ATPase. The flexibility of the hinge region allows the Smc homodimer to adopt either a V or a linear shape (Fig. 4B) (Melby et al 1998). It remains to be seen whether cohesin contains an Smc1/3 heterodimer or Smc1 and Smc3 homodimers.

Little is known about the properties of cohesin *in vitro*, except that fragments from the C-terminal domain of Smc3p and its coiled-coil region can bind DNA (Akhmedov et al 1999). Smc1 and Smc3 belong to a subfamily of eukaryotic Smc proteins, which includes Smc2 and Smc4. The latter two proteins are components of the condensin complex, which is necessary for mitotic chromosome condensation (Hirano & Mitchison 1994, Saka et al 1994, Hirano et al 1997). Condensin possesses ATPase activity and is capable of forming large supercoiled loops by introducing a global positive writhe (Kimura et al 1999). These positive supercoils might be the driving force for mitotic chromosome condensation. The presence of a pair of Smc proteins in both condensin and cohesin suggests that these two complexes might have similar though not identical activities. Cohesin might, for example, introduce large constrained supercoils, like those produced by condensin, at equivalent positions on each sister chromatid. Cohesin's Scc1 subunit might help link together equivalent coils from each sister (Fig. 4C). An ability to coil chromosomes would explain how cohesin contributes to chromosome compaction (Guacci et al 1997).

In animal cells, condensin binds to chromosomes at about the same time that most cohesin dissociates from them (Losada et al 1998), between prophase and pro-metaphase. It is possible that condensin's ability to condense chromosomes as cells enter mitosis depends on the prior dissociation of most cohesin. The connections between sister DNA molecules might otherwise interfere with the locally processive coiling of each chromatid on itself. Cohesin could also contribute to chromosome compaction during interphase and early stages of mitosis by providing longditudinal links along chromatids as well as horizontal ones between sisters (Guacci et al 1997).

Securin: a protein whose destruction by the APC controls sister chromatid separation

Destruction of mitotic cyclins occurs at or shortly before sister chromatid separation but is not required for this process (Holloway et al 1993, Surana et al 1993). The discovery that the ubiquitin protein ligase responsible for destroying cyclins was also required for separating sister chromatids (Irniger et al 1995) led to a hunt for other APC targets whose destruction might be necessary for sister separation. Two candidates soon emerged: Pds1 from budding yeast and Cut2 from fission yeast. Destruction of Pds1 and Cut2 proteins at the onset of anaphase depends on APC and is essential for sister chromatid separation (Yamamoto et al 1996a, Cohen-Fix et al 1996, Funabiki et al 1996a). Though these two proteins have rather dissimilar primary sequences, it appears that they are members of a class of anaphase inhibitory proteins existing in all eukaryotes and now called securins because of their role in controlling the onset of sister separation (Fig. 5).

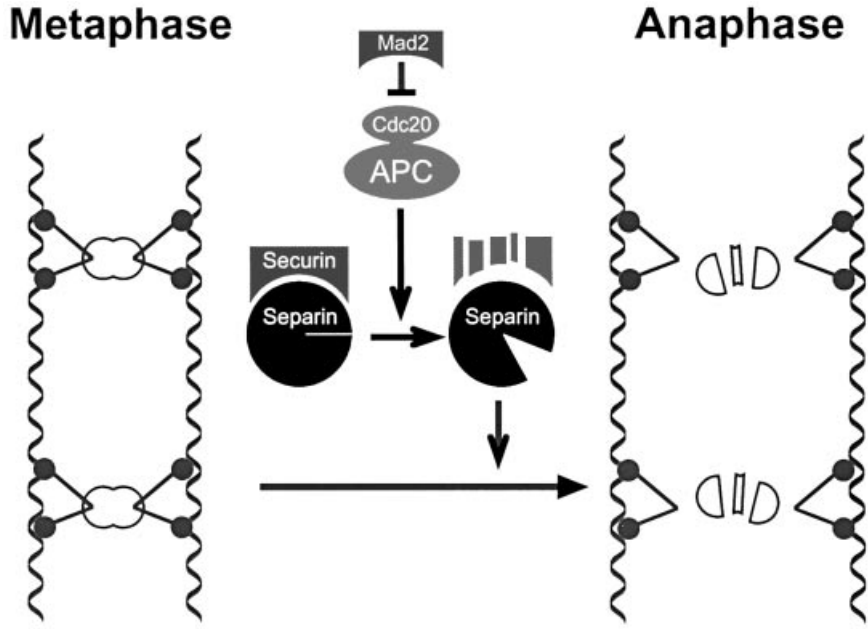

FIG. 5. The APC–separin pathway. A model illustrating how APC^{Cdc20} initiates anaphase through the activation of separin and subsequent cleavage of a cohesin subunit.

The human securin protein (Zou et al 1999) is overproduced in many tumour cells (Saez et al 1999) and is thought to be an oncogene (Pei & Melmed 1997). Increased securin levels might cause missegregation of chromosomes and thereby facilitate genome instability. A possible candidate for the securin homologue in *Drosophila* is the pimples protein, which like yeast and vertebrate securins is destroyed at the metaphase to anaphase transition (Stratmann & Lehner 1996). In budding yeast, Pds1 is only essential for proliferation at high temperatures (Yamamoto et al 1996b) and its elimination permits sister separation in the absence of APC activity (Yamamoto et al 1996a, Ciosk et al 1998). So, destruction of securin might be the APC's sole role in the triggering of sister separation, at least in yeast.

Separin: an endopeptidase necessary for separating chromatids?

The budding yeast securin has what appears to be a single stable partner, a 180 kDa protein called Esp1 (Ciosk et al 1998). In fission yeast, Cut2 had previously been found to be associated with Cut 1, an Esp1 homologue (Funabiki et al 1996b).

Vertebrate securins are likewise associated with an Esp1 homologue (Zou et al 1999). Esp1/Cut1-like proteins, now known as separins, are found in most if not all eukaryotes. They are usually large proteins, with molecular weights from 180 to 200 kDa, containing a conserved C-terminal 'separin' domain. In budding yeast (Ciosk et al 1998, McGrew et al 1992), fission yeast (Funabiki et al 1996b) and *Aspergillus* (May et al 1992), separins are essential for sister chromatid separation. Despite failing to separate sister chromatids, separin mutants proceed with most if not all other aspects of the cell cycle. It has been proposed that separins are dedicated 'sister-separating' proteins whose activity is held in check by their association with securins. According to this hypothesis, the APC mediates sister chromatid separation by liberating separin from its inhibitory embrace by securin (Fig. 5) (Ciosk et al 1998).

A clue to the mechanism by which separin splits sister chromatids was the observation that in budding yeast (contrary to most other eukaryotic cells) most Scc1 remains bound to chromosomes until the metaphase to anaphase transition (Michaelis et al 1997). Scc1's dissociation from chromosomes at the onset of anaphase depends on separin (Ciosk et al 1998) and is accompanied by Scc1's proteolytic cleavage, both *in vivo* and *in vitro* (Uhlmann et al 1999). Separin induces Scc1 cleavage at two related sites, each with an arginine in the P1 position. Mutation of either arginine to aspartic acid abolishes cleavage at that site but is not lethal to the cell. However, simultaneous mutation of both sites is lethal and prevents both sister chromatid separation and Scc1's dissociation from chromosomes (Uhlmann et al 1999). Similar potential cleavage sites are found in Rad21, the fission yeast Scc1 homologue, and their simultaneous (but not single) mutation also blocks chromosome segregation (Tomonaga et al 2000). Cleavage of cohesin's Sec1 subunit might therefore be a conserved feature of sister chromatid separation, at least in fungi (Fig. 5).

With the recent addition of several other separins to the databases, the conserved amino acid residues within the separin domain have been clarified. They include a universally conserved histidine and cysteine residue, which is a hallmark of cysteine endopeptidases (Barrett et al 1998). The sequences flanking these two residues are characteristic of cysteine endopeptidases of the CD subclass, which includes caspases, legumains, and two bacterial proteases, gingipain and clostripain (Chen et al 1998). Thus separin might indeed be the protease that cleaves Scc1. Whether cohesin's Scc1 subunit is separin's sole target is presently unclear but certainly possible, for the only other yeast protein to contain good matches to the Scc1/Rad21 consensus is Rec8, a related protein that replaces Scc1 in the cohesin complex during meiosis (Klein et al 1999). It will be crucial to address whether cleavage of Scc1 alone is sufficient to trigger anaphase in yeast and whether sister separation in animal and plant cells also depends on cleavage of cohesion proteins.

Separin's proposed C-terminal catalytic domain depends (at least for *in vivo* activity) on a long N-terminal domain, which is bound by its inhibitory securin chaperone (Kumada et al 1998). Securin must do more than just inhibit separin, because sister separation fails to occur in *cut2* (Funabiki et al 1996b) and *pimples* (Stratmann & Lehner 1996) mutants and is inefficient in *pds1* securin mutants (Ciosk et al 1998). Securin might either target separin to its future sites of action in the cell or help separin adopt a potentially active conformation, which is only unleashed on the cell when securins are destroyed by the APC.

Cutting the Gordian knot

Could proteolytic cleavage of a cohesin subunit really be a universal trigger for sister separation? If so, how does one explain the dissociation of the bulk of cohesin from chromosomes during pro-metaphase in organisms other than yeast (Losada et al 1998)? In vertebrates, this process clearly occurs in the absence of APC activity and is therefore presumably not due to separin activity (I. Sumara & J.-M. Peters, Personal communication). The implication is that there must exist two separate pathways for removing cohesin from chromosomes: one, thus far only detected in yeast, involving Scc1 cleavage at the metaphase to anaphase transition; and a second, possibly absent in yeast, that removes cohesin from chromosomes during pro-metaphase in the absence of cleavage (Fig. 6). It is of course possible that Scc1 is simply not cleaved at all by separin in animal cells and that some as yet unidentified cohesion protein that does indeed persist on chromosomes until metaphase, is separin's true target. Given the conservation of cell cycle mechanisms, it seems more likely that eukaryotic cells in fact possess both the cleavage and non-cleavage cohesin removal pathways and that separin's target is a residual amount of Scc1 associated with metaphase chromosomes, in particular in centromeric regions. Consistent with this hypothesis, a small fraction of cohesin remains associated with metaphase chromosomes in human cells and a similar fraction of Scc1 is cleaved around anaphase (Waizenegger et al 2000). Let us therefore explore this working hypothesis further, bearing in mind that what applies to cohesin could equally apply to other as yet unidentified cohesion proteins.

The non-cleavage pathway would remove most cohesin during prophase/pro-metaphase by an as yet obscure mechanism. This pathway could involve phosphorylation of a cohesin subunit by mitotic protein kinases, because vertebrate cohesins rebind to chromatin in telophase when mitotic kinases are inactivated and chromosomes decondense (Losada et al 1998). The dissociation of cohesin from chromatin during prophase coincides with, but does not depend on, the association of condensin with chromosomes. This first phase of cohesin removal may be crucial (possibly along with the arrival of

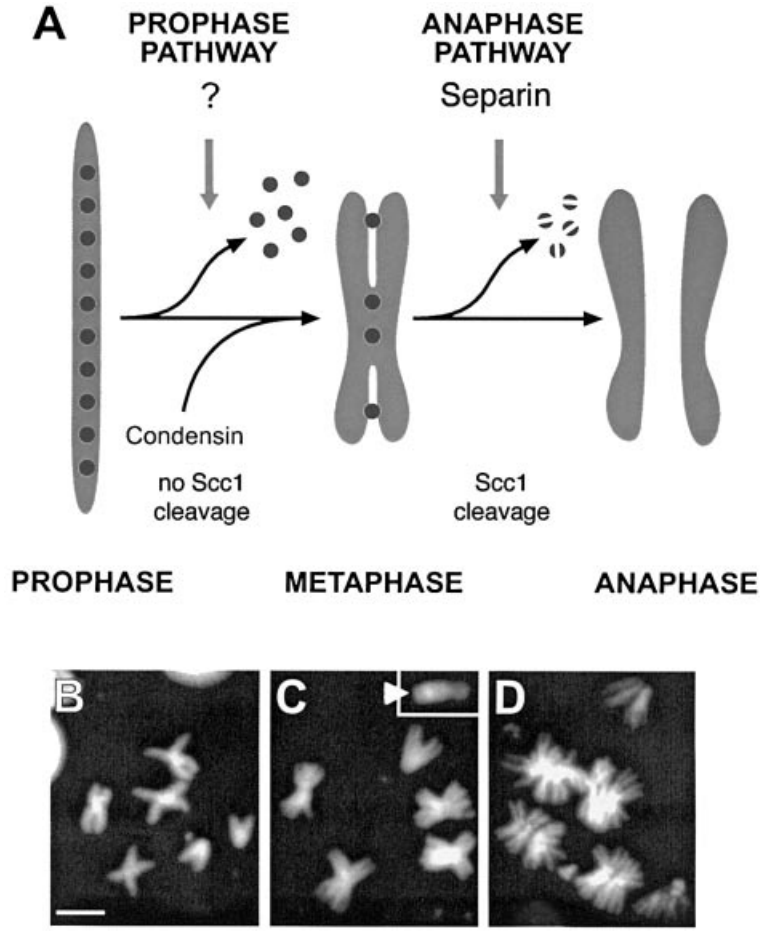

FIG. 6. (A) A two step model for the sequential loss of sister chromatid cohesion in animal cells. The bulk of cohesion proteins may be removed from condensing chromosomes during prophase by a separin-independent pathway, which might involve mitotic kinases such as Cdk1, Polo and Aurora. Activation of the separin pathway then initiates anaphase by cleaving residual cohesion proteins that remain on chromosomes, in particular at centromeres. (B–D) Mitotic chromosomes from wild-type *Drosophila* cells (B) and pimples mutant cells after one (C) and two (D) rounds of re-replication after possible failure of the separin pathway (Stratmann & Lehner 1996). Chromatids of autosomes are held together solely in pericentric heterochromatic regions but along the entire Y chromosome (inset C). Scale bar 5 μm.

condensin) for the initial splitting of chromosomes into two morphologically separable chromatids.

Though it commences during prophase, the non-cleavage pathway possibly does not complete its task before separins are activated after congression of all

chromatid pairs to the metaphase plate. This would explain why cohesion between chromosome arms is the last to be peeled away during undisturbed mitoses and why arm cohesion is apparently sufficient for orderly chromosome segregation when centromeric cohesion has been destroyed by a laser beam (Rieder & Cole 1999). Nevertheless, given sufficient time, the non-cleavage pathway is quite capable of removing all cohesin from chromosome arms, which explains why sister chromatid arms fully separate while centromeres remain connected in cells treated with spindle poisons (Rieder & Palazzo 1992) or in *Drosophila* mutants lacking either the APC activator Fizzy/Cdc20 (Sigrist et al 1995) or the putative securin pimples (Fig. 6B) (Stratmann & Lehner 1996).

According to our hypothesis, something prevents the full removal of cohesin from heterochromatic regions, including all centromeres, where the interface between sister chromatids during metaphase is far more extensive than along chromosome arms (Lica et al 1986, Rattner 1991). The final disentanglement of sister chromatids can only be achieved by cleavage of the 'Gordian knot' by separin. If as proposed by this hypothesis, cleavage of Scc1-like proteins is crucial for the final act of sister separation in all eukaryotic cells, this Achilles heal of the cohesion system deserves a nobler name ('gordin' for example) than the current ragbag of three letter words inherited from different organisms. It is currently unclear what property of heterochromatin might protect cohesin (or other cohesion proteins for that matter) from the non-cleavage dissociation pathway during metaphase. It is possible that the fairly widespread pathological phenomenon of premature centromere division (Fitzgerald et al 1975), which is thought to cause aneuploidy and is found in patients with Roberts syndrome (Tomkins et al 1979), might be caused by centromeric cohesion becoming susceptible to the non-cleavage pathway.

Controlled cutting

As Dan Mazia noted in 1961, 'Metaphase strikes us as an interruption of the flow of events, during which the mitotic apparatus is waiting for something to happen' and that, 'Chromosome splitting can be viewed as an event timed by a signal given by the cell and one that does not depend on the mitotic apparatus' (Mazia 1961). Mazia's 'signal' is presumably the liberation of separin from its inhibition by securin. If so, what initiates this process? Time-lapse photography of mitosis supplied the answer: 'Chromosomes that have already reached the equator wait for chromosomes delayed at one pole' (i.e. those that have not yet formed bipolar attachments to the spindle), 'And only when the metaphase plate contains all the chromosomes does anaphase begin' (Mazia 1961). This is a fairly clear description written over 40 years ago of the chromosome alignment surveillance mechanism, which is also called the spindle assembly checkpoint

(Amon 1999). In most, but not all eukaryotic cells, unaligned or lagging chromosomes transmit a signal via the protein Mad2, which inhibits the APC and its activator protein Cdc20 and thereby prevents the proteolysis of both B-type cyclins and securins. It is the block to securin destruction that prevents Scc1 cleavage and thereby sister chromatid separation (Fig. 5) (Alexandru et al 1999).

The Mad2 pathway is thought to be essential for regulating mitosis in somatic cells of many organisms. In its absence, chromatin bridges, lagging chromosomes, and chromosome fragmentation are observed during anaphase (Basu et al 1999). Most tumour cells are highly aneuploid and moreover have unstable karyotypes, which might be caused by defects in the Mad2 pathway (Cahill et al 1998). Nevertheless, destruction of securin by the APC is clearly tightly regulated by mechanisms that are independent of Mad2. These involve the accumulation of Cdc20 protein only as cells enter mitosis (Weinstein 1997, Shirayama et al 1998) and phosphorylation of APC by mitotic kinases, which enables the complex to respond to Cdc20 (Shteinberg et al 1999, R. Kramer & J.-M. Peters, Personal communication). Strikingly, sister chromatid separation remains tightly regulated in budding yeast mutants lacking securin (Alexandru et al 1999), suggesting that other mechanisms regulate cleavage of Scc1.

Summary

The pall of fog that has shrouded the sister separation process for over a century is starting to lift and this long mysterious process has started to reveal its secrets. There is now convincing evidence that the sudden movement of chromosomes to the poles at the onset of anaphase is triggered by cleavage of specific sister chromatid cohesion proteins. Future research must address the structural basis of cohesion and how it is established only at replication forks. It must also address the generality of mechanisms that dismantle cohesion at the metaphase to anaphase transition and how mistakes in this process contribute to human disease.

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DISCUSSION

Gönczy: Do you know whether the differences between mitosis and meiosis that you told us about in budding yeast also apply in mammals?

Nasmyth: In the second meiotic division the arms have come apart in every organism. As you saw in the yeast, they come apart at the first division because that is the mechanism by which chiasmata are resolved, but in humans the arms don't completely come apart. In a normal mitosis the arms are still glued together, but there is only a tenuous connection at the interface between those sort of sausages of condensed chromatin. The key thing is that blocking the APC, or activating the chromosome alignments surveillance mechanism or checkpoint blocks securin destruction. Consequently, this pathway that removes most of the cohesin from the arms goes on to completion, and the arms drift apart in all places except at places of heterochromatin. This includes, of course, the centromeres. It seems that arm separation can take place in mitosis without separin. The data are all consistent with this. The crucial question is whether in meiosis I, where it is arm separation that likely triggers the first division, this is mediated by separin or not. A student in my lab has knocked out the worm separin and has shown that it is needed for the first meiotic division. Arm separation in meiosis should be added to the list of major differences between mitosis and meiosis: in meiosis it is separin-dependent, whereas in mitosis it is not.

Vande Woude: What is activating the separin in meiosis I?

Nasmyth: We have looked at securin distribution during meiosis. From pre-meiotic DNA replication until meiosis I, securin is present all the way through, and Cdc20 is absent. Cdc20 rises dramatically just before the first division, and at this time securin decreases. Separin is needed for this first division. Our hypothesis is that in the first division securin destruction is crucial to activate the separin,

which then cleaves the Rec8. Then they come out of that division and Cdc20 goes down, securin goes back up and just before the second division Cdc20 goes back up and securin goes down. This is why we think that separin is cleaving the residual Rec8 that was retained in the centromeres at the second division. It is very difficult to do any biochemistry at that stage, because 95% of the Rec8 is dealt with at the first division: we are only looking at about 5%, and there is only about an hour between those divisions. The culture synchrony is just not good enough for us to be able to examine this closely.

Vande Woude: If you over-express separin, will that begin to target the Rec8 that is at the centromeres, or is there some post-translational modification of it?

Nasmyth: I suspect that there are two other classes of regulation of securin destruction by the APC. One is that in yeast mitosis, securin is not an essential gene. You can remove it and at low temperatures cells will proliferate fine, and cleavage is pretty well regulated. Something else is regulating the cleavage reaction other than APC-mediated destruction of securin. It is just not taking place in those cells. Our current working hypothesis is that it turns out that Scc1 and Rec8 are both hyperphosphorylated around the time of mitosis and the first meiotic division, respectively. Frank Uhlmann can make unphosphorylated Scc1 from baculovirus and phosphorylate it either *invivo* or *invitro*, and then it becomes a 5–10-fold better substrate for separin *invitro*. This is another mechanism, but I am not sure how important it is. We suspect that it is not absolutely required; it just helps it work better. But it might become absolutely crucial if securin is absent or the separin is not working terribly well. Phosphorylation of substrate is therefore a second mechanism that regulates Scc1 cleavage. Also, by analogy with caspases it is quite conceivable that separin, in addition to being released from securin, has to undergo some kind of autocatalytic cleavage to be activated. However, there is no evidence for this at the moment. If this were the case, this event could also be regulated.

Maller: What kinase do you think phosphorylates separin?

Nasmyth: It is Polo/Cdc5. And in a Polo/Cdc5 mutant, most cleavage still takes place, which is why we have been so slow to realise that it was actually Polo that was responsible. If you knock out both Polo and securin (Pds1), then the cleavage is very inefficient.

Maller: Kinase-dead Polo blocks the metaphase/anaphase transition, so this might be the mechanism.

Nasmyth: In the yeast, most of the cleavage takes place in a Polo mutant, and the centromeres go to the poles quite normally. But the chromosomes don't fully disengage. We suspect about 10% of the Scc1 may not be coming off. If you make a Pds1/Polo double mutant (which is difficult because they are almost synthetic lethal), then it looks like there isn't much anaphase at all. But these are recent, preliminary results.

Maller: This suggests that blockade by kinase-dead Polo is not acting directly on the APC.

Nasmyth: In this case, this is completely independent of the APC.

Nurse: That is in mitotic cells. In meiotic cells, is there a role for Polo?

Nasmyth: Almost certainly, in phosphorylating Rec8.

Kubiak: Listening to your paper I understood that mitotic, not meiotic, cohesins could be the ideal targets for cytostatic factor (CSF) in vertebrate cells.

Nasmyth: CSF has to affect more than just cohesins, because it has to block Cdk1 down-regulation. I think the APC is the target for CSF. It is no good just going for separin or the cohesins, because this will not explain why Cdk1 stays high. Through the APC it prevents securin destruction, which then prevents separin activation. This is why sister chromatids don't come apart and why cells don't do anaphase.

Kubiak: If CSF acts on cohesins, this could concern only the mitotic version of cohesin. We introduced CSF into metaphase I oocytes of mice, and it didn't disturb the metaphaseI/anaphase I transition (Ciemerych & Kubiak 1998).

Nasmyth: I'm not sure that it is necessarily cohesins. The APC probably changes between the first and second division. I am sure that there are a lot of differences in the activity of the APC between meiosis I and meiosis II. There are all sorts of things that have to be destroyed in the first division in order to do the second division.

Lehner: There is a paper by Goldstein (1981) in which he describes electron microscope (EM) studies of meiotic chromosomes. At meiosis I, chromosomes do not have two sister kinetochores. There is a maturation into two kinetochores between the two meiotic divisions. Have comparable EM analyses been done in yeast?

Nasmyth: There is nothing to see in yeast.

Hunt: I thought you could see *Saccharomyces cerevisiae* meiotic chromosomes quite nicely.

Nasmyth: Yes, but in the EM you can't see the structure. There is no electron-dense material to see; it is just too small.

Harper: Is there any evidence that Esp1 localization might be regulated?

Nasmyth: There is evidence in both fission and budding yeast. It tends to hang around in the cytoplasm and go to the nucleus around the time of its activation. Yenagida has shown that quite a lot of it is associated with the mitotic spindle in fission yeast. He thinks that separin has nothing to do with cohesins or cleavage, and that its main function is to regulate the spindle. There are two very firmly held views on this! But, in the human cell you never see separin anywhere near a spindle, according to Jan Michael Peters.

Harper: In the Pds1 deletion, is the ability of Esp1 to ultimately allow for separation altered?

Nasmyth: It is definitely compromised in yeast. But thankfully they are not completely dead, which allowed Doug Koshland and us to do the critical experiment which really pinpointed securin as the key target. In this we took an APC mutant, knocked out securin, and showed that then they would separate sister chromatids.

Hunt: There are differences between budding and fission yeast in the importance of Pds1.

Nasmyth: Yes, in fission yeast securin is essential.

Hunt: Cut2 (Pds1) looks like some kind of a chaperone-like protein that sets up Cut1 (Esp1) to do its stuff.

Nasmyth: My guess is that it is an inhibitor and something that targets separin to the right place.

Hunt: Although it doesn't look like it is going to the right place. Not much of the Cut2/Cut1 complex is where you would expect it to be.

Nasmyth: Cytologically we don't see it associated with chromosomes. From an indirect assay, if we isolate yeast chromosomes there is Esp1 there. We have a biological assay for that.

Nurse: Another difference is that the mitotic cohesin persists in fission yeast meiosis, which is not the case in budding yeast. I think what is exciting about this, however, are the similarities, so we shouldn't get too worried by the differences. We have talked a lot about cohesin, but I wondered whether it might be worth having some discussion of the S phase suppression.

Maller: Kim Nasmyth mentioned that perhaps Cdc6 was missing, but this is clearly not the case in *Xenopus*, because you can add cycloheximide and you immediately go into S phase. The only means of suppressing S phase is the presence of cyclin B/Cdc2, which is what Paul Nurse found in *S. pombe* some years ago.

Nurse: Actually, Cdc18 is present in fission yeast meiosis, which is a Cdc6 equivalent. I think there is more of a story here.

Hunt: In frogs, if you remove the B type cyclins using antisense RNA, this allows you to go straight into a premature S phase.

Nasmyth: If you reactivated the kinase, given that the kinase inhibits pre-replication complex formation, this would do the job. There are two ways of preventing replication: one is not to make the components of the pre-replication complex, the other is to keep the kinase high, which inhibits that formation. This could be an explanation of the *Xenopus* work.

Maller: I don't think anyone has looked at the origins during the meiosis I to II transition to see what is there.

Nasmyth: Again, this is a difficult thing to do, even in yeast.

Nurse: We are trying to do it.

Lehner: I wonder how complete the inactivation of MPF is between meiosis I and meiosis II.

Maller: It is only 50% in *Xenopus*.

Hunt: In frogs we never get it down to baseline.

Kubiak: In mouse it is definitely 100% inactivation.

Nurse: Work in starfish suggested that Cdk activity remained quite high, so that is the simplest model, and that is likely to be the case in fission yeast as well. We are cataloguing all the different components to see if we can work out the regulation of Cdk. It is consistent with but not yet proven that cdk regulation could regulate S phase between meiosis I and meiosis II. But I am worried about this mouse observation. Did you look at total H1 histone kinase?

Kubiak: Yes, we observed the interphase level for 30 min.

Maller: What about blottable cyclin B? Is it all completely gone?

Kubiak: Almost completely. We find only traces of cyclin B1, like just after mitosis.

Maller: There are some stories coming out that Wee1 comes up in meiosis II. There might be low H1 kinase activity, but there is still cyclin B present in a tyrosine-phosphorylated Cdc2 complex, and this might or might not be able to signal something about cell cycle phase.

Nasmyth: We know that in G2, where H1 kinase is not high, we know that there is sufficient cdk activity to inhibit pre-replication complex formation. If it has gone down at interphase, there still could be a lot of cdk present.

Gautier: As far as the pre-replication complex is concerned, just looking at the level of Cdc6/Cdc18 is not going to tell us anything, because in *Xenopus* eggs all the maternal protein is already made for 10 divisions.

Hunt: Take Orc1, for example: there is a lot of Orc1 synthesis in that interval. There isn't very much in a stage 6 oocyte. You don't need many of those proteins because there is only one set of chromosomes.

Nasmyth: How much do the chromosomes decondense?

Hunt: Not at all.

Nasmyth: That would explain things quite nicely! What I was trying to say is that there are a lot of mechanisms we can envisage that would deal with this problem.

Maller: We think S phase suppression after anaphase I is just a secondary effect of having significant Cdc2 kinase activity still around. The MAP kinase pathway is unable to suppress S phase in the absence of cyclin B.

Nasmyth: It probably has nothing to do with Cdc2 kinase.

Kubiak: It's rather MAP kinase. In mouse oocytes it is MAP kinase.

Nasmyth: It may be Cdc2; it may not.

Hunt: In *Xenopus* oocytes the maintenance of high MAP kinase levels depends on maintaining a significant level of cyclin B-dependent kinase. If you do away with the cyclin B kinase, then the Mos becomes unstable and goes away, and then MAP

kinase turns off. There is a complicated mutual positive feedback mechanism going on in that interval which is important and which is not fully understood. One other thing goes on at this time, which is the splitting or duplication of the spindle pole that has to take place. The cell has to reassemble a bipolar spindle between meiosis I and meiosis II.

Vande Woude: There are suppositions about a half spindle regenerating to a full spindle.

Kubiak: That is probably the reason why there are no centrioles in oocytes.

Hunt: All I know is what happens in clams, because I have seen it with my own eyes: you do not duplicate the spindle pole if you inhibit protein synthesis, so you get a monopolar second meiotic spindle, which causes chaos.

Nasmyth: These things can be quite specific to each system.

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The cell cycle machinery and asymmetric cell division of neural progenitors in the *Drosophila* embryonic central nervous system

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Abstract. Asymmetric cell divisions can be mediated by the preferential segregation of intrinsic cell fate determinants into one of two sibling daughters. In dividing *Drosophila* neural progenitors the apical–basal orientation of the mitotic spindle, the basal cortical localization of the cell fate determinants Numb and/or Prospero as well as the coordination of these events are mediated by several proteins which include Bazooka (Baz), Inscuteable (Insc) and Partner of Inscuteable (Pins) which localize as an apical cortical complex starting at interphase. Here I will summarize data which suggest that the formation of this apical complex involves two distinct steps: (1) during the initiation of apical complex formation in interphase neuroblasts, there appears to be a hierarchical relation amongst these components where Baz recruits Insc and Baz/Insc in turn recruit Pins to the apical cortex/stalk; (2) while in delaminated mitotic neuroblasts the maintenance of the apical cortical localization of these proteins is dependent on the presence of all three components. Moreover, we show that the maintenance of this apical protein complex is essential for the correct execution of asymmetric division. Finally, the localization of the various asymmetrically localized proteins shows cell cycle dependence; however, the involvement of the cell cycle regulator in asymmetric cell divisions has not been previously shown. Here we present evidence from ongoing experiments which suggest a requirement for the key cell cycle regulator *cdc2* in asymmetric cell divisions.

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Asymmetric cell division is a universal mechanism utilized for the generation of cellular diversity during development (for reviews see Horvitz & Herskowitz 1992, Guo & Kemphues 1996, Shapiro & Losick 1997, Jan & Jan 1998). Asymmetric cell divisions during *Drosophila* embryonic development involve both extrinsic cues mediated through Notch and Delta and intrinsic cell fate determinants and play a major role in producing the distinct cell types which are

organized into the stereotypic patterns seen in the central (Spana & Doe 1996, Skeath & Doe 1998) and peripheral (Guo et al 1996, Dye et al 1998) nervous systems (CNS and PNS) as well as the somatic musculature (Ruiz Gómez & Bate 1997, Carmena et al 1998).

Neuroblasts are neural progenitor cells which form by delamination from the ventral neuroectoderm. They undergo repeated divisions to produce several ganglion mother cells (GMCs) from their dorsal/lateral surfaces; each GMC divides once to generate two progeny neurons. CNS neurons are derived largely from neuroblast lineages (Bossing et al 1996, Schmidt et al 1997, Schmid et al 1999). Both neuroblast and GMC cell divisions can be asymmetric; however, they apparently rely on different intrinsic cell fate determinants. *prospero* (*pros*) encodes a homeo-domain protein (Doe et al 1991, Vaessin et al 1991, Matsuzaki et al 1992) and acts as a cell fate determinant for the neuroblast divisions; *pros* protein as well as RNA are asymmetrically localised as basal cortical crescents in dividing neuroblasts (Hirata et al 1995, Knoblich et al 1995, Spana & Doe 1995, Li et al 1997, Broadus et al 1998, Schuldt et al 1998), segregating only to the GMC daughter where Pros protein translocates to the nucleus and acts to promote the expression of GMC specific genes and repress the expression of neuroblast specific genes (Doe et al 1991, Vaessin et al 1991). *numb* (Uemera et al 1989) encodes a membrane associated protein which down-regulates Notch signalling. Numb localizes as a basal crescent in both neuroblasts (Rhyu et al 1994) and some GMCs but does not appear to play a role in the neuroblast asymmetric divisions (Lear et al 1999). However, in some GMC divisions, Numb is preferentially segregated to one daughter and acts as a cell fate determinant to mediate the distinct fates of sibling neurons (Buescher et al 1998).

inscuteable (*insc*) encodes a protein which is apically localized (Kraut & Campos-Ortega 1996). In neuroblasts, Insc is apically localised during interphase, prior to the formation of the mitotic spindle and prior to the basal localisation of Numb, Pros and *pros* RNA (Kraut et al 1996, Li et al 1997). *insc* function is required in dividing neuroblasts and cells of mitotic domain 9 to mediate and to coordinate the basal localisation cell fate determinants as well as the orientation of the mitotic spindle along the apical/basal axis. In addition, ectopic expression of Insc in ectodermal cells, which normally divide with their mitotic spindle parallel to the epithelial surface, causes an extra 90° spindle rotation, resulting in cells which divide with their spindle oriented along the apical/basal axis (Kraut et al 1996, Kaltschmidt et al 2000). Insc is also localized as an apical crescent in dividing GMCs; *insc* is required for the asymmetric localization and segregation of Numb and for the resolution of distinct fates for the sibling neurons derived from (at least some) GMC divisions; moreover, the nuclear and cell size asymmetry exhibited by some sibling neurons also require *insc* (Buescher et al 1998). These observations suggest that *insc* might act by providing positional information necessary to

coordinate and mediate the processes which together ensure the correct execution of asymmetric cell divisions.

Miranda (Mir; Shen et al 1997, Ikeshima-Kataoka et al 1997), Staufen (Li et al 1997, Broadus et al 1998, Schuldt et al 1998) and Partner of Numb (Pon; Lu et al 1998), act as a link between the apically localized Insc and the basally localized cell fate determinants. These adaptors act downstream of *insc* and are also asymmetrically localised, similar to the cell fate determinants they help to localize, in an *insc*-dependent manner.

bazooka (*baz*), a *Drosophila* homologue of the *Nematode par3* gene (Etemad-Moghadam et al 1995), encodes a multiple PDZ domain protein which is required for the apical/basal polarity of the neuroepithelium (Kuchinke et al 1998, Muller & Wieschaus 1996). It interacts/complexes with Insc and is required for Insc asymmetric localization. Baz is localized apically in the neuroepithelium as well as in dividing neuroblasts and may act to link neuroblast polarity to the (Kuchinke et al 1998, Schober et al 1999, Wodarz et al 1999) apical–basal polarity of the epithelium by recruiting Insc to the apical cortex during delamination.

A schematic summary of the localization of the key components of the asymmetric division process is given in Fig. 1.

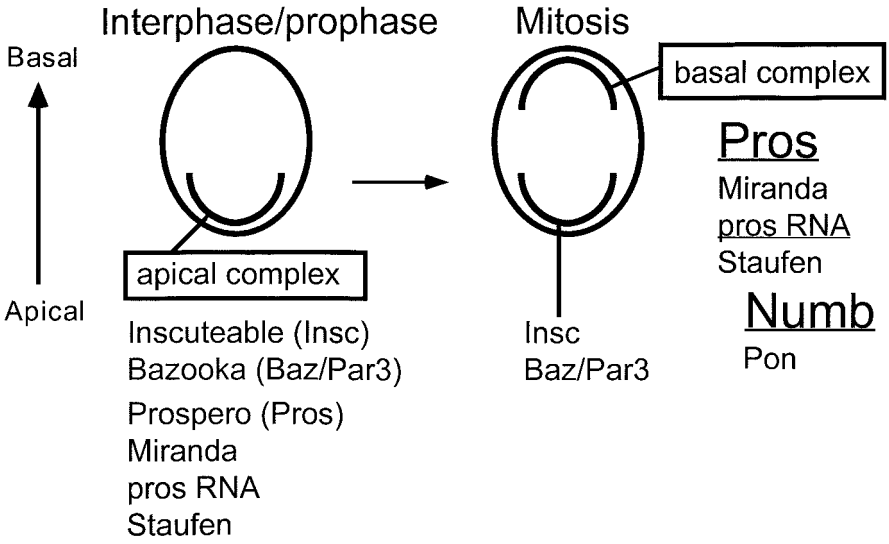

FIG. 1. Localization of key proteins involved in the neuroblast asymmetric cell division. During late interphase a complex of proteins including Insc, Baz (and Pins) are localized to the apical cell cortex. This complex acts to mediate the basal cortical localization of the cell fate determinants Numb (and its partner Pon), Pros (and its partner Miranda) and *pros* RNA (and its partner Staufen) during mitosis. During interphase, Numb is cytoplasmic and Pros is localized to the apical cortex.

Results and discussion

Pins interacts and co-localizes with Insc

We have previously shown that a 209 amino acid region (aa288–497, asymmetric localization domain) of Insc is necessary and sufficient for apical cortical localization and for mitotic spindle orientation along the apical–basal axis (Tio et al 1999). In a yeast two-hybrid screen we identified Partner of Inscuteable (Pins), a novel 658aa protein with multiple repeats of the Tetratricopeptide (TPR) motif. Affinity purification experiments using embryonic extracts demonstrate that Pins complexes with Insc *in vivo*. *In vitro* protein interaction assays demonstrates that Pins interacts with the Insc asymmetric localization domain (see Yu et al 2000).

Antibodies were raised against Pins fusion proteins which specifically recognize Pins. In neuroblasts Pins is localized as a crescent to the apical cortex starting at interphase following delamination. More intensely labelled Pins apical crescents can be seen during mitosis from prophase to anaphase. Double labellings with anti-Insc indicate that Pins and Insc are largely co-localized during the neuroblast cell cycle. The exception being in delaminating neuroblasts where high levels of Insc staining can be seen on the apical stalk which extends from the neuroblast towards the surface of the neuroectoderm; in contrast, high levels of apical Pins are detected only following neuroblast delamination. Hence although Insc and Pins are co-localized in fully delaminated neuroblasts, the initial localization of Insc to the apical stalk during delamination (interphase) may precede that of the Pins apical localization.

Apical cortical localization of Pins, Insc and Baz in mitotic neuroblasts is codependent

In order to assess the function of *pins*, we generated several small deletions which removed all or part of the *pins* coding region. Two alleles which are antigen negative were viable and allowed us to obtain Pins$^-$ embryos which lacked both the maternal and zygotic components of *pins*. The availability of embryos lacking *baz*, *insc* or *pins* enabled us to assess the localization of each of the three apical components in embryos lacking either of the other two functions. In wild-type mitotic neuroblasts, Baz, Insc and Pins co-localize as apical crescents. However, in mitotic neuroblasts lacking either *baz* or *insc*, Pins becomes distributed throughout the cortex. In *baz* or *pins* mutant embryos, Insc is localized to the cytoplasm of mitotic neuroblasts. Similarly, in mitotic neuroblasts lacking either *pins* or *insc*, Baz apical crescents are either undetectable or its levels are drastically reduced. Hence in neuroblasts which have completed delamination or are undergoing mitosis, the asymmetric localization/stability of Baz/Insc/Pins are

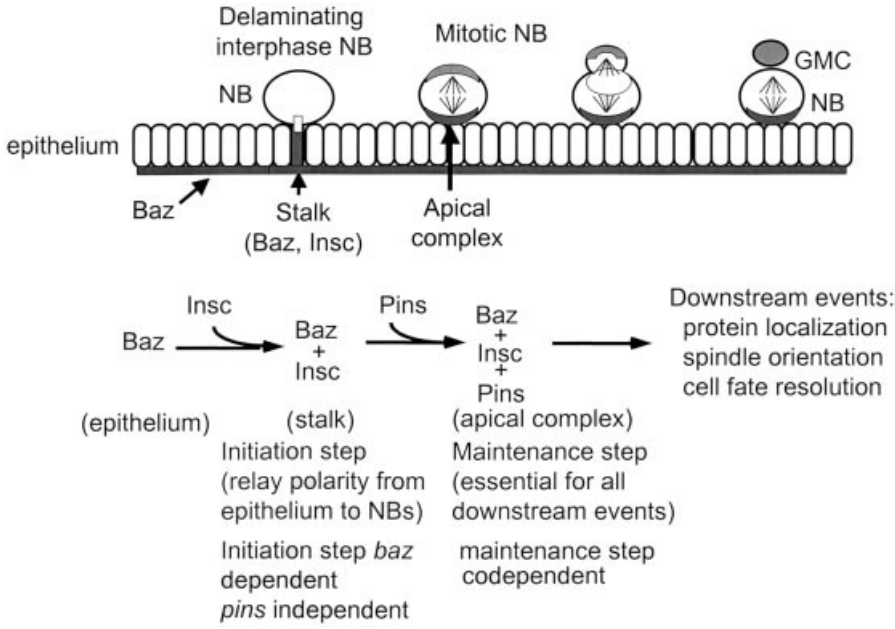

FIG. 2. The formation of the apical protein complex involves two distinct steps. Bazooka is localized apically in the epithelium from which neuroblasts are derived. In the interphase (G2), delaminating neuroblast formation of the apical complex is initiated. It is thought that Baz acts to allow neuroblasts to retain the apical/basal polarity inherent in the epithelium. Baz recruits Insc to the neuroblast apical stalk during delamination before Pins becomes part of the complex. During this initiation step Baz, Insc and Pins are part of a linear hierarchy. However following delamination and during mitosis, the maintenance of the apical localization of each of these proteins requires all three proteins.

mutually dependent. Loss of any single component will result in defective localization or instability of the remaining two components (see Fig. 2).

baz, insc and pins share a hierarchical relationship during the initiation of apical complex formation in interphase neuroblasts

However, in interphase delaminating neuroblasts, which are known to have completed S-phase and are at the G2 stage of the cell cycle, this codependence of Baz/Insc/Pins seen in mitotic neuroblasts does not apply. Delaminating neuroblasts possess an apical membrane stalk which retains contact with the epithelial surface and this is where apical cortical localization of Insc is initially seen (see Fig. 2). This initial localization of Insc to the apical stalk occurs

normally in Pins$^-$ embryos. In contrast, it is not seen in delaminating neuroblasts of *baz* mutants. Moreover, the localization of Baz to the stalk does not depend on either *insc* or *pins*. These results indicate that the initial localization of Baz to the stalk of delaminating neuroblasts requires neither *insc* nor *pins*; the initiation of Insc apical localization to the stalk requires *baz* but not *pins*; however, the maintenance of apical Baz/Pins/Insc following delamination and in mitotic neuroblasts is mutually dependent, requiring all three components. Hence during the initiation of apical complex formation, Baz/Insc/Pins appear to have a hierarchical relationship with Baz being the most upstream and Pins being the most downstream component. However for the maintenance of this apical complex during mitosis, they are codependent. A schematic summary of these conclusions is shown in Fig. 2.

Insc plays a role in the recruitment of Pins to the apical cortex

Epithelial cells express but do not apically localize Pins, and do not express Insc. We have previously shown that ectopically expressed Insc localizes to the apical cortex in wild-type epithelial cells (Kraut et al 1996). Interestingly, ectopic Insc expression causes Pins, which is normally localized to the lateral cortex, to localize to the apical cortex. Conversely, apical localization of ectopically expressed Insc is dependent on *pins*. Insc ectopically expressed in Pins$^-$ epithelial cells does not localize as an apical crescent; it adopts a cytoplasmic distribution which is enriched towards the apical side of the cell during interphase and is undetectable during mitosis, presumably due to rapid degradation. This instability of ectopically expressed Insc may be why the 90° rotation in the mitotic spindles which occurs as a consequence of Insc ectopic expression in the wild-type epithelial cells no longer occurs when Insc is expressed in Pins$^-$ embryos. These results indicate that Insc is necessary and sufficient for the recruitment of Pins to the apical cortex of wild-type epithelial cells.

Maintenance of the apical complex is necessary for all aspects of asymmetric cell division

In Pins$^-$ embryos the initiation steps of apical complex formation occur normally. However, this complex cannot be maintained in mitotic neuroblasts. Hence, the importance of the maintenance of this complex for asymmetric cell division can be ascertained by assessing how Pins$^-$ neural progenitors divide. Pins$^-$ embryos exhibit all of the defects seen in *insc* mutants. Mitotic spindle orientation is defective. In the cells of mitotic domain 9 the 90° reorientation, which normally occurs in wild-type resulting in the orientation of the spindle along the apical–basal axis (Fig. 3A), fails to occur in the mutant (Fig. 3B). Mitotic spindle orientation of neuroblasts in the segmented CNS, deduced from DNA staining, also often fails to

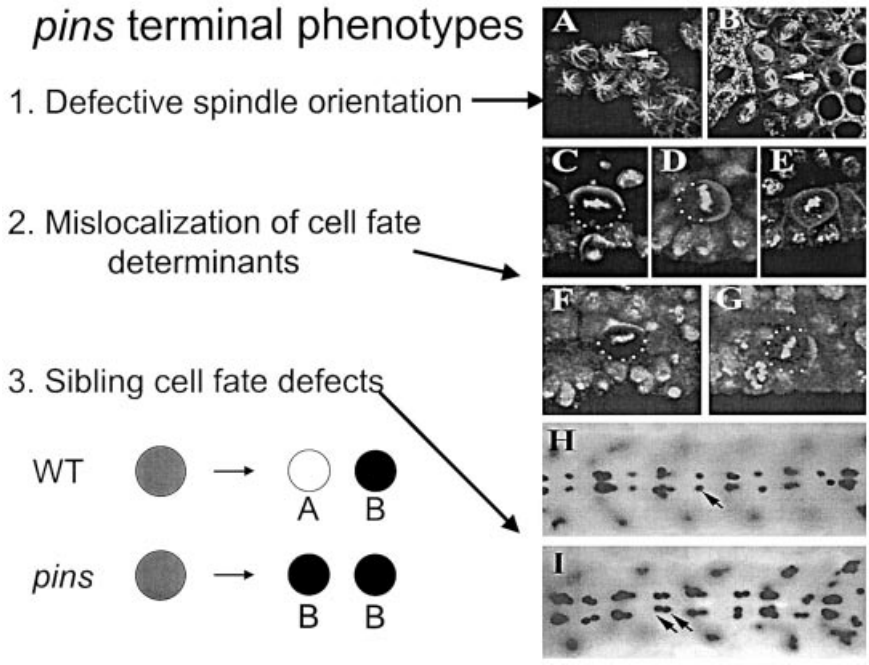

FIG. 3. Loss of *pins* function results in defects similar to those seen in *insc* mutants. In wild-type the mitotic spindle in Insc expressing cells, visualized by anti-β-tubulin staining, undergoes a 90° reorientation and adopts a apical/basal orientation perpendicular to the surface of the embryo (A). In *pins*$^-$ embryos this 90° spindle reorientation does not occur and the mitotic spindles are oriented parallel to the surface (B). In wild-type embryos Pros (C) and Pon (F) are localized as basal cortical crescents in mitotic neuroblasts. In *pins*$^-$ neuroblasts, Pros (D,E) and Pon (F) are often mislocalized, showing cortical distribution (E) or are localized as misplaced crescents (D,G). In wild-type embryos GMC4-2a divides asymmetrically to produce the sibling neurons RP2 (arrow) and RP2sib in each hemisegment (H). In *pins* embryos GMC4-2a often divides symmetrically to produce two RP2 neurons (I).

adopt an apical–basal orientation (e.g. Fig. 3D, 3G). Mir/Pros and Pon/Numb normally localize as basal crescents in wild-type metaphase neuroblasts (Fig. 3C, 3F). However, in Pins$^-$ metaphase neuroblasts these proteins often show defective localization, in the form of mislocalized crescents (Fig. 3D, 3G) and cortical localization (Fig. 3E). Resolution of distinct fates for the sibling neurons RP2 and RP2sib also frequently fail to occur. In $\sim$60% of the mutant hemisegments, duplicated RP2 neurons are found at the expense of the RP2sib (Fig. 3I). Moreover, the two RP2 neurons appear to have indistinguishable nuclear size, a phenotype also seen in *insc* mutants (Buescher et al 1998). This

similarity in the *pins* and *insc* mutants across a range of phenotypes indicates that the maintenance of the apical complex is necessary for the correct execution of neural progenitor asymmetric cell divisions. Expression of a wild-type *pins* transgene in neural tissues of Pins$^-$ embryos rescues all of the defects associated with *pins* loss of function.

A role for cdc2 in asymmetric cell divisions

The localization of the cell fate determinants show striking cell cycle dependence. For example, Pros is localized as an apical crescent at G2 but quickly adopts a basal localization upon entry to mitosis. Hence it seems likely that there is a connection between how cell cycle and asymmetric cell division are regulated. In a screen for mutants in which normally asymmetric cell divisions divide symmetrically, we identified a *cdc2* allele, $cdc2^{E51Q}$, which was caused by a single E to Q amino acid change at the conserved residue 51. We focused on the well characterized GMC4-2a sublineage in the embryonic CNS for the analysis of the mutant phenotype. In wild-type embryos, GMC4-2a divides to produce the RP2 neuron and its sibling, RP2sib; however, $cdc2^{E51Q}$ homozygous embryos, 33% of the GMC4-2a divides to produce two RP2 neurons at the expense of RP2sib. Hence the normally asymmetric division of GMC4-2a (GMC4-2a > RP2+RP2sib) can be converted to a symmetric division (GMC > RP2+RP2) in the mutant. In addition about 14% of the GMC4-2a fail to divide (see Fig. 4 for a schematic summary).

$cdc2^{E51Q}$ neural progenitors exhibit defects in asymmetric protein localization

Not surprisingly, $cdc2^{E51Q}$ homozygous embryos also exhibit defects in the asymmetric localization of Insc, Partner of Numb (Pon, which always colocalizes with Numb) and Miranda (which always colocalizes with Pros). Dividing GMC4-2a in wild-type embryos always localizes Insc as an apical cortical crescent and Pon as a basal cortical crescent. In dividing $cdc2^{E51Q}$ GMC4-2a defective localization of Insc and Pon, in the form of cortical distribution or misplaced crescents is seen. Mislocalization of Insc, Pon and Miranda can also be seen in mutant mitotic neuroblasts. These data suggest that the underlying cause of the abnormal symmetric GMC4-2a divisions seen in $cdc2^{E51Q}$ is the failure to maintain apical localization of Insc (and Pins) during mitosis, and consequently, localization of the basal determinants is also defective. Consistent with this notion, the duplicated RP2 neurons show identical nuclear size, a phenotype which is characteristic of *insc* and *pins* mutants.

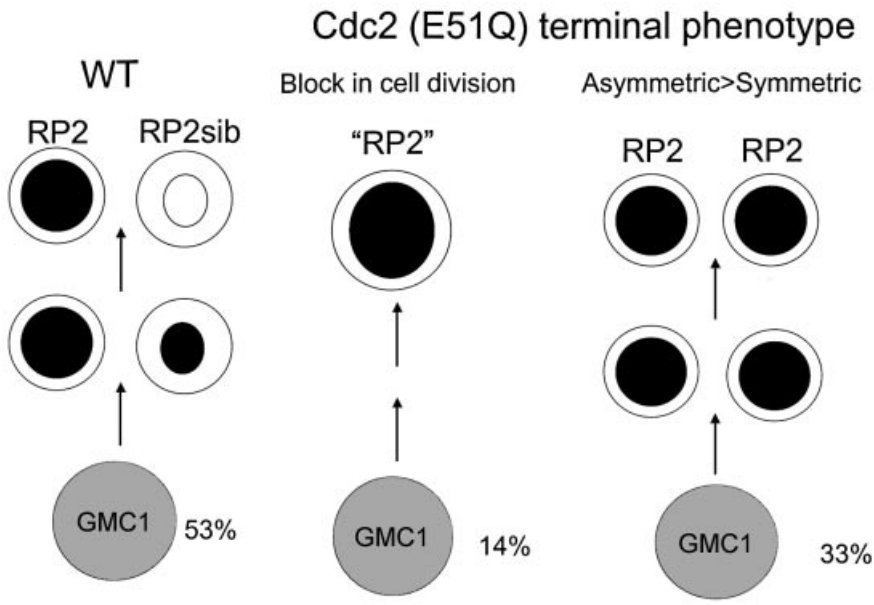

FIG. 4. In *cdc2*^{E51Q} embryos progenitor cell divisions which are normally asymmetric can become symmetric. In wild-type GMC4-2a normally divides asymmetrically to produce two distinct neuronal daughters RP2 and RP2sib. In *cdc2*^{E51Q} embryos two types of defects are seen. GMC4-2a can fail to divide and differentiate into a 'large RP2-like' cell; GMC4-1a can divide symmetrically to produce two RP2 neurons.

cdc2^{E51Q} *acts as a maternal effect dominant negative*

Although embryonic lethality and defects in asymmetric division are seen in *cdc2*^{E51Q}, embryos homozygous for null/amorphic *cdc2* alleles develop essentially normally, presumably due to maternally inherited wild-type *cdc2*. Several observations indicate that *cdc2*^{E51Q} acts as a maternal effect dominant negative allele and can neutralize the function of the maternally inherited wild-type *cdc2*, perhaps by titrating out some component necessary for *cdc2* function. The asymmetric cell division and cell fate defects are seen at high frequency in genotypically hemizygous (*cdc2*^{E51Q}/deficiency) embryos only if the *cdc2*^{E51Q} allele is inherited from the (*cdc2*^{E51Q}/CyO) mother but not if it comes from the father. Moreover an earlier arrest of cell divisions can be seen by over-expressing the *cdc2*^{E51Q} mutant product (from a uas- *cdc2*^{E51Q} transgene) in *cdc2*^{E51Q} homozygous embryos.

To see whether similar defects in asymmetric cell divisions and protein localization can be seen in other *cdc2* mutant combinations, we used a stock

which is homozygous for an amorphic allele, *cdc2*^{B47} and, in addition, contains four copies of a transgene carrying a temperature-sensitive allele, *cdc2*^{A171T}, (henceforth referred to as *cdc2*^{ts}*4X*) (Sigrist et al 1995). The 5′-splice consensus site in the first intron is mutated in *cdc2*^{B47} and does not make a full length products; it has been shown that immunoprecipitates from *cdc2*^{ts}*4X* embryonic extracts contain exclusively *cdc2*^{A171T} kinase whose activity is highly temperature sensitive *in vitro* with histone H1 as substrate. Using appropriate temperature shift conditions these embryos exhibit all of the phenotypes seen in *cdc2*^{E51Q} including RP2 duplication and defective protein localization in both neuroblasts and GMCs.

Cdc2 kinase activity is required during mitosis but not during interphase for apical Insc localization

To ascertain whether Cdc2 kinase activity is required for asymmetric cell divisions, a binary expression system was used to express wild-type and mutated forms of *cdc2* in the neural progenitors of *cdc2*^{E51Q} homozygous embryos. Expression of a wild-type *cdc2* transgene rescued all aspects of the *cdc2*^{E51Q} phenotypes whereas the expression of a kinase-dead version of Cdc2 which does not exhibit dominant negative properties, e.g. T161A which affects cyclin binding, did not rescue any aspect of the phenotype, suggesting that Cdc2 kinase activity is involved in mediating neural progenitor asymmetric division. Since Cdc2 kinase activity appears to be required to maintain apical Insc localization during mitosis, is it possible that it is also required for the apical localization of Insc during interphase? In embryos lacking zygotic *string* (the *Drosophila cdc25* homologue), all neuroblasts arrest at G2 and fail to enter mitosis because Cdc2 kinase activation does not occur. Insc and Pins form normal apical crescents indicating that the initial apical localization of Insc (and Pins) during interphase does not require Cdc2 kinase activation. Moreover, appropriate temperature upshifts will induce similar *string*-like phenotypes in *cdc2*^{ts}*4X* embryos and in these embryos, the neuroblasts arrested at G2 show normal localization of Insc. These data suggest that Cdc2 kinase activity is required only to maintain Insc/Pins apical localization during mitosis.

Differing levels of cdc2 activity determines whether a progenitor divides at all, divides symmetrically or divides asymmetrically

Our results indicate that the key cell cycle regulator *cdc2* plays a role in asymmetric cell divisions. Through the use of maternal dominant and temperature sensitive alleles, the levels of *cdc2* activity can be manipulated such that a neural progenitor has sufficient activity to undergo and complete mitosis but insufficient *cdc2* function to divide asymmetrically. Like entry to mitosis, Cdc2 kinase is

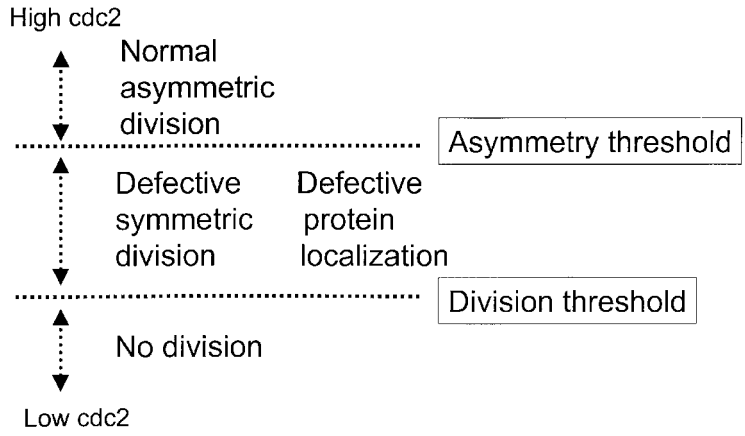

FIG. 5. The level of *cdc2* activity determines whether a progenitor divides, divides symmetrically or divides asymmetrically. A schematic model is shown.

apparently required to mediate the asymmetry related functions because kinase dead versions of *cdc2* cannot rescue these defects. Phosphorylation mediated by Cdc2 is likely to play an important role in maintaining the correct localization of the apical complex of asymmetry proteins during mitosis. However, Cdc2 probably does not act directly on Insc since the putative Cdc2 phosphorylation sites of Insc can be removed without affecting its function in over-expression paradigms. Since the functional regions of the other known apical components have not been defined, it remains to be determined whether Cdc2 acts directly to phosphorylate apical component(s) or indirectly through as yet undefined intermediate(s). Consistent with previous observations and proposals that different aspects of *cdc2* function can be mediated through distinct levels of *cdc2* activity, our data suggest that different levels of Cdc2 kinase activity are required for mitosis and for making the cell division asymmetric (see Fig. 5 for schematic model).

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DISCUSSION

Gönczy: This may be a semantic question, but is it possible that what is happening is that things are simply slowed down in the *cdc2* mutant, and that this is the primary reason why asymmetric determinants cannot get in place at the proper time?

Chia: If it is just a timing issue we wouldn't expect the asymmetrical protein localization to be affected.

Gönczy: But you are looking later, aren't you?

Chia: An interesting experiment in which we blocked division with String and then kicked the cells into mitosis again with heat-shock String, to see whether the

divisions which have been delayed are normal. They appear to be, at least with respect to the fate of the sibling neurons we are assaying.

Edgar: In relation to that, once the apical complex is set up, is it stable? Does it stay apical for ever?

Chia: It stays apical from G1 to at least anaphase.

Hunt: What happens in the *cdc2* hypomorph? Does it then diffuse around?

Chia: A certain proportion of the time Insc failed to localize and subsequently downstream events such as spindle orientation and Pros and Numb asymmetric localization were abnormal.

Hunt: Although it was localized, you think, in G2 before that.

Chia: What we are saying is that you need the mitotic kinase in order to maintain the localization of the apical components during mitosis.

Nasmyth: Have you been able to look at Insc localization in the mutant in G2?

Chia: Yes, we can look at Insc localization in G2. With String, all the cells are in G2, so you can just look at the embryo. In some ways it is a more convincing result. The defects that we see are seen during mitosis.

Nurse: Didn't someone suggest that cyclin A/Cdc2 activity which comes up earlier in the cell cycle might have a role in spindle orientation?

Hunt: It wasn't orientation so much. Rather, it had to do with catastrophe rates.

Nurse: I thought they were arguing that a first stage is formed which couldn't go any further with a cyclin A-induced activity, and this would allow the spindle to orient before you got too far into mitosis.

Newport: I think that was what it was. Cyclin A stabilizes spindles. In this case, with low Cdc2 it was actually going through mitosis a little bit faster than normal, and the spindle itself might not have time to orient properly, or it might rock a bit. This would result in an asymmetric division.

Chia: It is difficult for us to dissociate that effect from the failure to localize Insc.

Newport: That would determine where the cleavage plane is going to occur.

Chia: We see a defect in spindle orientation when there is a failure of localization of the apical complex. This is also what is seen in the *cdc2* alleles.

Nurse: Do the cyclin A mutants also have a specific defect?

Chia: The problem with cyclin A is that there is a maternal component, and the GMC division is blocked. The problem here is that the reason why these phenotypes are seen is because you have to hit it just right, with just enough kinase activity to divide but not enough to do it right.

Nurse: So you need a paternal dominant negative cyclin A mutant.

Chia: Or a temperature-sensitive mutant.

Hunt: I think that E–Q mutant you discovered inadvertently could have differential effects on the binding of cyclin, because that helix is a major point of cyclin/Cdc2 contact. We certainly have mutants which map in different places that affect the relative strength of binding of cyclins A and B.

Chia: Is there evidence that the difference between cyclin A and B is important?

Lehner: Pierre Gönczy's comment concerning timing might well be relevant. If you look in *cyclin B* mutants, they still go through mitosis. Cyclin B is not necessary to go through mitosis. But this mitosis is abnormal: it is four times as long as a normal division and the spindles are all wobbly. With regard to timing, I think that during an abnormally slow mitosis you might lose the Insc localization prematurely.

Hunt: Normally cyclin A goes away very early in mitosis — almost as soon as the nuclear envelope breaks down. If it doesn't go away, what seems to happen is that the spindle doesn't organize itself properly, yet it goes through anaphase. It is peculiar; the spindles look horrible. These are spindles with both cyclin A and cyclin B. The cyclin A seems to be necessary to get it up to that point, but then you need to get rid of it in order for the cyclin B to take over.

Chia: What is clear is that the neurons that are made look normal. If these abnormalities are occurring during mitosis, the daughter cells produced still appear normal.

Hunt: But if there was no Cdc2 you wouldn't have any mitosis at all. You are dealing with a level that is enough to get you through, but not enough to do it quite right. The question is what aspect of 'not quite right'-ness we are looking at.

Raff: Are any of the components of the apical complexes regulated by phosphorylation?

Chia: We have looked at the potential phosphorylation sites of Insc, and this is the only apical complex component for which the functional domain has been defined. All the putative Cdc2 phosphorylation sites lie outside the region required for function (at least using an over-expression paradigm). For the other two known components, Baz and Pins, we don't know which the functional parts of the molecule are. It seems more appealing to think in terms of effects on the cytoskeleton, and in particular actin.

Raff: Is the same apical complex involved in all asymmetrical divisions in *Drosophila*?

Chia: Absolutely not. To my knowledge this complex is involved in all divisions which are oriented along the apical–basal axis in the CNS. It doesn't apply for the peripheral nervous system, which involves Wingless signalling and Frizzled. Insc is involved in asymmetrical divisions in the mesoderm which do not have a fixed orientation. It is very interesting because although the cells don't divide with a fixed orientation and Insc crescents can be in any position on the cortex of dividing mesodermal progenitors, Numb crescent is always on the opposite side. This is consistent with the notion that wherever this complex is, which is not apical in the mesoderm, it defines all subsequent steps in that division.

Nurse: Would it therefore be fairer to say that the complex has a greater role in generating asymmetry, and is not itself a reflection of a particular polarity? In

other words, it is generating asymmetry in the mesoderm, but there is no clear polarity.

Chia: It is not clear that it is the exact same complex present in the mesoderm. We know Insc is present in the mesoderm progenitors, but there is no indication of Baz. Perhaps components of this complex are involved.

Nurse: If they were, there wouldn't be a polarized asymmetry in that system, it would simply be asymmetry. Therefore the role of Insc might be better thought of in terms of generating asymmetry in response to no polarity in the mesoderm, but in this system in response to another polarity.

Bryant: I have a question about the localization of those proteins. You said that in the epithelium, Pins is membrane-associated.

Chia: It is between cells.

Bryant: Does that mean it is associated with a membrane protein? Then, when you go to the apical crescent stage is that apical crescent membrane-associated, or is it a cytoskeletal structure?

Chia: I can't tell from the immunofluorescence. The work that has been done with other molecules of this sort suggests that these molecules are anchored to the membrane.

Bryant: Does that mean that there is a known anchor? What are the data?

Chia: The data are that you can create conditions in which you form a crescent, but this crescent is not fixed, and can move around and occupy different positions in the cell cortex.

Hunt: So the crescent is a sort of sliding structure.

Chia: Normally it is a basal crescent during mitosis, but Yuh-Nung Jan's lab has shown that in *insc*$^{-}$ mutants, green fluorescent protein (GFP)–PON can form cortical crescents but these are not fixed to the basal cortex and can move.

Nurse: Does the subsequent asymmetrical division always reflect the crescent, even though its position has moved?

Chia: No, what determines fate in the daughter cells is whether or not they inherit the cell fate determinants which are normally localized as basal crescents. If the crescents of cell fate determinants are not fixed, they will not always overlie one of the spindle poles. Consequently, their segregation to the daughter cells will not always be asymmetric.

Schaar: In the epithelial cells that don't normally express Insc, does ectopic expression of Insc not only result in relocalization of Pins but also reorientation of the spindle?

Chia: Yes.

Schaar: That is interesting, because in normal epithelium vertical divisions usually occur. Would you think that there could be some sort of epistasis that would put Pins closer to being able to anchor these astral microtubules from the spindle? Have you tried to mislocalize Pins?

Chia: I can't think of a good way of mislocalizing Pins. With Inscuteable, early on in embryogenesis it is expressed in a single domain and nowhere else. The cells give rise to some of the cells that become the larval brain. The spindle in this region forms parallel to the surface and reorients at 90°, perpendicular to the surface of the embryo. These cells which don't express Insc set up their spindle parallel and divide parallel to the surface. If you force Insc expression in these cells they will reorient.

Schaar: My question was, if expressing Insc in those cells relocalizes Pins, then would you say that there is some kind of epistasis where Pins is more responsible for anchoring the spindle?

Chia: This is hard to say. But probably not, because in Pins$^-$ embryos epithelial cells still orient their spindles parallel to the surface.

Hunt: One further question is what is responsible for those spindle attachments?

Schaar: In epithelial cells where Pins is lateral, the aster is always oriented towards the lateral surfaces. That is what is being buried when neuroblasts are being made.

Chia: There is another experiment that is relevant to your question. If Pins is overexpressed in the epithelial cells in a Pins$^-$ mutant, there is no longer any apical Insc. But this distribution is bizarre: it is in the cytoplasm, but it is preferentially in the apical cytoplasm, as if apical Baz were trying to recruit Insc. One might speculate that the transient Baz–Insc complex can't be stabilized in the absence of Pins, so Insc keeps falling off. In this situation you don't get spindle reorientation.

Gönczy: I have a question regarding 'spindle reorientation'. Do you mean actual spindle reorientation, or rather centrosome repositioning prior to mitosis?

Chia: It is actually spindle reorientation. It occurs during mitosis. The spindle actually sets up, and the whole thing rotates.

Edgar: Could you clarify the difference between polarity and asymmetry. Most of the mutants you talked about didn't destroy symmetry, they just randomized it and gave you randomization of what was still asymmetric.

Chia: Not necessarily. For a certain proportion of the time, the distribution is throughout the cortex. The cell fate determinants were totally randomly distributed throughout the cortex, with no crescent formation. However, at other times we still get crescents, which are mislocalized.

Edgar: So why is there that variability?

Chia: Perhaps there is something else that works in this system which we don't know anything about. The terminal phenotype of these mutants is never 100% penetrant.

Nurse: In an individual cell, once it has dispersed does it remain dispersed, or does it oscillate between being localized and being dispersed? I know you need to look at living cells to determine this.

Chia: The only molecule that has been looked at in living cells is Partner of Numb (PON), which has been looked at by Kuh-Nung Jah's lab. They see with GFP–PON that it is cytoplasmic during interphase and goes to the cortex in early prophase. Then the molecules appear to migrate to the basal cortex later on in prophase and are anchored there.

Nurse: I was wondering whether if you have different Cdk levels the cell might switch from one state to another, or whether there are cell autonomous effects: once you are in a certain condition you are either one state or the other?

Newport: The way that spindles and microtubules are normally reoriented is by the stabilization of dynamic instability at the plus-end of the microtubule. So if microtubules were to embed in this apically localized complex, they would effectively be capped and this would reorient the spindle. One would expect that this would happen to the centriole prior to mitosis, so that the interphase microtubules would be stabilized at that location as well. Are you saying this doesn't happen? If it doesn't happen, perhaps Cdc2 is necessary to activate this apical region for stabilizing plus ends, and this would explain why it rocks about. Are any of these molecules potential candidates for capping microtubules at the plus-end, for instance?

Nurse: There are yeast mutants that capture the astral microtubules and orient the nucleus. It may be interesting to look at homologues of these.

Schmidt: If this is tissue specific, are there family members in other tissues?

Chia: I'm not aware of other family members of Pins or Insc.

Schmidt: So you don't know how generalizeable the mechanism is to asymmetric division in lungs or other branching-type structures?

Chia: I have no idea.

Nurse: What about mammals or worms?

Chia: There is extremely good homology between the worm asymmetry proteins and those of *Drosophila* and mouse. There are homologues of Pins and Baz. The homology with Pins is in excess of 60%, and it is expressed in the appropriate tissues.

Hunt: Are these also in the nervous system of the mice?

Chia: We are neophytes with mice. We have looked, using elecroporation and GFP fusions, but we are not convinced enough to say whether there is asymmetric localization. There is certainly expression in the neural tube.

Lehner: Neuroblast divisions are asymmetric not just because the cell determinants are distributed asymmetrically, but also the size of the GMC is very different, as is the nuclear size. Is nuclear size difference present from the outset? If you look at cells in telophase, are the nuclei the same size?

Chia: For the neuroblasts it is obviously different right from the start. It is clear that the GMC is much smaller. The division that we looked at carefully is the GMC division to produce two neurons. This division produces sibling neurons that have

different nuclear size, and this nuclear size is generated during the division itself. In the absence of inscuteable function, the two nuclei are equal sized. The same phenotype is seen in the *cdc2* allele. Two neurons of identical nuclear size are obtained, which normally are not seen in wild-type. Insc and Pins are the only two molecules that we know of which equalize the nuclear size of the two sibling neurons. All the downstream molecules cause cell fate changes, but don't equalize this nuclear size difference.

Raff: What about the asymmetric divisions in the mesoderm? Where is the cell polarity originating?

Chia: We don't really know.

Raff: Is it known which molecules are involved?

Chia: The ones we know of are Insc and Numb. We don't think that Baz is expressed in the mesoderm.

Raff: Can you reorient the polarity by changing the source of the signals?

Chia: We haven't tried to address this. One would imagine that there has to be some type of signalling. Different muscle progenitors have different orientation of divisions, but the same muscle progenitors that give rise to the same muscles, they retain the same orientation. There must be some information feeding into this.

Vande Woude: Is it generally understood that nuclear size is regulated by asymmetrical divisions?

Nurse: In fission yeast we can get asymmetrical divisions and make cells different sizes in various ways. Nuclear volume is influenced by subsequent cytoplasmic volume, and of course it is also directly influenced by ploidy. In an asymmetric division there will be a small nucleus and a big nucleus.

Nasmyth: You need more polymerase in a big cell to produce more RNA from the same number of genes.

Hunt: The most extreme case is an oocyte, which has a giant nucleus and very little DNA. This must just be because there are an awful lot of proteins that belong in the nucleus, and it fills up.

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General discussion III

Determining organ size

Nurse: I wondered whether it might be worth revisiting the issue of what determines organ size, because this is obviously relevant to a number of issues that have risen. Martin Raff, do you have any thoughts about overall organ size and how that is regulated?

Raff: I think there are several things to be said. First, the extent to which an organ depends on local controls versus systemic controls for its final size varies greatly. The thymus is apparently dependent on local controls. Don Metcalf did an experiment in the 1960s in which he put multiple thymus lobes from newborn mice into another newborn mouse, and each thymus grew to a normal adult size (Metcalf 1963). With spleen, the result was the opposite. When he put multiple newborn spleens into a newborn mouse, the total mass of spleen in the adult was equal to one normal adult spleen (Metcalf 1964). But most organs are like the thymus in this respect and so the real mystery here is how a primordial organ or limb knows what its final size should be. How much of this is cell–cell signalling and how much depends on intrinsic cell programmes of the kind I was talking about? How important are intercalation mechanisms of the kind that Peter Bryant described years ago and Antonio Garcia-Bellido still studies?

Nurse: Peter Bryant, didn't you take bits out of the imaginal disc and then found that it reconstructed the cells in between?

Bryant: Yes, but the experiment is a little more complicated than that. You can take an entire imaginal disc and cut it into two pieces. Usually, one piece is larger than the other, and in general the large piece will regenerate the missing piece (Bryant 1975). The smaller piece will duplicate and be stable at a much smaller final size than the normal disc. What is stabilized in that final structure is a pattern in which every cell has a normal neighbour, which is true of a duplicate as well as a regenerate. The cells are not sensing the final size in any way at all, but each cell is just sensing whether it has normal neighbours or not. This is the model, and even with the genetic experiments that have been done since then, it is still pretty much an accurate statement of what happens. In his paper, Bruce Edgar described a wing in which there had been ectopic Dpp expression. The result was massive intercalation which gives a triplicated wing. There you have a final organ that is much bigger than it should be, but all of the local conditions are satisfied.

Raff: What are the local conditions that are satisfied?

Bryant: Appropriate nearest neighbours. Presumably 'appropriateness' translates into patterns of expression of molecules such as Dpp and Wingless. There is still a way in which cells can sense appropriate neighbour relationships. I don't know how they do it.

Nurse: In the intercalation experiments, when you did it with a smaller bit and just reconstructed the lower number of intermediates, you ended up with a smaller imaginal disc compared with when you did it with a bigger bit which constructed the complete series. If you have a complete series of fates you seem to make a bigger organ than if you only have a limited series of fates. If this is true, wouldn't it be telling us that something contributing to overall organ size requires all the different cell fates to be in one organ.

Bryant: That is how you get an organ of completely normal size, but you can also get stable end products that are not the normal final size. I don't know any reason to think that cells within that population are sensing the size of the whole population.

Nurse: If instead of a whole dozen fates you have, let's say, just five, you end up with an imaginal disc that is smaller over all, compared with the one with all dozen. This suggests that determining the overall proper organ size requires a proper mixture of cells with all the cell fates, rather than a limited set. Would this be consistent?

Bryant: I think so.

Nasmyth: Is the critical question not what size you get when you do these intercalation experiments, but rather what is the difference between a small fly and a big fly?

Raff: Not necessarily. In poodles, for example, differences in size between miniatures, toys and regulars apparently mirror levels of insulin-like growth factors (IGFs). To me that is not especially interesting. We know that if you put in more growth hormone (GH) or IGF, you get a proportionally bigger animal. What interests me more is how a few hundred cells in a limb primordium know what the approximate final size of the limb should be. This seems to me to be a more fundamental question than how growth hormones adjust the final size of an animal.

Nasmyth: The bar can be moved up and down by GH, but what is the bar?

Raff: You can't make a mouse the size of a human by increasing GH, but you can make a mouse almost twice as big in this way.

Nasmyth: That bar may have nothing to do with intercalation: those experiments may not be terribly informative.

Raff: But is the bar read out by intercalation? An intercalationist might say that an imaginal disc is initially set up with extreme positional values, and proliferation continues until the missing intermediate values are filled in. The question then becomes how the initial values are set. Patterning genes presumably play a major role in determining those values. But if this is what is really going on, one needs to

know how a disparity in positional values stimulates cell proliferation. What are the mitogenic molecules involved? Are patterning molecules like Dpp and Wingless the mitogens? This doesn't seem to be the case, as there is little relationship between the levels of Dpp or Wingless in a disc and bromodeoxyuridine (BrdU) incorporation, for example, which tends to show a salt-and-pepper distribution throughout the disc, as I understand it.

Edgar: But the proliferation rates are different in different regions.

Raff: Why are they different? Is it because Dpp and Wingless are mitogens?

Nasmyth: Let's not get away from the fact that they are key regulators in this process. Of course they are not the whole story.

Nurse: Is there a sensible model that could explain why a liver or a wing should be the size that they are?

Bryant: At the end of his talk, Martin Raff said something provocative that I would like him to elaborate on. He said that if you have excess cells all over the body, it does not cut off the excess ones by cell death. But if this only happens in one organ, it does.

Raff: No. If you increase one cell type in an organ, the prediction is that cell death will bring that population back to normal size. If you increase every cell type within the organ, however, the different cell types would support one another's survival, so that you end up with more cells of various types and a bigger organ.

Thomas: What happens in the experiments where activated Cdk4 is expressed in the β cells of the pancreas, which results in huge islets? Do these cells go through apoptosis?

Raff: I don't know. But the islets contain more than one cell type, and these may support one another.

Goodwin: I have a general point about this question of boundaries and relationships with cell neighbours. To some extent it depends on the dimensionality that is discriminated within that organ. If you have an organ that consists of cells that are all the same, the neighbour relationships are trivially satisfied because they are all the same. If you want to control size, it has to be done systemically. But if you have a two-dimensional organ or a three-dimensional organ, so that the neighbourhood relationships have to be satisfied along different dimensions, then of course you are going to get the kind of phenomenon that occurs in the imaginal discs. That is, if you cut a piece and it regenerates symmetrically, then the neighbourhood relationships are satisfied one way, but there is a large piece that is at the boundary for which the neighbourhood relationships have to be satisfied in another way. This is the difference between a small organ and a large organ. If there is a higher degree of spatial information in an organ, it will force the thing through the neighbourhood relationships to reestablish its original size, whereas when you break the symmetry into small fragments there are smaller elements that are stable. There is something to do

with dimensionality that is important in terms of the fields that are being established. What information is there about different organs having different dimensionalities in the sense of being organized in some coherent way along different axes?

Vande Woude: It would be interesting to do organ-specific targeted disruption of p27. Does this change the size of specific organs, or is an organ restricted to a regular size?

Raff: Going back to Paul Nurse's original question of what determines the final size of an organ, the myostatin story in muscle is very interesting. Myostatin is a transforming growth factor (TGF)β family member. When the gene encoding it is inactivated, muscles grow to be much larger than normal. As far as I know the rest of the animal is normal. Does anyone know more about how myostatin works? Does it affect muscle cell survival, proliferation or growth?

Nasmyth: Is it cell size or cell division?

Raff: Both. You get bigger cells and more cells.

Hunt: But muscles are syncytia.

Raff: The reason this is such an important example is because it is the only clear example in which an extracellular inhibitor is known to play a crucial part in determining organ size.

Nasmyth: What happens with grafting experiments with chicks, in which a little chick wing is grafted onto a bigger chick? What do those experiments tell us?

Raff: There have been a lot of these kinds of experiments. Generally, the limb or organ grows to pretty much its normal size, but there is some regulation. If it is transplanted into a bigger animal, for example, it grows a little bigger than it otherwise would have done.

Nasmyth: So there is autonomy of the limb and regulation.

Reik: I would like to counteract Martin Raff's statement that IGFs are not interesting. One of the models that I think is particularly relevant is that of compensatory growth, or catch-up growth. This is when an organism is smaller than it should be and catches up. We have done a placental-specific knockout of an *Igf2* transcript. These mice are born smaller, and then they catch up at weaning. This is associated with over-expression of *Igf2* during that period. We don't have genetic evidence that this overexpression of *Igf2* controls the catch-up, but increased protein is there. During this period the organs grow faster and express more *Igf2*. The obvious question is what is the sensor: what tells that animal that is too small and that it needs to increase its growth?

Leevers: Is it definitely at the levels of the ligand and not also the amount of receptor?

Reik: It is possible; we haven't looked. The increase of ligand may be a red herring; we don't have the ultimate genetic evidence that this is important. It

could be that the ligand is there, but that the receptor concentration is the actual regulator.

Kozma: In the mouse, if one performs a two-third hepatectomy, the remaining liver mass proliferates and then stops at the weight corresponding to the liver initially. The liver is very complex. It consists of multiple lobes with specific shapes, but if part of these lobes is taken away, the liver does not regrow its initial shape, but it certainly recovers its initial mass. There is precise mass control.

Raff: This also works in the other direction. If one stimulates hepatocytes to go through a round of division in the adult, programmed cell death will bring the liver back to its normal size within a week. Thus, survival control seems to contribute to size control in the adult liver. But the big question remains: how does an organ know what size it should be?

Nurse: I think it has to stop being homogeneous. If you have a homogeneous tissue, it won't be able to measure size.

Bryant: There are important issues concerning the relationship between local and systemic controls of growth. Some classic experiments on insects show that growing imaginal discs send an inhibitory signal to the rest of the organism. When an imaginal disc is taken from a lepidopteran larva, the missing disc is regenerated, but during this time the growth of the rest of the animal is held back, and the regenerating disc catches up. It is only when the regeneration is finished that the animal can go into metamorphosis (Madhavan & Schneiderman 1969, Pohley 1965). Implanted disc fragments will also regenerate, and block pupation of the host until regeneration is complete (Rahn 1972, Dewes 1975, 1979). Mutations that keep imaginal discs in a constantly growing state, also prevent the animal from entering metamorphosis, associated with a failure of the ecdysteroid titres to reach the level where they induce pupariation (Sehnal & Bryant 1993). Apparently the growing imaginal discs send a signal to the endocrine system to indicate that they are not yet ready to move on to metamorphosis. This is an elegant way of coordinating the growth of peripheral tissues. Is there any similar system in vertebrates?

Schmidt: There is a coincidence between those two comments. Hepatocyte growth factor (HGF) is actually produced by the lung, so what results when you do a two-thirds hepatectomy is that the HGF secreted by the lung brings the liver back up to its size.

Kozma: How does the liver stop?

Schmidt: It stops as a result of TGFβ signalling. It actually overshoots by about 5–10% and then comes back down.

Bryant: Is that restricted to liver and lung, or can it be generalized even more?

Schmidt: HGF has pleiotropic effects on essentially everything, but the clearest example of it being secreted in response to the damage of one organ is the liver. It is

also a terrific trophic factor for the kidney, but I am not really aware of the same kind of damage models in the kidney.

Vande Woude: In ischaemic kidneys there is induction of HGF effects. When cytotoxic agents are used it protects.

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Spindle positioning during the asymmetric first cell division of *Caenorhabditis elegans* embryos

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Abstract. Cell division during development in many cases generates daughter cells that differ not only in fate, but also in size. We investigate the mechanisms that ensure proper spindle positioning during such asymmetric divisions using the one-cell stage *Caenorhabditis elegans* embryo as a model system. We utilized a UV laser microbeam as an *in vivo* microtubule-severing device to probe the forces driving spindle positioning. Our results indicate that extra-spindle pulling forces acting on the spindle poles dictate spindle position along the anterior–posterior embryonic axis. Importantly, forces acting on the posterior spindle pole appear more extensive than those acting on the anterior one, thus explaining the overall posterior spindle displacement that leads to the asymmetric division of the wild-type one-cell stage embryo. In separate work, we analysed a locus called *zyg-8*, which plays a key role in ensuring proper spindle positioning. Our data show that *zyg-8* is required to promote microtubule growth and/or stability during anaphase. We identified the molecular nature of the *zyg-8* locus in the course of a large-scale RNAi-based functional genomics screen. ZYG-8 harbours two notable protein domains: a Ca^{2+}/calmodulin-dependent kinase domain, and a domain related to doublecortin, a human microtubule-associated protein involved in neuronal migration.

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Asymmetric divisions are central to the generation of cell fate diversity (for review see Horvitz & Herskowitz 1992). During development, asymmetric divisions often give rise to daughter cells that differ not only in fate, but also in size. For instance, in the developing *Drosophila* nervous system, neuroblasts divide asymmetrically to generate a large neuroblast and a small ganglion mother cell (for review see Doe 1996). Similarly, at the fourth cleavage division of sea urchin embryos, vegetal

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tier cells undergo an asymmetric division that gives rise to a macromere and a micromere, which differ markedly in size (for review see Hörstadius 1973). In animal cells, for such divisions to take place, the mitotic spindle must be asymmetrically localized by the end of anaphase, when the cleavage furrow is specified so as to bisect the mitotic spindle (for review see Rappaport 1971). The mechanisms which allow the anaphase spindle to be asymmetrically positioned within the three dimensional space of the cell remain poorly understood.

In this paper, we discuss how we have begun to investigate this question in the one-cell stage *Caenorhabditis elegans* embryo. First, we review how cell polarity is established along the anterior–posterior (AP) embryonic axis, and how anaphase spindle positioning is achieved in wild-type embryos. Second, we discuss experiments in which we have used a UV laser microbeam to probe the forces that act on spindle poles to dictate anaphase spindle positioning. Third, we report our cell biological and molecular analysis of *zyg-8*, a locus that plays a crucial role in ensuring proper anaphase spindle positioning. In conclusion, we summarize our findings and mention some future directions.

Cell polarity and anaphase spindle positioning in the wild-type one-cell stage *C. elegans* embryo

In *C. elegans*, polarity along the AP embryonic axis is established shortly after fertilization (Goldstein & Hird 1996). A sperm component, which remains to be identified, provides an initial polarity cue that determines the future posterior of the embryo. This initial cue is then translated by the concerted action of six maternally required *par* genes (for *par*titioning-defective) to establish polarity along the AP axis (for review see Kemphues & Strome 1997). All six *par* genes have been cloned, and antibodies have been raised to most of the corresponding proteins (Boyd et al 1996, Etemad-Moghadam et al 1995, Guo & Kemphues 1995, Hung & Kemphues 1999, Levitan et al 1994, Watts et al 2000, K. Kemphues, personal communication). Strikingly, several of the PAR proteins have a polarized distribution along the AP axis in the one-cell stage embryo. Thus the PDZ-containing proteins PAR-3 and PAR-6 both localize to the anterior cortex (Etemad-Moghadam et al 1995, Hung & Kemphues 1999). Conversely, PAR-2, a ring-finger containing protein, and PAR-1, a Ser/Thr protein kinase, both localize to the posterior cortex (Boyd et al 1996, Guo & Kemphues 1995). These observations indicate that the polarized distribution of PAR proteins at the cell cortex is essential for establishing proper polarity along the AP embryonic axis.

Interestingly, homologues of some of the PAR proteins have been found in other metazoans where they also have a polarized distribution. For instance, in *Drosophila*, *bazooka* encodes a PAR-3 homologue that localizes to the apical surface of epithelia cells and neuroblasts; moreover, *bazooka* is required for

proper polarity in these cells (Kuchinke et al 1998). In mammalian epithelial cells, the PAR-3 homologue ASIP is present in apically located tight junctions, while a PAR-1 homologue localizes to the basolateral domain (Bohm et al 1997, Izumi et al 1998). These observations suggest that the function of PAR proteins in establishing cell polarity may have been significantly conserved across metazoan evolution.

In the one-cell stage *C. elegans* embryo, establishment of polarity by the PAR proteins leads in turn, among other things, to an asymmetric position of the anaphase spindle along the AP axis. Spindle positioning can be followed with great spatial and temporal resolution in living embryos using time-lapse differential interference contrast (DIC) microscopy (Fig. 1). The spindle is initially set up roughly in the cell centre (Fig. 1, top panel). Then, as the spindle elongates during anaphase B, the anterior spindle pole stays relatively put with respect to overall AP polarity, while the posterior spindle pole is displaced slightly towards the posterior. This results in an asymmetric spindle position along the AP axis by the end of anaphase (Fig. 1, middle panel). As a result, the one-cell stage embryo divides asymmetrically, into a larger anterior blastomere and a smaller posterior one (Fig. 1, bottom panel). In embryos derived from *par* mutant hermaphrodites (hereafter referred to as *par* mutant embryos), posterior displacement does not take place and the first division is symmetric, most likely as a consequence of the earlier defects in establishing polarity along the AP axis (Kemphues et al 1988).

Pulling forces acting on the spindle poles dictate spindle position along the AP axis

The mechanisms by which overall cell polarity, as established by the PAR proteins, is communicated to the cytoskeleton to mediate proper spindle positioning are not understood. Below we discuss two experimental approaches that we have taken to address this question. In the first set of experiments, we sought to identify the forces that act on spindle poles to drive spindle positioning during anaphase. Experiments in other systems have revealed that two types of microtubule-dependent forces can contribute to spindle elongation during anaphase B (Aist & Berns 1981, Aist et al 1993, Leslie & Pickett 1983). First, forces that act on overlapping spindle microtubules can 'push' spindle poles apart. Second, forces that act on astral microtubules can 'pull' spindle poles apart. In some cases, both types of forces may contribute to spindle elongation. Spindle-severing experiments are instrumental for testing whether pulling forces acting along astral microtubules play a role in driving anaphase B. Indeed, if solely intra-spindle pushing forces drive anaphase B, then the two spindle poles should not separate or even collapse onto one another after severing of the spindle, as was

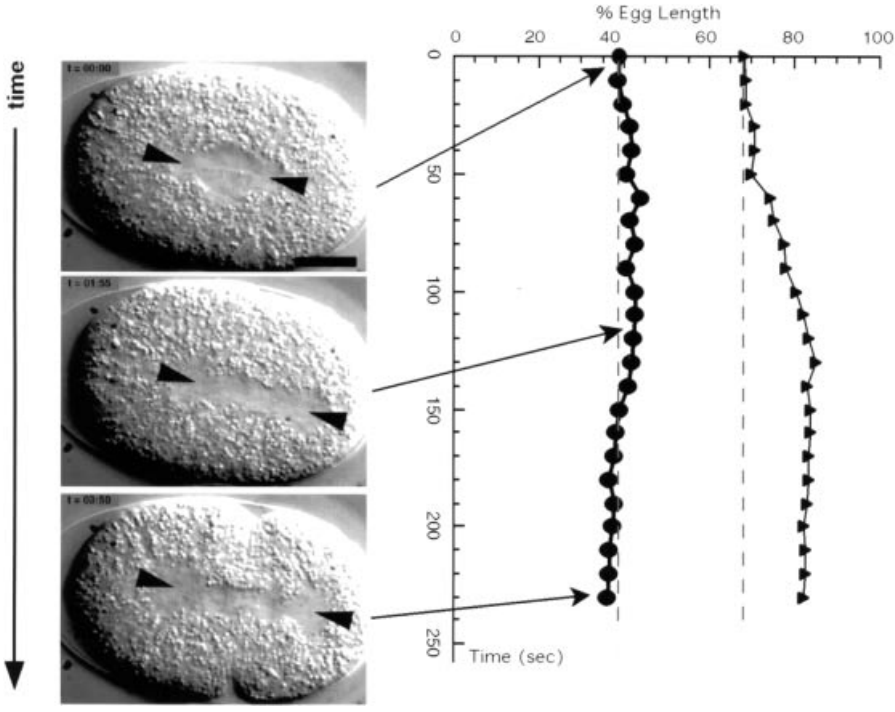

FIG. 1. Anaphase spindle positioning in wild-type embryo. Left: three images taken from a time-lapse DIC recording; arrowheads point to centrosomes and spindle poles. Anterior (0% egg length) is to the left, posterior (100% egg length) to the right in this and all other figures. Time elapsed since the beginning of the sequence is indicated in minutes and seconds. Right: corresponding tracings of aster positions over time. During anaphase, the anterior spindle pole hardly moves with respect to overall AP polarity. In contrast, the posterior spindle pole is displaced slightly towards the posterior. This results in the asymmetric division of the one-cell stage embryo into a larger anterior blastomere and a smaller posterior one. Bar = 10 μm. A Quicktime movie of this sequence can be viewed at *http://www.embl-heidelberg.de/ExternalInfo/hyman/Data.htm*.

observed in diatoms (Leslie & Pickett 1983). In contrast, if extra-spindle pulling forces participate in driving anaphase B, then the two spindle poles should keep moving or even accelerate after severing of the spindle, as was observed in vertebrate Ptk2 cells (Aist et al 1993).

Spindle-severing experiments in wild-type embryos

We conducted spindle-severing experiments in the wild-type one-cell stage *C. elegans* embryo to investigate whether extra-spindle pulling forces participate

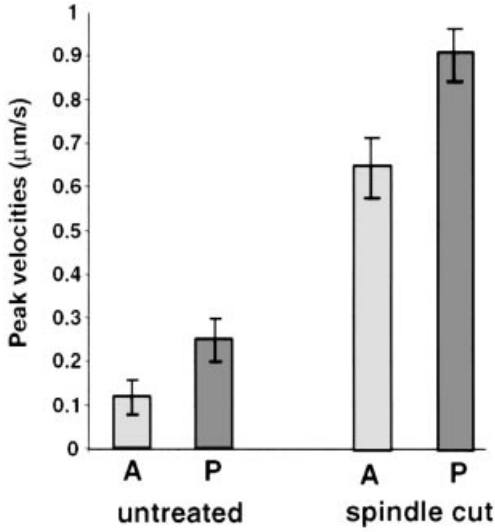

FIG. 2. Peak velocities achieved by the anterior (A) and posterior (P) spindle poles during anaphase. Left: wild-type untreated embryos; $n=5$. Right: wild-type embryos whose spindle has been severed during early anaphase by the UV laser microbeam; $n=34$. The peak velocities of both spindle poles are higher in embryos whose spindle has been severed than in untreated embryos, indicating that extra-spindle pulling forces play an important role in driving anaphase B. Moreover, after severing, the peak velocity of the posterior spindle pole exceeds that of the anterior one, suggesting that a larger pulling force is acting on the posterior spindle pole.

in driving anaphase B. A 337 nm laser microbeam was used as a local *in vivo* microtubule-severing device. By using a moderately well focused laser microbeam (focal diameter $\sim 3\,\mu$m), spindle microtubules could be destroyed selectively, without affecting astral microtubules. The spindle was severed with the UV laser microbeam during early anaphase, and the position of the spindle poles was monitored using time-lapse DIC microscopy. Strikingly, both spindle poles kept moving, and the peak velocity of each spindle pole was significantly higher than that observed in untreated embryos (Fig. 2). This demonstrates that extra-spindle forces pulling on the spindle poles, presumably along astral microtubules, play an important role in driving anaphase B in the one-cell stage *C. elegans* embryo. It should be noted, however, that these experiments do not rule out the possibility that intra-spindle pushing forces may also contribute to spindle elongation.

Importantly, we observed that the two spindle poles behaved differently following spindle severing: the posterior spindle pole had a higher peak velocity than the anterior one (Fig. 2). This suggests that a more extensive vector sum is

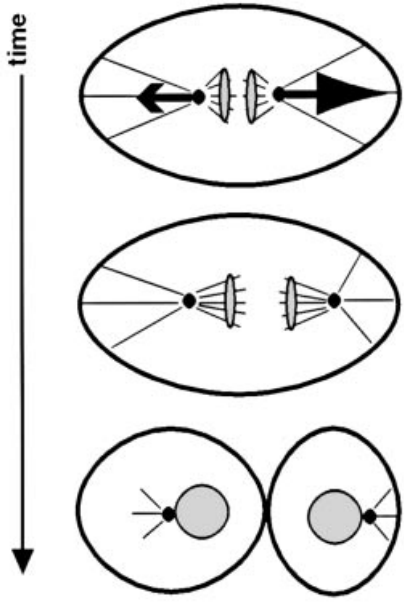

FIG. 3. Model of anaphase spindle positioning in the one-cell stage *C. elegans* embryo. Filled circles, spindle poles and centrosomes; black lines, microtubules. Segregating sets of chromosomes are also indicated, as are nuclei in daughter cells. Spindle-severing experiments indicate that the extent of extra-spindle pulling forces acting on the spindle poles determines spindle position along the AP axis. Forces acting on the posterior spindle pole are more extensive than those acting on the anterior one, thus generating a slight posterior spindle displacement during anaphase. This results in the asymmetric division of the one-cell stage embryo into a larger anterior blastomere and a smaller posterior one.

pulling on the posterior spindle pole than on the anterior one. This leads us to propose a model in which the asymmetric elongation of the spindle during anaphase results from differential extra-spindle pulling forces acting on the spindle poles (Fig. 3). Because forces acting on the posterior spindle pole are more extensive than those acting on the anterior one, a slight posterior displacement of the spindle is achieved during anaphase in wild-type embryos.

Spindle-severing experiments in par mutant embryos

We conducted spindle-severing experiments in *par-2* and *par-3* mutant embryos to test the validity of this model. In wild-type, PAR-3 protein is restricted to the anterior cortex and PAR-2 protein to the posterior cortex (Boyd et al 1996, Etemad-Moghadam et al 1995). In *par-2* mutants, PAR-3 expands to fill the

entire circumference of the embryo, which can be thought of as having anterior character throughout (Etemad-Moghadam et al 1995, Kemphues & Strome 1997). Conversely, in *par-3* mutants, PAR-2 expands to fill the entire circumference of the embryo, which can be thought of as having posterior character throughout (Boyd et al 1996). The first division is symmetric in both *par-2* and *par-3* mutant embryos, presumably as a consequence of the absence of polarity along the AP axis. According to our model, the first division should be symmetric in *par-2* and *par-3* mutant embryos due to distinct alterations in forces pulling on the spindle poles, both of which result in equal forces being exerted on either side. In a *par-2* mutant embryo, forces acting on both spindle poles should be weak, since the embryo has anterior character throughout. Conversely, in a *par-3* mutant embryo, forces acting on both spindle poles should be strong, since the embryo has posterior character throughout.

We tested these predictions by conducting spindle-severing experiments in *par-2* and *par-3* mutant embryos (data not shown). In severed *par-2* mutant embryos, both spindle poles had a velocity that resembled that of the anterior spindle pole of irradiated wild-type embryos. Conversely, in severed *par-3* mutant embryos, both spindle poles had a velocity that resembled that of the posterior spindle pole of irradiated wild-type embryos. These results lend strong support to the model presented in Fig. 3, in which the extent of pulling forces acting on the spindle poles dictates spindle position along the AP axis during the first cleavage division of *C. elegans* embryos.

zyg-8 is required for proper anaphase spindle positioning and encodes a protein kinase related to Doublecortin

In separate work, we screened large collections of maternal-effect embryonic lethal mutations by time-lapse DIC microscopy to identify novel components required for proper anaphase spindle positioning and other cell division processes in the one-cell stage *C. elegans* embryo. In our initial study with a collection of mutations on chromosome III, we identified mutations in a locus called *zyg-8* which gave rise to a spectacular defect in anaphase spindle positioning (Gönczy et al 1999). In most *zyg-8* mutant embryos, the spindle sets up roughly in the cell centre as in wild-type (Fig. 4, top panel). However, during anaphase, both anterior and posterior spindle poles are displaced in an exaggerated manner towards the posterior (Fig. 4, middle panel). As a result, the spindle is located too far towards the posterior by the end of anaphase, and the cleavage furrow is aberrantly placed (Fig. 4, bottom panel). The anaphase spindle positioning phenotype is likely due to a reduction in *zyg-8* function, because it was observed in five out of eight *zyg-8* alleles examined and in the progeny of animals transheterozygous for a *zyg-8* mutation and a deficiency uncovering the region. These observations indicate

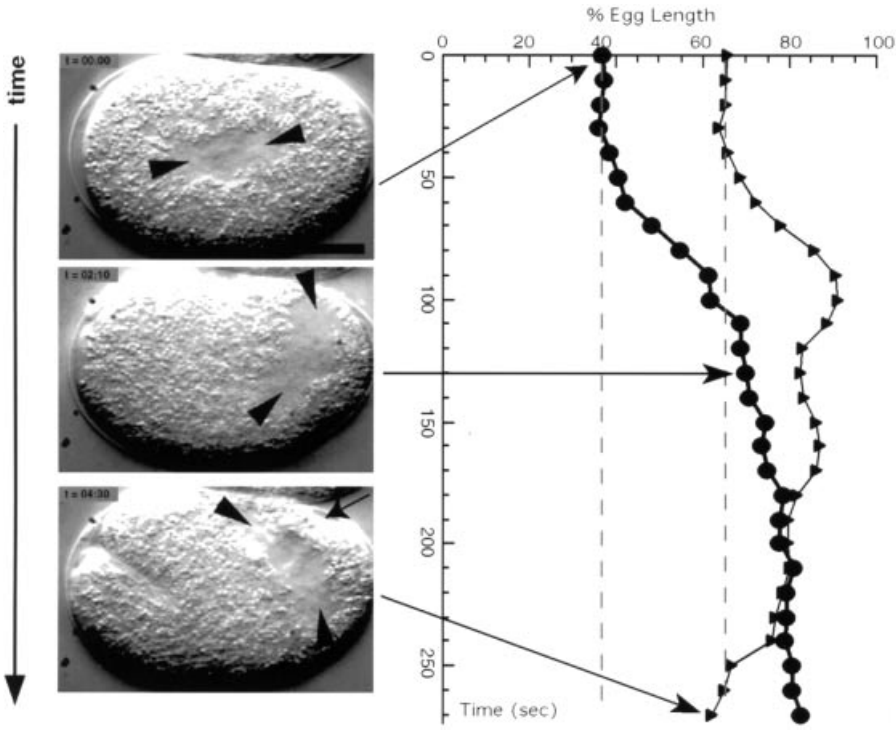

FIG. 4. Anaphase spindle positioning in *zyg-8* (*t1650*) mutant embryo. Left: three images taken from a time-lapse DIC recording; arrowheads point to centrosomes and spindle poles. Time elapsed since the beginning of the sequence is indicated in minutes and seconds. Right: corresponding tracings of aster positions over time. Both anterior and posterior spindle poles move in an exaggerated manner towards the posterior during anaphase. As a result, the cleavage furrow (arrow) is specified too much to the posterior along the AP axis, and has an aberrant orientation. Bar=10 μm. A Quicktime movie of this sequence can be viewed at *http://www.embl-heidelberg.de/ExternalInfo/hyman/Data.htm*.

that *zyg-8* must normally act to somehow restrict the extent of posterior spindle displacement during anaphase.

zyg-8 regulates microtubule behaviour during anaphase

How could *zyg-8* achieve this function? One possibility is that *zyg-8* could be required to set up proper AP polarity. In this scenario, a polarity defect in *zyg-8* mutant embryos would result, as a consequence, in the spindle going to the wrong position along the longitudinal axis. To address this possibility, we examined the distribution of PAR-1, PAR-2 and PAR-3, as well as P granules,

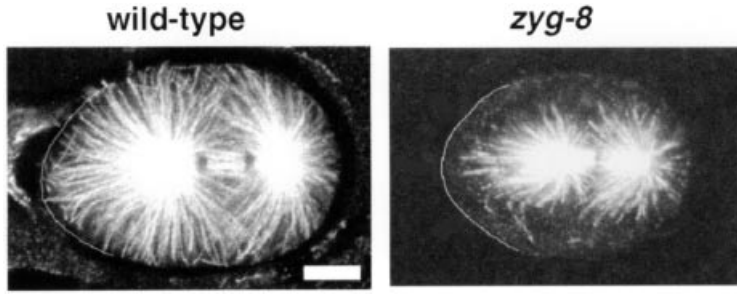

FIG. 5. Distribution of microtubules during anaphase in wild-type and *zyg-8*(*t1650*) mutant embryos as revealed by staining with anti-tubulin antibodies. White contour marks the anterior cortex. In wild-type, astral microtubules are long, extending all the way to the cell cortex. Astral microtubules in *zyg-8* mutant embryos are shorter, and do not reach the vicinity of the cell cortex. Bar = 10 μm.

which are cell fate determinants normally segregated to the posterior of the embryo (Strome & Wood 1983). We found that all these markers of polarity were correctly localized in *zyg-8* mutant embryos (data not shown). Thus, *zyg-8* does not appear to play a role in setting up overall embryonic polarity.

Another possibility is that *zyg-8* could be required in some manner for the integrity of the microtubule cytoskeleton, in particular during anaphase. To test this, we examined the distribution of microtubules in fixed wild-type and *zyg-8* mutant embryos (Fig. 5). Strikingly, we found that astral microtubules during anaphase were shorter in *zyg-8* mutant embryos than in wild-type; as a result, a few microns separated the tip of the longest astral microtubule from the cortex in *zyg-8* mutant embryos. Moreover, spindle microtubules appeared altered as well. Taken together, these observations suggest that *zyg-8* normally acts to somehow, directly or indirectly, promote anaphase microtubule growth and/or stability.

We next addressed whether shorter microtubules during anaphase were actually causing exaggerated posterior spindle displacement in *zyg-8* mutant embryos. To this end, we asked whether exaggerated posterior displacement could be generated by shortening microtubules in wild-type embryos during anaphase using low doses of the microtubule-destabilizing agent nocodazole. We found that embryos treated in this manner displayed a *zyg-8*-like anaphase spindle positioning phenotype (data not shown). This leads us to conclude that shorter microtubules during anaphase indeed cause excess posterior displacement and, therefore, likely explain the *zyg-8* mutant phenotype.

zyg-8 encodes an evolutionarily conserved kinase related to Doublecortin

The molecular nature of *zyg-8* was identified in the course of a genome-wide functional genomics screen for cell division genes that was initiated in the Hyman laboratory in 1999. This screen makes use of RNA mediated interference (RNAi). With RNAi, the expression of a given gene in the early embryo can be abolished in a sequence-specific manner via microinjection of corresponding double-stranded RNA into the gonad of the mother (Fire et al 1998). RNAi is very efficient and phenocopies the null phenotype of the vast majority of genes acting in the early *C. elegans* embryo (C. Mello, personal communication; P. Gönczy, unpublished observations). In our screen, dsRNAs were generated, injected, and the resulting one-cell stage embryos analysed by time-lapse DIC microscopy for potential defects in cell division processes. We initially investigated ~2200 genes located on chromosome III using this screening paradigm. We found a single gene whose RNAi phenotype resembled that of *zyg-8* mutant embryos, and this gene was located in the chromosomal interval to which *zyg-8* had been mapped genetically (C. Mello, personal communication). Therefore, we sequenced this gene in three *zyg-8* mutant alleles, and found three distinct point mutations which all result in premature STOP codons. This indicates that the gene identified during the RNAi-based screen corresponds to the *zyg-8* locus.

The ZYG-8 protein is predicted to be 802 amino acids long and has homologues in *Drosophila* and mammals. ZYG-8 harbours two notable protein domains: a Ca^{2+}/calmodulin-dependent kinase domain, as well as an approximately 220 amino acid domain similar (47% amino acid identity) to human Doublecortin. The *doublecortin* gene is mutated in patients with X-linked lissencephaly and double cortex syndrome (des Portes et al 1998, Gleeson et al 1998). These diseases result from defects in neuronal migration, a process which is normally accompanied by translocation of the centrosome and the associated nucleus through an elongating neuronal process, and which may require proper modulation of the microtubule cytoskeleton (for review see Hatten 1999). Compatible with this view, Doublecortin is a microtubule-associated protein that stimulates polymerization of microtubules *in vitro* (Francis et al 1999, Gleeson et al 1999). This fits well with the phenotype of *zyg-8* mutant embryos, in which anaphase microtubules are short. These observations raise the possibility that the function of protein domains that modulate microtubule dynamics to ensure proper centrosome and spindle positioning may have been significantly conserved across metazoan evolution.

Conclusion and prospects

Asymmetric divisions that give rise to daughter cells of different sizes contribute to the generation of cell fate diversity during development. In this paper, we have discussed what we are in the process of learning about the mechanisms that position the mitotic spindle during the asymmetric division of the one-cell stage *C. elegans* embryo.

We have demonstrated that one of the consequences of establishing polarity along the AP embryonic axis is the generation of differential pulling forces that act on the spindle poles. Forces acting on the posterior spindle pole are more extensive than those acting on the anterior one, thus explaining the slight overall posterior spindle displacement observed in wild-type embryos. The mechanisms by which the action of PAR proteins results in such differential pulling forces remain to be elucidated. This may include local depolymerization of microtubules to generate spatially restricted depolymerization-coupled movements, or differential action of motor proteins at the anterior versus the posterior cortex.

Our work with *zyg-8* has revealed a requirement for long and/or stable microtubules to prevent exaggerated posterior spindle displacement during anaphase. Why this is the case remains to be determined. The simplest interpretation in light of the model presented in Fig. 3 is that, in wild-type, astral microtubules may need to contact components located at the anterior cortex to generate pulling force on the anterior spindle pole. If these microtubules are too short, as in *zyg-8* mutant embryos, pulling force would not be exerted on the anterior spindle pole. This would result in exaggerated posterior spindle displacement, provided some pulling force is still acting on the posterior spindle pole. Whether this or other scenarios are at play can be tested by conducting spindle-cutting experiments in *zyg-8* mutant embryos.

In conclusion, these studies have shed light on the mechanisms by which animal cells manage to position their spindle in an asymmetric manner, thus contributing to a better understanding of how cell diversity is generated during development.

Acknowledgements

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DISCUSSION

Simon: What determines the initial cell polarity?

Gönczy: Elegant experiments by Goldstein & Hird (1996) have shown that a sperm component, whose nature remains to be shown, is what determines the posterior of the embryo. This sperm component then somehow leads to polarized distribution of PAR proteins along the anterior–posterior (AP) embryonic axis.

Raff: Does the location of sperm entry determine the polarity?

Gönczy: No. If this were the case, you would expect the side of the embryo to assume posterior character in the rare cases where sperm entry is lateral. Goldstein & Hird (1996) showed this not to be the case. Instead, what they observed is that the male pronucleus and associated centrosomes move along the cell cortex towards the nearest pole, which then becomes the posterior of the embryo. This movement does not require cytoplasmic dynein function (P. Gönczy, unpublished observations) and may also be microtubule-independent.

Raff: So the AP axis is already set up, in the sense that there are poles.

Gönczy: Poles are present indeed.

Nurse: And does it always move to the nearest one?

Gönczy: That is correct.

Raff: How are the poles set up?

Gönczy: That is not known. In a way, this results from the topology of the ooctye, which already has an oval shape.

Reik: Can you parthenogenetically activate the oocyte?

Gönczy: Not that I know of, although I am not sure how much people have tried. One interesting observation related to the origin of polariy in *C. elegans* embryos is that sperm lacking DNA is still able to fertilize the oocyte and set up proper AP polarity in the embryo (Sadler & Shakes 2000). This rules out the male genetic material being required for setting up polarity. However, this leaves open the possibility that the centrosome plays an essential role in this process.

Vande Woude: How did they exclude microtubules from being involved in this activity?

Gönczy: I was referring earlier to the movement of the male pronucleus and associated centrosome towards the nearest pole. The data concerning a potential role for microtubules in setting up embryonic polarity are as follows. If you subject early embryos to the microtubule-destabilizing agent nocodazole, AP polarity does not appear to be affected (Strome & Wood 1983, Hird & White 1993). But we know that nocodazole does not get rid of all microtubules. However, we have identified a number of β-tubulin genes required for microtubule-based processes in the early embryo during the course of our large-scale RNAi-based screen, and it will be interesting to examine AP polarity in β-tubulin RNAi-embryos, which may lack microtubules entirely.

Nurse: Are there mutants which alter either the shape of the ovary or the relationship of the ovary to the AP axis of the worm? If so, are there any effects of this on the polarity of the eggs?

Gönczy: Varying ovary morphology does not seem to have an effect. In fact, Goldstein & Hird (1996) utilized mutants with altered ovary morphology in which oocytes enter the spermatheca in unusual ways, to ascribe the role of a sperm component in determining the posterior of the embryo.

Nurse: What I was really trying to get at were the external developmental cues that might be important for establishing the poles. Presumably polarity is imposed by this organ on the overall animal: is there something you could do to manipulate this to see where the axis comes from?

Ambros: Before the oocyte finally gets cellular it has cytoplasmic bridges to the rest of the gonad. These bridges are at right angles to the AP axis, and could provide a source of polarity.

Chia: How do you rationalize the generation of the posterior movement in the *zyg-8* mutants or nocodazole treated embryos?

Gönczy: There are three scenarios that I can envisage, which are not mutually exclusive. One possibility is that forces acting on the anterior aster are diminished compared to wild-type, but that forces acting on the posterior aster are unchanged. The second possibility is that forces acting on the posterior aster are increased compared to wild-type, but that those acting on the anterior aster are unchanged. The third possibility it that forces acting on both spindle poles are just like in wild-type, but that there is a problem in spindle elongation, which as a secondary consequence leads to the spindle zooming to the posterior. We will be able to distinguish between these three possibilities by severing the spindle in *zyg-8* mutant embryos, as the three scenarios have different predictions in terms of the velocity of each spindle pole after spindle severing.

Leevers: Won't the treatment with nocodazole have cut the spindle?

Gönczy: No, because we are using doses of nocodazole that don't completely abolish the spindle. We are hitting the embryo during anaphase, and the spindle shrinks a bit but not too much during the course of the experiment. We exclude from our analysis those embryos in which the spindle is completely gone. One point that I would like to make is that we don't know anything about the mechanisms of force generation at this point. Force generation could be coupled to depolymerization of microtubules, or result from the work of minus-end directed motor proteins anchored at the cell cortex, which would pull on astral microtubules.

Schaar: Is the pronucleus in the initial spindle set up in the centre?

Gönczy: Yes, the spindle assembles in the cell centre.

Schaar: How does that come about? Are there any astral interactions that this process depends on?

Gönczy: After pronuclear migration and meeting of the pronuclei in the posterior half of the embryo, the pronuclei and associated centrosomes undergo a 90° rotation while going towards the centre of the embryo. This centration/rotation process requires intact microtubules, as well as the function of cytoplasmic dynein and dynactin components (Hyman & White 1987, Gönczy et al 1999a).

Schaar: Do you need *zyg-8* for this process?

Gönczy: To a large extent you don't. However, in about 20% of *zyg-8* mutant embryos, rotation is incomplete, suggesting a partial requirement for *zyg-8* function.

Schaar: But the zipping to other poles occurs in 100%.

Gönczy: That is true in embryos that manage rotation as in wild-type. But in those 20% of embryos that don't, the spindle usually drifts slowly towards the anterior pole. We think this is because astral microtubules are not long enough to reach the posterior cortex.

Kozma: Did your screen uncover other genes with a similar phenotype?

Gönczy: In our earlier mutational analysis of chromosome III, we identified another locus, called *apo-1*, which mutates to a *zyg-8* like phenotype (Gönczy et al 1999b).

Newport: One way to explain your results would be to say that there is a protein which stabilizes microtubules on one side, resulting in more on one side than the other.

Gönczy: This is very difficult to quantitate. As you have seen from the immunofluorescence images, there are many microtubules on either side in wild-type; moreover, anaphase B takes places within a couple of minutes. Therefore, it will be difficult to uncover potential transient changes in microtubule numbers using fixed specimens. However, we have generated a green fluorescent protein

(GFP)–tubulin fusion construct in the laboratory which should be appropriate to address these kinds of questions.

Newport: In the absence of PAR-2, PAR-3 can spread uniformly and vice versa. Do you think they bind competitively to the same sites?

Gönczy: Yes, that is a possibility, but we don't know which sites they may bind to. The Kemphues laboratory has done a thorough analysis of the distribution of PAR proteins in various *par* mutant backgrounds, and this may shed light on this question (Kemphues & Strome 1997).

Nurse: Does PAR-2 or PAR-3 start from one pole and spread, or does it just invade?

Gönczy: Initially, PAR-3 has a wider domain of expression, which becomes restricted to the anterior cortex over time, presumably by the action of PAR-2 (Etemad-Moghadam et al 1995).

Vande Woude: Is this correlated with regulated expression, with *par-3* expressed first and then *par-2*? For this to occur, are they simultaneously expressed and you get free assortment, or is there some time-dependent regulation of translation?

Gönczy: The regulation of PAR-2 and PAR-3 translation is an underexplored topic of investigation. I should add that everything that happens in the one-cell-stage embryo is thought to be driven by the maternal and parental genomes, as there appears to be no zygotic transcription at that stage (Edgar et al 1994, Seydoux & Fire 1994).

Nurse: With respect to the astral microtubules emanating towards the cortex, which are shorter in the *zyg-8* mutant, are the pulling forces a consequence of the microtubules in wild-type being attached to the cortex, or are they due to attachment sites in the cytoplasm and associated motors, which means that if you extend longer into the cortex you would have a bigger pulling force than if you are shorter?

Gönczy: We don't know. Indeed, there could be either length-dependent forces which do not require astral microtubules touching the cortex, or forces that require such contact.

Lehner: Have you looked at *par-2*|*zyg-8* double mutants?

Gönczy: Yes. Because AP polarity appears normal in *zyg-8* mutant embryos, we expected that *zyg-8* mutant embryos would be able to respond to changes in AP polarity. In other words, if a *zyg-8* mutant embryo is also lacking *par-2* or *par-3* function, the spindle should no longer shoot to the posterior, but instead go to random locations around the circumference of the embryo. This is exactly what we observed, at least to a first approximation.

Edgar: What happens to the spindle?

Gönczy: The spindle is not stable, contrary to the situation in a *par-2* or *par-3* single mutant.

Ambros: Do you have any evidence for the *par*s or *zyg-8* acting later in development, for example in postembryonic asymmetric cell divisions?

Gönczy: par genes are required strictly maternally.

Ambros: In your RNAi experiments with *zyg-8*, do you see animals that survive embryogenesis, and then show nervous system defects?

Gönczy: We haven't looked.

Vande Woude: What is the similarity in the structure between PAR-2 and PAR-3 proteins?

Gönczy: Their molecular nature is completely different. PAR-3 is a PDZ-containing protein that has *Bazooka* and ASIP as *Drosophila* and mammalian cousins, respectively (Izumi et al 1998, Kuchinke et al 1998). PAR-2 is a ring-finger containing protein that does not have a characterized *Drosophila* or mammalian orthologue.

Nurse: Are the attempts to furrow failing because the spindle is moving all the time? Could it be that it sends a signal, furrowing starts and then the spindle moves, so that the signal is lost?

Gönczy: Indeed, these observations suggest that cleavage furrow specification does not occur at a single time point, but instead that the signal might be needed for a while.

Raff: In relation to Bill Chia's results, can you summarize in a few sentences what is known about the 90° turn in the spindle?

Gönczy: What Bill showed is that Bazooka, Inscuteable and Pins are required for a 90° spindle rotation in *Drosophila*. In the one cell stage *C. elegans* embryo, it appears that PAR-3, the Bazooka homologue, is not required for the 90° rotation of centrosomes that precedes spindle assembly (Cheng et al 1995).

Raff: What is required?

Gönczy: We know of a requirement for astral microtubules, cytoplasmic dynein, the dynactin components p50 and p150, as well as a protein called LET-99 (Hyman & White 1987, Gönczy et al 1999b, Rose & Kemphues 1998).

Raff: Is the original spindle vertical to the AP axis?

Gönczy: No. There is a 90° rotation of the centrosome pair and associated pronuclei that precedes spindle assembly.

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Growth factors controlling imaginal disc growth in *Drosophila*

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Abstract. In the imaginal discs of *Drosophila*, contact-dependent cell interactions are important both for promoting cell proliferation and for limiting it at the end of the growth period. However, recent work indicates that diffusible growth factors are also important in regulating growth and proliferation. We have identified a family of five imaginal disc growth factors (IDGFs) by purifying mitogenic proteins that accumulate in conditioned medium during culture of imaginal disc cell lines. These proteins cooperate with insulin to stimulate not only proliferation, but also polarization and motility of imaginal disc cells. They are produced by the fat body and are probably active on a variety of peripheral tissues. The IDGFs are structurally related to chitinases, but they show an amino acid substitution that is known to abrogate catalytic activity and to transform chitinases into lectins. We suggest that these proteins act as endogenous mitogenic lectins and mediate nutritional effects on tissue growth, possibly by interacting with the insulin receptor pathway. Glycoproteins similar to the IDGFs are found in mammals and may constitute a novel class of growth factors and/or inflammatory cytokines.

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The production of the appropriate number of cells at different times and places in the body is obviously crucial to normal development, and to controlling whether the embryo becomes a fly, a mouse or a human. Growth is controlled by factors that are both intrinsic and extrinsic to each developing organ or tissue (Bryant & Simpson 1984), and the extrinsic factors almost certainly include the polypeptide growth factors that are best known by their ability to stimulate serum-starved cells *in vitro* to re-enter the cell cycle. Alterations in the production of growth factors, and especially changes in their receptors leading to activation of signalling pathways, are likely contributors to many kinds of cancer. However, we are still quite ignorant about which factors act in various sites *in vivo*, how production and response is regulated, over what physical and temporal range the factors are effective, and how gain or loss results in disease. The production and analysis of both gain- and loss-of-function mutants in genetically tractable organisms might provide some more definitive answers to such questions.

Growth factor homologues in *Drosophila*

Here we discuss some new findings that make it possible to make more use of *Drosophila* in the analysis of growth factor functions. The *Drosophila* genome contains homologues of several of the well-known mammalian growth factors, but in most cases these have not been shown to be mitogenic using traditional assays *in vitro*. However, genetic analysis is providing many new clues about the roles of these diffusible factors and the pathways they activate during development. The best-known examples are the pathways activated by the epidermal growth factor (EGF), the decapentaplegic gene product (Dpp) and insulin. We will discuss the evidence that these pathways function in imaginal discs, the undifferentiated epithelial sacs found in the larva that differentiate into adult structures during metamorphosis.

There are several *Drosophila* gene products with significant sequence similarity to both EGF and transforming growth factor (TGF)α. These gene products act as ligands for the ubiquitously expressed *Drosophila* EGF receptor, and the specificity of EGFR activation must result at least partially from localized production of the ligands (Schweitzer & Shilo 1997). The ligands include the products of *spitz* (Schweitzer et al 1995), which is required for embryonic muscle induction, *gurken* (Neuman-Silberberg & Schüpbach 1993), which is involved in axis determination by the follicle cells and oocyte (Sapir et al 1998), and *vein* (Wessells et al 1999), which is required for growth of the wing disc (Simcox 1997). Vein is the only one of these ligands that seems to act as an authentic mitogen *in vivo* although this function has not been confirmed by direct tests *in vitro*. Loss-of-function mutations in Drk, Sos, Ras1, Raf and rolled/Map kinase all show sets of phenotypes identical to those produced by loss of EGFR in homozygous somatic clones, suggesting that these gene products function in a linear pathway downstream of the EGF receptor (Diaz-Benjumea & Hafen 1994).

The *Drosophila* homologue of TGFβ is the product of the *dpp* gene (Gelbart 1989), in which loss-of-function mutations cause dramatic reductions of imaginal disc size. This may be mainly due to massive apoptosis in mutant discs rather than to a direct effect on proliferation (Bryant 1988), but the gene product may also be required for proliferation, since blocking Dpp function using loss-of-function mutations in its receptors does result in growth inhibition (Peifer et al 1991, Burke & Basler 1996). Ectopic Dpp expression leads to excess cell proliferation, including the production of excess cells outside the expressing region, but this is associated with large-scale duplications of the pattern produced by the affected imaginal disc (Edgar & Lehner 1996), suggesting that the growth effects may be secondary to the pattern alteration, rather than resulting from direct mitogenic action.

One of the reasons that *Drosophila* has lagged behind mammals in studies designed to identify mitogenic growth factors has been the lack of appropriate *in vitro* culture techniques and assays. Only a small number of cell lines have been isolated from imaginal discs, and they do not show the classical contact inhibition seen in mammalian cells that has provided the basis for *in vitro* assay of mitogens. Instead, when they reach confluence they continue to pile up into large aggregates (Peel et al 1990). But in spite of the lack of a conventional assay system for mitogens, the data indicate that imaginal disc cell lines, and probably other *Drosophila* cell lines, are dependent on soluble growth factors for their survival and proliferation. When they are cultured in medium lacking serum, insulin and fly extract (Shields & Sang 1970) the cells round up, fail to proliferate, and instead undergo apoptosis. Addition of serum, insulin and an extract of adult flies promotes proliferation, as well as motility and polarization (Cullen & Milner 1991). Similar effects can be produced by culturing the cells in medium that has been conditioned by incubating a population of growing imaginal disc cells in the serum-free medium (Kawamura et al 1999). These results indicate that imaginal disc cells secrete factors into the medium to promote their own growth. Many similar examples of medium conditioning have been reported using mammalian cells.

Imaginal disc growth factors

We have purified and identified the active fraction from medium conditioned by imaginal disc cells from the C1.8+ line (Kawamura et al 1999). After size-exclusion filtration, anion exchange chromatography, gel filtration HPLC and preparative electrophoresis, the protein was subjected to N-terminal microsequencing. The data led to the identification of a family of five genes encoding similar glycoproteins that we call imaginal disc growth factors (IDGFs). One of them is the 47 kDa glycoprotein Ds47 (Kirkpatrick et al 1995), which was already known to be abundantly secreted from a *Drosophila* embryo-derived cell line (S2), to be produced *in vivo* by the fat body and haemocytes, and to be secreted into the haemolymph. However, no growth-control function has been reported for this protein. Three family members (IDGF1, 2 and 3) are encoded by three genes in a tight cluster on chromosome 2, cytological region 36A2-4, and another (IDGF4) is encoded by a separate gene on the X chromosome at band 9A.

The predicted amino acid sequences of the IDGFs are about 50% identical to each other (Kawamura et al 1999). They all contain an N-terminal signal sequence, and a single consensus motif for *N*-linked glycosylation as previously reported for Ds47. A close homologue has been found in the lepidopteran *Manduca sexta*, and shown to inhibit haemocyte aggregation (Kanost et al 1994), and a similar sequence is present in an expressed sequence tag (EST) database from the

silkworm *Bombyx* (P. Bryant, unpublished results). These proteins are, surprisingly, closely related to chitinase enzymes rather than to known growth factors, but Ds47 has been shown to lack this catalytic activity and the other family members are almost certainly not active chitinases. Chitinases are enzymes with poly-*N*-acetylglucosaminidase activity that are involved in digestion of the carbohydrate component of cuticle during the molt cycle. Other animals, plants, fungi and bacteria also produce these enzymes. The catalytic mechanism of chitinases is well known, and the most critical residue is a glutamate (Kirkpatrick et al 1995) that provides the proton for the hydrolysis reaction (Watanabe et al 1993). This residue is replaced by glutamine in all of the IDGFs, and the same substitution is known to abolish catalytic activity in bacterial chitinase (Watanabe et al 1993). We conclude that these proteins are evolutionarily related to chitinases but that they have acquired a new growth-promoting function that does not require chitinase catalytic activity.

To confirm that the IDGFs are the growth-promoting factors of medium conditioned by imaginal disc cells, recombinant IDGF1 and IDGF2 were prepared using a baculovirus protein expression system. These recombinant proteins promoted cell proliferation when provided at concentrations above 0.2 μg/ml (4 nM), but only in the presence of insulin. Conversely, insulin (0.125 IU/ml) was required for the effect of IDGF1 and IDGF2 on cell growth and elongation (Figs 1 and 2) but did not show either activity by itself.

The IDGF genes show similar spatial patterns of expression at the RNA level (Kawamura et al 1999). Transcripts of IDGF1–4 (probably maternal in origin) are detected in the yolk cytoplasm of the early embryo, but they appear to be excluded from the blastoderm as it cellularizes. At the syncytial blastoderm stage the transcripts are detected over the entire embryo except for the pole cells. During gastrulation, a series of morphogenetic movements creates consecutively the ventral furrow, the posterior midgut primordium, the cephalic furrow and the anterior midgut primordium. At the beginning of gastrulation, a line of strong IDGF expression appears on each side of and parallel with the ventral furrow. The lines approach one another, but the zone of expression does not invaginate with the mesoderm cells. As with the ventral furrow, IDGFs are expressed in lines parallel to each of the subsequent invaginations. These expression patterns might reveal a function for IDGFs in controlling morphogenetic changes of cell shape, consistent with the dramatic effects of these proteins on the shape of cells *in vitro* (Kawamura et al 1999).

After a phase of expression in yolk cells, the IDGF genes show strong expression in the embryonic fat body (Fig. 3A) and the salivary glands. In larvae, all of the IDGF genes are strongly expressed in the fat body (Fig. 3B,C), as well as in the ring gland and the lymph glands. They are also expressed at low levels in variable patterns in the imaginal discs and other organs. These expression patterns are

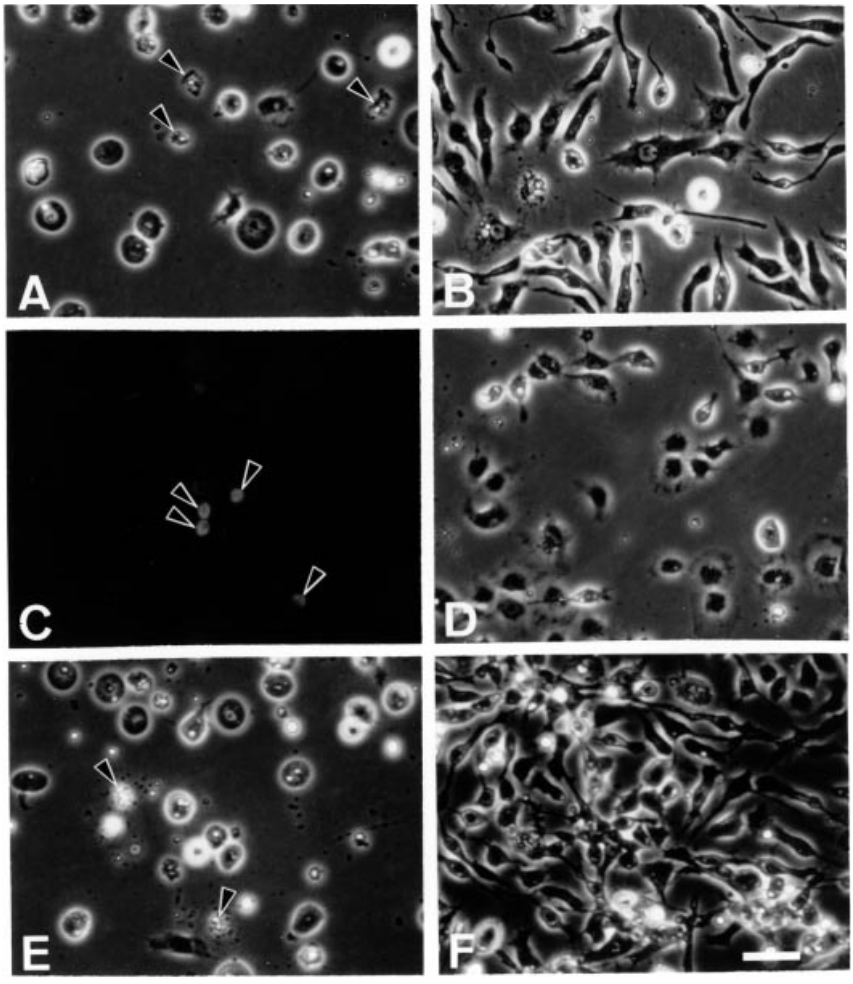

FIG. 1. Effect of conditioned medium, recombinant IDGFs and insulin on cell morphology and DNA synthesis in the imaginal disc cell line C1.8+. (A) Low-density cells (0.5×10^6 cells/ml) in supplement-free medium (SFM), two days after plating. They are round without any pseudopodia. Arrowheads indicate cell fragmentation. (B) Low-density cells in conditioned medium (CM), two days after plating. They elongate conspicuously and cell motility is also enhanced. (C) Cells were allowed to grow for one day in the CM and then prepared for anti-BrdU immunofluorescence. Labelling index was $35.1 \pm 9.9\%$. Note the labelled nuclei (arrowheads). Cells cultured in serum-free medium showed no nuclear BrdU staining. (D) Cells in SFM plus recombinant IDGF2 (0.2 μg/ml). Most cells have developed lamellipodia. (E) Cells in SFM plus insulin (0.125 U/ml). Most cells are flat and round, and apoptotic fragmentation is occurring (arrowheads). (F) Cells in SFM plus IDGF2 and insulin. Proliferation and elongation are evident. (D–F) were taken four days after plating. Bar = 25 μm. (Kawamura et al 1999.)

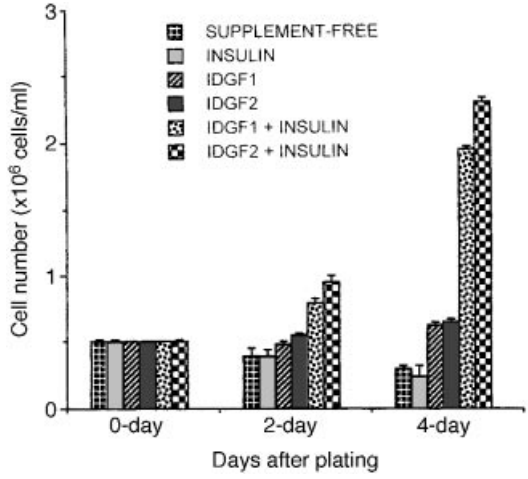

FIG. 2. Effect of recombinant IDGFs and insulin on cell proliferation in the imaginal disc cell line C1.8+. Cells were plated at 0.5×10^6 cells/ml in 24-well plates and allowed to proliferate for four days in the presence of each supplement, and cell number was estimated using the MTT method (Denizot & Lang 1986). Bars show the standard deviation. SFM was supplemented with IDGF1 or IDGF2 (0.2 μg/ml) in the presence and absence of insulin (0.125 U/ml). The results show strong molecular cooperation between IDGFs and insulin in promoting cell growth. (Kawamura et al 1999.)

consistent with previous experimental results showing that the *Drosophila* fat body produces mitogenic factors. In the larval stage, a supply of dietary amino acids is required to maintain cell proliferation in the larval brain. However, supplying amino acids directly to the brain *in vitro* does not stimulate cell proliferation. Instead, the results suggest that the effect of dietary amino acids is mediated by the production of a diffusible signal from the fat body (Britton & Edgar 1998). It seems very likely that the signal includes one or more IDGFs. This idea is supported by the finding that conditioning of medium by fat body, or co-culture with fat body, allows growth of imaginal discs *in vitro* by cell proliferation (Davis & Shearn 1977). These conditions also allow regeneration (Fain & Schneiderman 1979) and transdetermination (Shearn et al 1978) of imaginal disc fragments *in vitro*, and these events are generally thought to require cell proliferation. Our hypothesis is that the fat body exerts these effects by secreting IDGFs into the medium.

Possible interaction of IDGFs with the insulin pathway

Like other growth factors, the IDGFs presumably activate a signal transduction pathway that ultimately controls transcription and replication. One possibility is that they interact in some way with the insulin receptor (InsR) pathway, which has

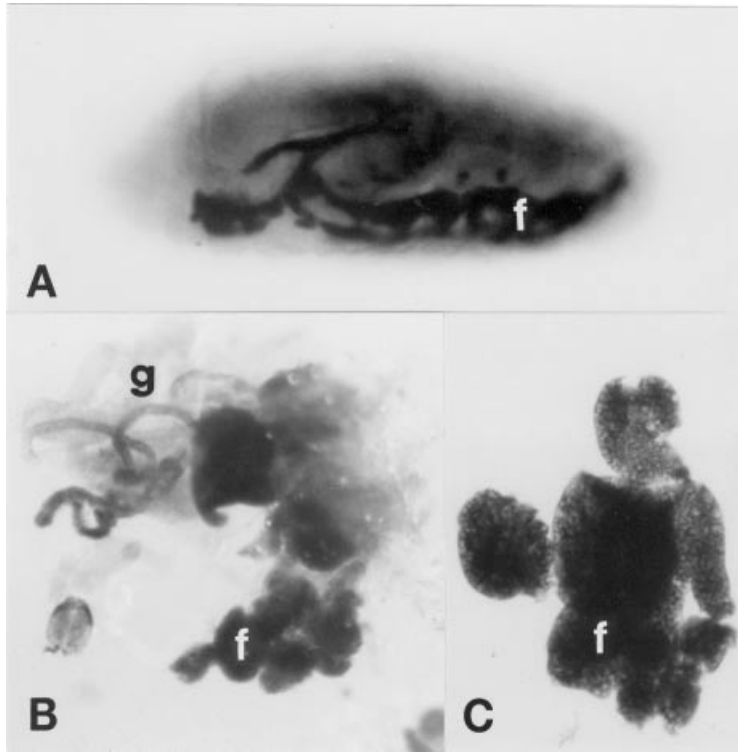

FIG. 3. Expression of IDGF genes in the embryonic and larval fat body. Embryos and larval tissues (wholemounts) were used for *in situ* hybridization using digoxigenin-labelled antisense RNA probes. (A) Late embryo showing fat body expression of IDGF2. (B,C) Third-instar larval tissues showing fat body expression of IDGF3. IDGF1, 2 and 3 show similar expression patterns at all three stages.

been shown by several genetic studies to be involved in controlling imaginal disc growth *in vivo* (Weinkove & Leevers 2000). Neither insulin nor the IDGFs are effective alone, but the combination of these factors is effective in stimulating growth of imaginal disc cells *in vitro*. Although the *Drosophila* equivalent of insulin has not yet been reported, an insulin-like molecule has been detected in larvae using antibodies against mammalian insulin (Seecof & Dewhurst 1974, Meneses & De Los Angeles Ortiz 1975). Mammalian insulin is one of the requirements for the culture of imaginal disc cell lines *in vitro* (Cullen & Milner 1991) and it promotes growth of *Drosophila* cell primary cultures (Echalier 1997). An insulin-like hormone (prothoracicotropic hormone) has been identified in other insects and is produced by the endocrine system, but this protein does not activate the *Drosophila* InsR (Fernandez-Almonacid & Rosen 1987).

More is known about the *Drosophila* InsR and the downstream elements of this pathway. The receptor is expressed in imaginal discs (Garofalo & Rosen 1988), and is required for their normal growth (Chen et al 1996). It is a tetrameric glycoprotein with two α and two β subunits (Fernandez-Almonacid & Rosen 1987) and as in the mammalian receptor, the β subunit contains a transmembrane domain and a ligand-activated tyrosine kinase domain (Petruzzelli et al 1986, Nishida et al 1986). In mammalian cells, and probably in *Drosophila* as well, activation by ligand results in receptor autophosphorylation as well as recruitment of insulin receptor substrate proteins (encoded by *chico* in *Drosophila*) (Böhni et al 1999) to the receptor and subsequent phosphorylation of multiple tyrosine residues on the receptor and the substrate proteins. This triggers the activation of several signalling pathways including the Ras/MAP kinase pathway and the PI3K/PKB pathway (Yenush et al 1996).

The signal transduction pathway downstream of InsR seems to be well conserved in *Drosophila* compared to mammals, and to play an important role in controlling growth and body size (Weinkove & Leevers 2000). Overexpression of the insulin receptor in the developing eye imaginal disc causes enlargement of the eye in the adult, suggesting an increased growth rate of this tissue during the larval or pupal period (Huang et al 1999). Overexpression of wild-type *Drosophila* PI3K *in vivo* also leads to enlarged organs, and at least in the wing this is partly due to increased cell number (Leevers et al 1996). In contrast, overexpression of the PI3K target Dakt1 leads to increased cell size but does not affect cell number (Verdu et al 1999), so it is possible that the insulin receptor pathway divides between PI3K and Dakt1 into a branch that regulates cell size (via Dakt1 and S6 kinase) and one that regulates cell number (via unknown targets).

Expression of an activated form of Ras can also cause hyperplastic imaginal disc overgrowth (Karim & Rubin 1998), which might involve activation of the MAP kinase pathway. However, as with mammalian Ras, *Drosophila* Ras could also activate the downstream elements of the InsR pathway through direct interaction with PI3K (Weinkove & Leevers 2000). Additional pathway components must be identified in order to understand exactly how InsR signalling functions in controlling both cell size and cell proliferation *in vivo*. Direct studies of the interaction of the IDGFs with this pathway are also needed in order to understand the relationship between insulin and the IDGFs in controlling these processes.

Mammalian homologues of IDGFs

Glycoproteins similar to the IDGFs are found in mammals and may constitute a novel class of cytokines, some of which are important in inflammation. The best characterized is the human glycoprotein HC gp-39 (=YKL40; 16–23% identical to IDGFs), which accumulates in the synovial fluid of rheumatoid arthritis

patients, and is one of the antigens stimulating the autoimmune response associated with this disease (Verheijden et al 1997). It is produced by inflammatory macrophages associated with the rheumatoid synovial membrane, and is a major secretory product of articular chondrocytes from patients with other forms of arthritis (Hakala et al 1993). A mouse homologue, the eosinophil chemotactic cytokine (ECF-L; identical to YM-1 and MCRP, and 16–20% identical to IDGFs) has been purified from medium conditioned by splenocytes and shown to be induced by parasitic infection (Owhashi et al 2000). The protein stimulates chemotactic activity of eosinophils, T lymphocytes and bone marrow polymorphonuclear leukocytes *in vitro*, and causes extravasation of eosinophils *in vivo*. Additional mammalian chitinase-related proteins include Brp-39 (14–19% identical to IDGFs) secreted by certain mouse mammary tumours (Morrison & Leder 1994), the heparin-binding glycoprotein gp38k, produced during differentiation of vascular smooth muscle cells (Shackelton et al 1995), and related proteins that are secreted by the oviduct (Buhi et al 1996). A closely related chitotriosidase accumulates in the bronchoalveolar fluid of rats with silicosis, and in this case the protein has been shown to be mitogenic on fibroblasts (Guoping et al 1997). All of these examples show intriguing connections between IDGF-like proteins and disease and are consistent with the suggestion that these proteins contribute to the disease processes by acting as cytokines or mitogens. Supporting the idea that these proteins are mitogenic is the finding that several of them accumulate in conditioned medium. For example, YKL40 and the closely related YKL39 (15–21% identical to IDGFs) accumulate in chondrocyte-conditioned medium (Hu et al 1996). As with *Drosophila* cells and IDGFs, these cells may adapt to culture *in vitro* by up-regulating the production of these mitogens to stimulate their own growth.

The mammalian homologues of IDGFs have the same relationship to chitinase enzymes that we described earlier for IDGFs. Substitution of the critical glutamic acid in the catalytic core of human chitotriosidase by leucine has been shown to eliminate catalytic activity; but it also increases the affinity of this protein for chitin, converting it from an enzyme into a lectin (Renkema et al 1998). The human IDGF relative HC gp-39 has exactly this substitution, so it might function as a lectin. In the murine IDGF homolog ECF-L, the glutamic acid is replaced by glutamine as in all of the IDGFs, and this protein also shows no chitinase activity but has strong affinity for chitin (Owhashi et al 2000). This suggests that the IDGFs and other chitinase-related proteins might be endogenous lectins with an affinity for chitin-like oligosaccharides.

IDGFs as lectins?

An intriguing possibility is that the IDGFs could interact as lectins with the insulin receptor itself, which is already known to bind other lectins. Wheat germ

agglutinin (WGA), a mitogenic lectin for which the receptor sugar is *N*-acetyl-glucosamine, binds to the mammalian insulin receptor (see *http://www.vectorlabs.com/frames/FRLectins.htm*), suggesting that its mitogenic activity could be mediated in part through the insulin receptor pathway. Further evidence supporting interaction between mitogenic lectins and the insulin receptor comes from demonstrations that some lectins compete with insulin for binding to cells expressing the insulin receptor (Rouiller et al 1986) and glycosylation mutants show altered insulin binding (Podskalny et al 1984). Furthermore, when one of the acceptor asparagines for glycosylation in the mammalian insulin receptor is altered by site-directed mutagenesis, the mutant receptor shows a defect in ligand-induced internalization, major alterations in tyrosine kinase activity and autophosphorylation, and is unable to transduce the signal for either glycogen or DNA synthesis (Leconte et al 1994). All *N*-linked oligosaccharides have the same pentasaccharide core Mannose α-1,3 (Mannose α-1,6) Mannose β-1,4 GlcNAc β-1,4 GlcNAc, which is recognized by numerous lectins (Di Virgilio 1997). Since chitin is a β-1,4-linked GlcNAc linear polymer, it seems likely that chitin-specific lectins might also interact with *N*-linked oligosaccharides.

A role for lectins in regulation of the *Drosophila* insulin receptor is suggested by the fact that the receptor showed increased basal autophosphorylation when purified using a lentil (mannose-specific) lectin column (Ruan et al 1995). However, the *Drosophila* insulin receptor has a different lectin-binding specificity than the mammalian homologue, showing a higher affinity for mannose-specific lectins than for WGA (Marin-Hincapie & Garofalo 1995).

Conclusions

Our work on the *Drosophila* IDGFs supports the idea that the mammalian relatives might contribute to inflammatory disease by acting as mitogens on fibroblasts and perhaps other cell types. If confirmed, this finding opens up major new opportunities for rational drug design for some common and debilitating disorders. As far as we are aware, none of the mammalian proteins has been tested for any interaction with the insulin pathway. The identification and analysis of the IDGFs should help in understanding the functions of these mammalian family members and their relationships to disease states.

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DISCUSSION

Raff: How long can you keep your cells dividing in culture?

Bryant: We find that they senesce after a certain number of divisions, so we keep a large stock of them frozen.

Nurse: Do different IDGFs have the propensity to transdetermine discs to different fates?

Bryant: Not as far as I know. We have started ectopic expression studies. When we express them in imaginal discs they don't show a growth phenotype. They don't seem to be acting on a local level in this way.

Nurse: They don't switch fate?

Bryant: No. We don't have any indication that they would do that, but it hasn't really been tested.

Leevers: Have you done any experiments that clearly distinguish whether IDGFs are acting as survival, proliferation or growth factors?

Bryant: The simple answer is no.

Leevers: I assume it isn't growth; instead it is probably proliferation and survival, but it is hard to study this. There are ways that people have dissected these out in other systems.

Bryant: As you know, our experiments so far are crude: we see an increase in cell number.

Thomas: Cbl is a ring finger protein which is involved in down-regulation of the receptor. This didn't fall out of the original screen. Are there mutants in Cbl as well?

Bryant: There are no mutants in Cbl but it has been tested in an ectopic expression assay, and if it is expressed in the eye disc it interferes with photoreceptor cell development (Meisner et al 1997).

Edgar: Is the interaction between discs-large (Dlg) and EGFR involved in speeding up growth during the proliferative phase, or just extending the proliferative phase longer? EGFR is necessary for growth in the early disc but it is also used to pattern the disc and give vein/intervein patterning. Where do you think your findings fit in?

Bryant: If the EGF pathway is the main target, then I would have to conclude that Dlg is negatively regulating the pathway, possibly by localizing EGFR and keeping it away from downstream targets. This is on the basis of the phenotype that we get from partial rescues.

Lehner: Can you grow imaginal discs with purified IDGF?

Bryant: Yes, although we didn't use purified IDGF, but recombinant IDGF produced from a baculovirus expression system. These are clone 8 cells from an imaginal disc; we haven't tried growing the whole imaginal disc.

Thomas: Have you tested the two PDZ domains independently?

Bryant: Yes. We find very strong co-operativity between those domains. The three PDZ domains of Dlg seem to have the same specificity when we test them against many different partners. If you have two of them, there is strong binding to EGFR, but with one of them it is much weaker. With all three of them it is very strong. This is not rigorous evidence for what is generally called co-operativity, but it means that there is a biologically meaningful difference in affinity, depending on the number of PDZ domains present.

Hunt: What do you know about the modality of signalling of these growth factors? What do they bind to and what pathways are they stimulating inside the cell?

Bryant: Perhaps it is the insulin receptor pathway.

Hunt: Insulin is already there, isn't it?

Bryant: Yes, they might act as cofactors with insulin.

Hunt: Are they competing for binding at the same receptor? I don't understand that.

Bryant: With the mammalian receptor, lectins will compete with insulin for binding to the receptor (Roullier et al 1986). I am thinking that the IDGFs may be co-operating, not competing. The insulin receptor is heavily glycosylated, one of the sugars is *N*-acetyl glucosamine, and it has been shown that this glycosylation is absolutely required for its function (Leconte et al 1994). Even with the *Drosophila* receptor, one of the ways of purifying it is by lectin-affinity chromatography. When you purify it this way, it ends up being activated (Ruan et al 1995).

Hunt: The other corollary is, have you tried putting other activated oncogenes of the signalling variety (such as *ras* and *src*) into these cells to make them independent of the exogenous signalling molecules?

Bryant: We haven't tested any of those. Bruce Edgar has shown that activated Ras does promote proliferation *in vivo*.

Edgar: We have looked at proliferating neuroblasts in the brain. When we co-culture the brain with fat body those cells proliferate, but when we express activated ras in them without fat body, they don't proliferate.

Nurse: Do these factors stimulate other *Drosophila* tissue culture cells, or are they specific for the imaginal discs?

Bryant: From what we have done with the genetics, it looks like they are required for larval growth in general. At least two other cell lines have been shown to condition the medium in this way.

Raff: Is it clear where the ligand Vein comes from?

Bryant: No.

Edgar: There are some pretty pictures showing the Vein expression patterns: mRNA is transcribed in really fancy patterns that change throughout development.

Raff: Do these patterns correlate in some way with the proliferation domains?

Edgar: Potentially.

Raff: So vein could be an important mitogen in the discs.

Edgar: Yes, the vein ligands are required for growth of the disc.

Raff: I have a question about the insulin receptor glycosylation. Is it needed for function, or is it just needed to get the receptor to the plasma membrane in a stable form?

Bryant: The work published on the mammalian insulin receptor shows that glycosylation is required for function. There is a mutagenesis study showing that there is a particular Asp residue, without which the receptor is expressed on the membrane but doesn't function (Leconte et al 1994). In other studies the biosynthesis of the oligosaccharides has been inhibited, and this has the same kind of effect (Podskalny et al 1984).

Raff: Is there any precedent for a lectin–ligand interaction on a receptor in any other system?

Bryant: There is some genetic evidence from *Drosophila* that the wingless signalling pathway requires glycosaminoglycans expressed on the cell surface (Haerry et al 1997).

Raff: I thought that the glycosaminoglycan is supposed to bind the ligand and present it to the real receptor.

Bryant: It could do.

Raff: Is Vein membrane-bound, or is it a secreted ligand?

Bryant: It is predicted to be secreted.

Schaar: In any of the EGF receptor mutants, is there an analogous story to *Caenorhabditis elegans* vulval development, where the receptor has to bind a PDZ domain and be localized in order to signal properly?

Bryant: This has not been studied in *Drosophila*. Several Dlg-like MAGUKs have been picked up in a yeast two-hybrid screen using as bait the Erb-B4 receptor (Garcia et al 2000), which is the only one of the mammalian EGF receptor family that has a C-terminus predicted to bind to PDZ domains. This interaction could be involved in controlling receptor localization. Potentially it is the same kind of interaction.

Raff: Does Vein go away when cell proliferation stops?

Edgar: No, it is used again in vein patterning.

Raff: What about in the disc itself?

Edgar: The protein doesn't go away. The whole system is used over and over again. The Ras pathway is required in growth in an early disc and is also used later in wings for vein–intervein patterning and in eyes for photoreceptor determination. This is all post-mitotic.

Raff: I'm just trying to figure out why, when the cells don't have Dlg, they don't stop dividing. Do you have an explanation for this?

Bryant: I have always thought that by looking at these kinds of mutations we would find elements of the pathway downstream from the pattern formation system. Perhaps this is where EGFR fits in: once there is a local signal for proliferation, it is mediated through that pathway, and discs-large is interfering with the way this is controlled. The other mutations that give these kinds of phenotypes might identify other elements of that pathway downstream from pattern formation.

Raff: Is this true in all discs?

Bryant: Yes, until the animal dies. This is related to the other feedback mechanism that we talked about earlier. All of these overgrowth mutants have a prolonged larval life, and during that time you get the overgrowth. I think that the reason that larval life is so prolonged is that there is continuously growing disc tissue in the animal, and it sends out a signal. There were some experiments done

where those kinds of mutants were combined with mutants that eliminate imaginal discs, and then you don't get the protracted larval life. Or if you wipe out the discs with X-rays you shorten the life as well. Their phenotype is consistent with the idea that growing tissue in the periphery blocks the endocrine system from initiating progression to the next stage of the life cycle (for review see Bryant & Schmidt 1990).

Raff: Do you need the endocrine system — for example, the fat body — to stop disc growth at the right time?

Bryant: No, you can put these mutant imaginal discs into an adult fly as a culture medium, and they will continue growing, whereas the wild-type disc will stop growing at the normal final size (see Sehnal & Bryant 1993 for review). I don't think there is a systemic signal for shutting down growth.

Edgar: I had a comment about why things stop and how signalling is tied up in this. One thing we see with the EGF/vein pathway and also the wingless pathway is that the patterns of expression of the ligands go from rather low level diffuse expression during the proliferation stage to really sharp on–off patterns in the differentiation stage. One explanation that has been offered is that low uniform signalling gives proliferation, and on–off states of high or low signalling effect differentiation.

McMahon: Also, if the signals that are regulating growth come from a fixed position, and the system is growing in response to that, then cells are being pushed from that position and can commit to terminal fates. So you only have to control very locally the action of a local proliferative signal.

Raff: The problem with this is that it is not what is seen with BrdU labelling. If these signalling centres are the source of mitogen, you might expect to see most proliferation near the centres, and apparently you don't.

McMahon: I think there is evidence for that. In the development of the midbrain in mammals, differentiation occurs relative to the midbrain–hindbrain junction, which is the source of signals shown to regulate proliferation. As it gets larger, the zone of commitment to post-mitotic fates gets closer and closer to that junction. In the end, the problem could be just how do you finally extinguish that mitogen there.

Bryant: The first place that shuts down growth is along the prospective wing margin (O'Brochta & Bryant 1985). This is not generally considered as distant from signalling sources.

Raff: I wonder if the reason that there are relatively few cell lines in flies is that if they don't make IDGFs you don't get them as lines. Have you looked to see whether all *Drosophila* cell lines make IDGFs?

Bryant: I haven't looked at all of them, but it is already known that several of them do. We also know that they are not universal. For example, the medium that is

conditioned by S2 cells does not permit clone 8 cells to proliferate (K. Kawamura, personal communication). So there may be some interesting tissue specificity.

Raff: Would it be worthwhile to make more lines, adding all the known IDGFs to the culture medium?

Bryant: We are collecting lines; we are not making more. There are plenty already.

Raff: How come, then, that everyone seems to use the same cell line when they do transfection experiments?

Leevers: I think it is the only line for which there is an established transfection protocol.

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General discussion IV

Spatial organization and the cell cycle

Nurse: In this general discussion I would like us to address some of the issues relevant to spatial organization. We have heard several papers concerning asymmetrical divisions or specific orientation of the spindle. It seems that we can ask a number of questions. When a spindle is lined up in a particular direction, is there a common mechanism in the different cases where this occurs? Could we imagine a common mechanism involving the core machinery of microtubule organization? Does this intimately involve the cortex, and how does it relate to external signals in the organism? In terms of asymmetrical determinants, how often is this cortical? And how can we relate this symmetry with the overall polar axis in the organism?

Nasmyth: A lot of the original asymmetry of the embryo is historical. The nurse cells are concentrated on one side. This is not dissimilar from epithelial delamination: you start off with asymmetry there.

Nurse: You are drawing attention to the fact that usually asymmetries are associated with asymmetry in the environment. Does it mean anything to imagine asymmetry outside this?

Edgar: What about lateral inhibition in neuroblast determination? This generates asymmetry that is presumably not dependent on any cell division. This is not intrinsic; it relies on cell communication. There must be countless examples of this kind of asymmetry being set up.

Nasmyth: There are many examples where this is a stochastic event. 'Stochastic' can refer to genes flicking on and off, or it can be choosing a position on the cell and marking that point — budding in yeast is a good example of this. Also, in the lateral inhibition, it is which one of the neuroblasts will win out. Once you have established this, you have created a focus for generating asymmetry.

Nurse: Then lateral inhibition is required to reinforce that.

Nasmyth: Presumably it is a sort of intracellular version of that that gives rise to a bud in one position inside a yeast cell. This is completely unsolved: starting from a completely symmetrical cell you get a bud at one side.

Goodwin: That is similar to what happens in *Fucus*. There is a symmetrical cell, and even in the absence of any polarization due to light, it will break symmetry and produce an axis. There is probably a similar sort of stochastic event that triggers some kind of polymerization or pattern.

Nasmyth: Presumably the same thing is taking place in the mammalian embryo, when it suddenly breaks symmetry.

Raff: It also seems to be true in nerve cells, in the formation of dendrites and axons. This seems to be a stochastic process.

Gönczy: Similarly, the 90° rotation of centrosomes that precedes spindle assembly in the *Caenorhabditis elegans* embryo can be cell intrinsic. This is obviously the case in the one cell stage, but also in P1, the posterior blastomere of the two cell stage embryo. If you isolate P1 shortly after the first cleavage division, 90° rotation of centrosomes still takes place (Goldstein 1995). However, cell-extrinsic influences are crucial in other blastomeres. For instance, repositioning of centrosomes in EMS, one of the daughters of P1, depends on a signal emanating from P2, the other daughter of P1 (Goldstein 1995). It is tempting to think that there is a basic cell intrinsic machinery that can be used either as such or be regulated by signalling, depending on the cellular context in the organism.

Nurse: In this particular case you are arguing that the actual centrosomal rotation may be common, but the developmental signal that generates this rotation is in one case cell intrinsic, and in the other case is dependent on external signals.

Gönczy: That is right.

Nurse: What is the likely molecular mechanism for the orientation of the spindle in those circumstances?

Gönczy: One of the popular models to explain the 90° rotation of centrosomes in P1 invokes capture of astral microtubules at an anterior cortical site by a localized minus-end directed motor. This would generate a torque on the centrosome/nuclear complex and reel one of the centrosomes in the direction of the cortical site (Hyman 1989).

Schaar: Isn't the cortical site a remnant of the first cleavage furrow? This is set up from the previous division.

Gönczy: I am not saying that the process is random. But it remains that rotation of centrosomes still takes place when P1 is isolated from its sister blastomere early in the two cell stage (Goldstein 1995). In contrast, if the same experiment is carried out with EMS, centrosome repositioning no longer takes place (Goldstein 1995).

Nurse: In that context, in budding yeast there is nuclear migration and there are astral microtubules captured by the bud tip. The bud tip will have also been marking where cytokinesis will have been initiated. There might be some links there.

Schaar: Another connection between budding yeast and metazoan systems is that Eb1 and its homologue in budding yeast, and also APC, are dynamically localized to the growing tips of microtubules. The connection isn't certain, but people place APC in the Wnt signalling pathway. Perhaps there is a non-nuclear pathway that is

occurring that is involved in spindle positioning. Certainly, the components are where you would expect them to be at the dynamic ends.

Nurse: Mal3, an Eb1 homologue in fission yeast, interacts with a collection of other proteins that we have identified which are all localized at the ends of microtubules. They target the growth of microtubules to the cell ends in fission yeast; in their absence, microtubules just keep growing.

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The temporal control of cell cycle and cell fate in *Caenorhabditis elegans*

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Abstract. The nematode *Caenorhabditis elegans* develops through two major phases: the first phase, embryogenesis, consists of a rapid series of cleavage cell divisions leading to morphogenesis of a first stage larva. The second phase is postembryonic development, which consists of developmentally regulated cell cycles that occur during the four larval stages leading to the adult. Precursor cells set aside during embryogenesis divide through stereotypical cell lineage patterns during the four larval stages to generate larval and adult structures. The precise timing of the postembryonic cell divisions is under strict control, in most cases with a developmentally regulated G1. In certain postembryonic cell lineages, various aspects of the cell division cycle, including cell cycle exit, or G1/S progression, are controlled by temporal regulatory genes of the heterochronic gene pathway. Heterochronic genes also control the timing of numerous other developmental events, indicating that this pathway functions to coordinate the schedule of cell division and cellular differentiation throughout the animal. Some choices of cell fate that occur in response to inductive or lateral signals are linked to cell cycle progression, suggesting that cell cycle phase can confer a critical period for developmental potential in certain cells.

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The relationship between the cell cycle and the developmental program of the nematode *Caenorhabditis elegans* has been viewed from shifting perspectives over the years. After the publication of the complete cell lineage of *C. elegans* (Sulston & Horvitz 1977, Sulston et al 1983), a conventional wisdom emerged wherein a cell's developmental fate was thought to be strictly governed by its lineage history. The cell lineage of this animal was discovered to be essentially invariant; each particular cell is generated by precisely the same pattern of cell divisions from animal to animal. So early thinking about *C. elegans* development was dominated by the concept of 'mosaic development' where individual differentiated cells were generated by some kind of internal programming linked to cell division history. This was in contrast to other animals, such as flies and humans, which were known to develop by much more flexible developmental strategies, where large fields of

cells with indeterminate fates become specified to particular developmental pathways by extracellular signals.

This perspective shifted substantially in response to the results of experiments where the positions of two blastomeres of the *C. elegans* four-cell embryo were reversed, and their fates were switched (Priess & Thomson 1987). These two cells always express distinct and specific fates when left undisturbed — an invariance in developmental fate that was now understood to stem not from invariant cell lineage history, but from invariant cell contact history. Indeed, genetic analysis of developmental decisions at various steps of *C. elegans* development began to reveal that inductive interactions among cells are widespread in *C. elegans* — despite the invariant pattern of normal development (Mello et al 1994). What had seemed at first to be a developmental strategy whereby cell fates were coupled to cell cycle history is now understood to involve an interplay between intrinsic determinants and extrinsic signals.

Curiously, once emphasis shifted away from cell lineage as a dominant determinant of cell fate, the mechanisms of cell cycle control in the nematode was, for a time, given little emphasis. This is despite the fact, of course, that the complete cell lineage of this animals was known — and hence represented a precise spatial and temporal pattern of cell cycles that was, in principle, accessible to genetic and molecular analysis. Pioneering work in *Drosophila*, showing how the temporal and spatial patterns of cell cycles in the fly embryo are coupled to developmental regulators (for reviews, see Edgar & Lehner 1996, Lehner & Lane 1997), established a paradigm for thinking about how the *C. elegans* cell lineages are generated. Recently, *C. elegans* has become increasingly employed to address questions of how the cell cycle machinery is coupled to developmental regulators, particularly on the level of individual cells. The completion of the genomic sequence of *C. elegans* (The *C. elegans* Sequencing Consortium 1998) has greatly stimulated the identification of *C. elegans* cell cycle components and their regulators. This review will briefly cover some of the recent advances in our understanding of how the cell cycle interacts with developmental regulatory pathways in *C. elegans* to produce the right numbers and types of cells at the right times in development.

Developmental patterns of *C. elegans* cell cycles

C. elegans embryonic development (Fig. 1), like that of many animals embryos, involves a series of rapid cleavage cell divisions which generate the various cells of the newly-hatched L1 larva (Sulston et al 1983). Postembryonic development differs substantially from embryonic development, chiefly in that postembryonic cell cycle progression requires growth. In the absence of food, the hatchling does not develop and postembryonic cell cycles are suspended in G1 until food is

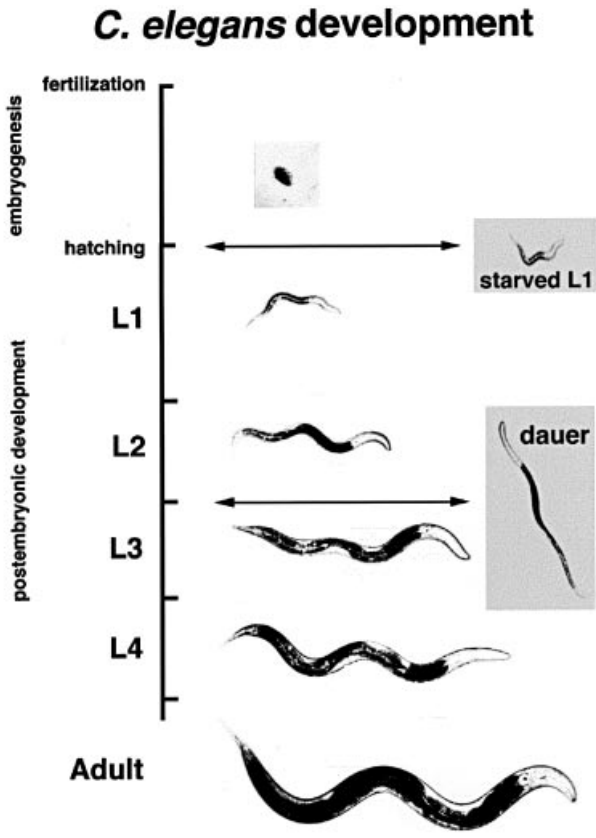

FIG. 1. Phases of *C. elegans* embryonic and postembryonic development. Embryonic development involves cleavage cell cycles and no increase in mass. Embryogenesis is separated from larval development by an extended G1 phase that spans hatching. After hatching, subsequent cell cycle progression depends on, in addition to the intrinsic cell cycle machinery, other factors such as cellular growth and developmental signals. Postembryonic cell divisions occur during all four larval stages (L1–L4). Animals can develop continuously, or they can arrest as newly-hatched L1 larvae if food is absent. Starvation and crowding induces L2 animals to molt to the developmentally arrested dauer larva (Riddle & Albert 1997). Arrested L1 larvae or dauer larvae will resume development if transferred to favourable culture conditions.

encountered (Hong et al 1998). Postembryonic development can also be temporarily suspended at the end of the second larval stage, when harsh environmental conditions can cause the animal to arrest development as the so-called 'dauer larva' (Riddle & Albert 1997). Dauer larva cell cycles also seem to be arrested in G1 (Hong et al 1998). When dauer larvae are returned to favourable conditions, they resume development through the L3 and L4 stages to the adult. Although dauer larvae can only arrest at the second moult in the

wild type, in certain mutants, the arrest can occur at the L1 or L3 moults (Liu & Ambros 1989), indicating that cells throughout larval development may have the capacity to suspend cell cycling. Many postembryonic cell lineages exhibit lengthy G1 phases during continuous development (Hedgecock & White 1985), suggesting that cell cycle progression in the *C. elegans* larva is chiefly regulated via the G1 cell cycle machinery.

C. elegans cell cycle machinery

Components of the conserved cell cycle machinery are rapidly being identified in *C. elegans* and their roles are being tested in the context of the developing worm. For example, a number of Cdc2-related protein kinases were identified from the genomic sequence, and the gene encoding one of these, *ncc-1* was found by RNA-mediated interference (RNAi) experiments, and by mutant phenotypes, to be required for mitosis in embryonic and postembryonic cell divisions (Boxem et al 1999). *ncc-1* is not required for S phase, and this M-specific role indicates that *ncc-1* encodes an orthologue of Cdk1/Cdc2. Other members of the *C. elegans* Cdc2-related class of kinases do not seem to have critical roles, as judged by gene inactivation experiments, suggesting that they may act redundantly.

C. elegans cyclin A and B have been identified, and functional characterization of them suggests roles in oogenesis and early embryogenesis (Kreutzer et al 1995). The *C. elegans cyd-1* and *cdk-4* genes encode cyclin D and a cyclin kinase related to Cdk4/Cdk6, respectively (Park & Krause 1999). Both *cyd-1* and *cdk-4* are expressed during embryonic development, and during many postembryonic cell lineages with a temporal pattern coincident with postembryonic G1/S progression. Loss-of-function phenotypes of *cyd-1* and *cdk-4* indicate that cyclin D and CDK-4 are required for postembryonic development, but not for embryonic cell cycles (Park & Krause 1999), perhaps consistent with an apparent lack of G1 phases in the worm embryonic cell cycles. It is not known what developmental regulators govern the timing of *cyd-1* and *cdk-4* expression during the larval stages and to what extent cyclin D and CDK-4 are limiting factors controlling timing of cell cycle progression. In contrast to cyclin D, which may be specific to postembryonic development, the *C. elegans* cyclin E seems to be required for both embryonic and postembryonic cell cycle progression (Fay & Han 2000).

Cullins were identified in *C. elegans* by mutations of *cul-1* and *cul-2* that cause an over-proliferation of postembryonic cell divisions (Kipreos et al 1996). They define a protein family that is widely employed for targeting proteins for ubiquitin-mediated proteolysis. The CUL-1 and CUL-2 proteins appear to control the number of cell divisions in numerous *C. elegans* larval cell lineages by targeting cyclins and other cell cycle components (Kipreos et al 1996, Feng et al 1999). The developmental regulation of *cul-1* and *cul-2* activity is likely to underlie

at least some of the strict control of cell division timing and cell cycle number in *C. elegans*.

Cyclin kinase inhibitors (CKIs) of the p21/p27 class (Sherr & Roberts 1999) are represented in *C. elegans* by the products of the *cki-1* and *cki-2* genes (Hong et al 1998, Feng et al 1999). *cki-1* is the best characterized of these two. Inactivation of *cki-1* results in extensive postembryonic over-proliferation phenotypes, suggesting that *cki-1* is widely employed throughout *C. elegans* embryonic and postembryonic development to limit the number of cell cycles (Hong et al 1998).

cki-1 is expressed in differentiating cells, and in numerous resting progenitor cells at various stages of *C. elegans* development, and also in larvae developmentally-arrested as dauer larvae or starved hatchlings (Hong et al 1998; Table 1). This developmental pattern of expression is displayed by transgenes with the *cki-1* promoter fused to *gfp*, indicating that *cki-1* transcription is developmentally regulated. The *cki-1* expression patterns in resting progenitor cells are consistent with the *cki-1* over-proliferation phenotypes, and suggest that *cki-1* inhibits G1 cyclin/Cdk complex(es).

In some cases, such as the hermaphrodite vulval lineage, *cki-1* loss-of-function defects include an extra round of precursor cell division, without any appreciable change in the timing of subsequent vulval differentiation. Although the timing of vulva differentiation is normal in *cki-1* loss-of-function animals, the vulva is often non-functional, suggesting that *cki-1* is required for the completion of proper vulval differentiation. *cki-1* is expressed in differentiating neurons, muscle and hypodermal cells, and the *cki-1* loss-of-function phenotype includes uncoordinated behaviour (Hong et al 1998), which may result from defects in neuronal or muscle differentiation.

C. elegans lin-35 encodes a protein closely related to retinoblastoma (Rb) tumour suppressor (Lu & Horvitz 1998). The *lin-35* gene product, together with the product of *lin-53*, which is similar to the RbAp48 Rb-binding protein, antagonizes the Ras signalling involved in vulval cell fate determination. *lin-53* may also have essential roles as well (Lu & Horvitz 1998), but it is not clear if *C. elegans* Rb and its partners are involved in controlling cell cycle progression, as in vertebrates.

Developmental pathways controlling cell cycle progression

Work from *Drosophila* (Edgar & Lehner 1996, Lehner & Lane 1997) established the paradigm that the timing of cell cycle progression during development could result from the coupling of limiting components of the cell cycle machinery to the activity of developmental signals or regulatory molecules. In *C. elegans*, genetic pathways affecting cell cycle progress have been identified, and in some cases, the targeted cell cycle machinery has been identified.

TABLE 1 Stage- and tissue-specific expression of a *cki-1::gfp* reporter gene during *C. elegans* development

Developmental stage	*cki-1::gfp expressing cell type*	
Mid-embryogenesis	Pharynx primordium	
Late embryogenesis	numerous cells	
Early L1	Z1/Z4 gonad cells	Hypodermal V cells Hypodermal P cells
	Q cell	M cell
L2	Pn.p cells	Sex myoblasts (SM)
L2 moult	Pn.p cells	Distal tip cells
L3 moult	Vulva cells	Sex muscles
L4 moult	Seam cells	
All larval stages	Neurons	Intestine cells
Dauer larva	Lateral hypodermis, SM,	VPC, intestinal cells

From Hong et al (1998).

Global cell cycle arrest in dauer larvae occurs in response to crowded environmental conditions, and involves converging extracellular signals via transforming growth factor (TGF)β, insulin and cyclic GMP pathways (Riddle & Albert 1997). The observation that *cki-1* is up-regulated in dauer larvae, and that depletion of *cki-1* activity allows cell cycling in dauer larvae, suggests that *cki-1*, a p27 homologue, is an effector of G1 arrest in dauer larvae (Hong et al 1998). The involvement of a TGFβ pathway in dauer larva arrest (Ren et al 1996) and in the regulation of p27 in mammalian cells (Polyak et al 1994), suggests that a TGFβ–p27 link may be evolutionarily conserved.

The heterochronic gene pathway affects the time of expression of diverse stage-specific events during *C. elegans* larval development (Ambros 1997), including stage-specific cell cycle behaviour (Euling & Ambros 1996). Two examples of cell cycle behaviour that are affected in heterochronic mutants include cell cycle exit accompanying hypodermal cell terminal differentiation, and G1/S progression in vulva precursor cells (VPCs). These cell cycle effects are highly cell type-specific, and hence likely reflect specific interactions of the heterochronic gene pathway with components of the cell cycle machinery in these particular cell types.

In the lateral hypodermis, the timing of terminal differentiation, which normally occurs at the L4 moult, is controlled by the activity of the heterochronic gene *lin-29*. *lin-29* encodes a zinc-finger transcription factor, and thus likely affects terminal differentiation in this cell type by controlling the transcription of genes associated with aspects of differentiation, including cell cycle exit (Rougvie & Ambros 1995). *lin-29* is required for the up-regulation of *cki-1* in these terminally

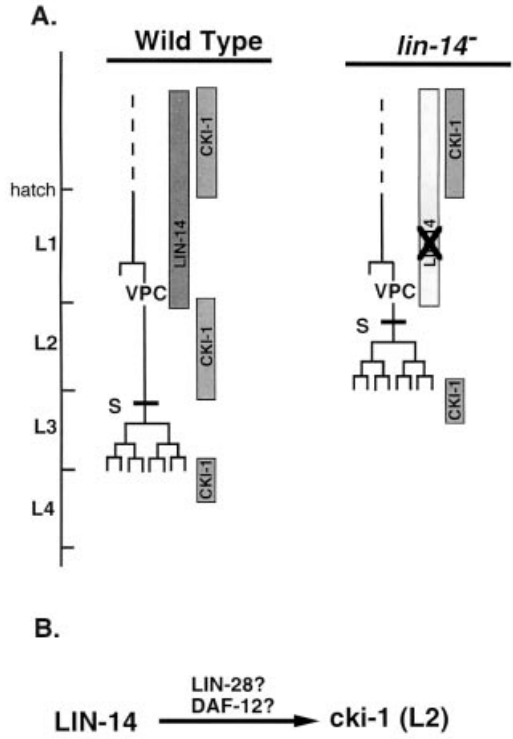

FIG. 2. Cell-specific developmental regulation of *cki-1* in the *C. elegans* vulva cell lineage. (A) Vulval precursor cells (VPCs) are born in the late L1 stage and normally they do not divide again until the L3, after an extended G1. In the L2, VPCs are unable to express differentiated vulval fates, even when induced to divide by removal of *cki-1* (Hong et al 1998). In late G1, VPCs acquire the capacity to select one of the two induced vulval fates (1° or 2°) or the uninduced fate (3°) depending on the nature and amount of Ras and/or LIN-12 signalling (Kenyon 1995), and cell cycle phase (Ambros 1999; see Fig. 3). LIN-14 expression (Ruvkun & Guisto 1989) begins during embryonic development, and is down-regulated after the L1 stage. *lin-14* loss-of-function mutations cause loss of *cki-1* expression specifically in the L2. Note that expression of *cki-1* in this lineage prior to the L2, or during the L3, is not affected by *lin-14*. (B) *lin-14* is proposed to specify a VPC resting state, which may in part involve expression of G1 arrest factors including *cki-1*. Epistasis (Antebi et al 1998) suggests that *lin-14* performs these functions indirectly, perhaps via *lin-28* and/or *daf-12*.

differentiating hypodermal cells, suggesting that *cki-1* is a *lin-29* target in the differentiating hypodermis (Hong et al 1998).

lin-14, which encodes a novel nuclear protein (Ruvkun & Guisto 1989), plays a central role in controlling the timing of events throughout development (Ambros 1997), including cell cycle progression in the hermaphrodite vulva cell lineages (Fig. 2A). The VPCs are born in the L1 stage and traverse a cell cycle with a long

G1 phase and then divide in the mid L3 (Sulston & Horvitz 1977, Euling & Ambros 1996). In *lin-14* mutants, VPCs divide early, in the mid L2, owing to a shortened G1 (Euling & Ambros 1996). Significantly, the VPCs are essentially the only cell types with a shortened cell cycle length in *lin-14* mutants, indicating that LIN-14 regulates the cell cycle machinery specifically in the VPCs. Presumably, this specificity reflects a situation where control of some critical cell cycle component requires an interaction between heterochronic gene products and VPC-specific factors. *lin-14*, *lin-28* (Euling & Ambros 1996) and *daf-12* (Antebi et al 1998) seem to all be involved in controlling the timing of G1/S in VPCs, and epistasis experiments suggest that *lin-14* could act indirectly, perhaps via *daf-12* (Antebi et al 1998), and possibly *lin-28* also (Fig. 2B).

One key effector of VPC cell cycle control is *cki-1* (Hong et al 1998). As a Cdk inhibitor, *cki-1* is a candidate negative regulator of G1/S in VPCs. Consistent with this model, *lin-14(lf)* mutations specifically abolish *cki-1::gfp* transcription in VPCs, and inactivation of *cki-1* causes precocious VPC cell divisions (Hong et al 1998). Thus *lin-14* likely controls the developmental timing of G1/S in VPCs by directly or indirectly activating *cki-1* transcription in these cells during the L2 (Fig. 2B). Cyclin D (*cyd-1*) and CDK-4 (*cdk-4*) expression in VPCs is approximately coincident with the time of down-regulation of *cki-1* transcription (Park & Krause 1999). It is not known whether these reciprocal events associated with VPC G1/S occur in response to the same regulatory pathway. Both the down-regulation of *cki-1* and the activation of *cyd-1* expression are blocked by abnormally late expression of *lin-14* (V. Ambros, unpublished results), suggesting that the developmental timing of these G1/S events is specified by the dynamics of *lin-14* (Fig. 2B).

As discussed above, *cki-1* loss-of-function animals do not execute vulval cell fate commitment or differentiation precociously, despite executing VPC divisions early (Hong et al 1998). This result suggests that *lin-14* controls two separable cell cycle events in VPCs—acting through *cki-1*, it prevents G1/S during the L2; independently of *cki-1* it affects a further delay of VPC cell cycle and differentiation until the mid L3. It is not known to what extent these distinct effects are mediated by distinct *lin-14* partners or downstream regulators, such as *lin-28* and *daf-12*. *daf-12* encodes a nuclear receptor homologue (Antebi et al 2000), and *lin-28* encodes a cytoplasmic RNA binding protein (Moss et al 1997), suggesting potential roles for post-transcriptional and/or hormonal mechanisms in the temporal control of VPC cell cycle and differentiation.

Cell cycle-dependent cell fate choices

In many developing systems, decisions about cell fate are influenced by cell cycle status (McConnell & Kaznowski 1991, Weigmann & Lehner 1995, Lehner & Lane

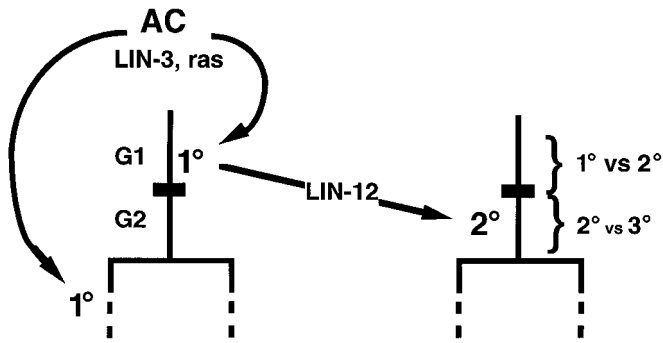

FIG. 3. Two cell cycle phase-dependent choices of cell fate for VPCs. Two VPCs are shown, one near to the anchor cell (AC) the source of inductive signal, and one further away. The VPC closest to the AC receives a high LIN-3 inductive signal (transduced by the Ras signal transduction pathway) and initiates expression of the 1° fate prior to S phase. The presumptive 1° cell signals laterally to its neighbour via LIN-12 to repress the 1° fate. Commitment to the 2° fate by the neighbour occurs only after S phase (Ambros 1999). (VPCs more distant from the 1° cell [not shown] receive no LIN-12 signal, and adopt the 3° fate). A repeated sensitivity to LIN-3 signalling at subsequent cell cycle(s) permits a reinforcement of the 1° fate in the AC-proximal VPC lineage (Wang & Sternberg 1999).

1997). During *C. elegans* larval development, there are also certain situations where the competence of a cell to choose its fate is influenced by cell cycle phase. This phenomenon may represent strategies for ordering or prioritizing cell fate choices using a convenient timing mechanism — the cell cycle.

One example of a linkage between cell cycle phase and cell fate determination in *C. elegans* can be seen in the selection of VPC fates (Fig. 3). The six equally potent VPCs are exposed to multiple extracellular signals that determine their individual fates and generate an organized pattern of so-called 1°, 2° and 3° vulval fates (for review, see Kenyon 1995). A high level of LIN-3 (EGF-like) signal, acting via the LET-23 receptor tyrosine kinase and Ras pathway, specifies the 1° fate in one VPC. The 1° VPC signals laterally to its neighbours via the LIN-12/Notch receptor to specify that they adopt the 2° fate (Sternberg 1988). The 1° fate takes precedence over the 2° fate (Simske & Kim 1995), in part owing to a cell cycle-based sequencing of the 1° and 2° cell fate choices; the 1° fate is chosen in late G1, while commitment to the 2° fate choice is restricted to G2 (Ambros 1999; Fig. 3). Another factor reinforcing the prioritization of the 1° fate is a cell cycle-associated reinforcement of LIN-3 signalling during successive cell cycles. Specifically, LIN-3 signals can act repeatedly — apparently during corresponding phases of the cell cycle in the 1° VPC and again its daughters — to over-ride the effects of LIN-12 signals (Wang & Sternberg 1999). It remains to be determined what molecular components of the cell cycle machinery are responsible for

modulating the sensitivity of vulval cells to developmental signals, depending on cell cycle phase.

Conclusions

C. elegans offers a genetically and developmentally accessible system for exploring how the positive cell cycle components (such as the cyclins and their Cdk partners), and the negative components (for example, cullins and CKIs) can be linked to developmental pathways in specific cells. The pathway regulating dauer larva formation, and the heterochronic gene pathway, represent examples of pathways affecting global and tissue specific cell cycle control, respectively. One challenge will be to understand the sometimes reciprocal interplay between developmental pathways and the cell cycle machinery. We wish to know how developmental regulators interact with cell cycle machinery to control developmental patterns of cell division. We also want to understand how the cell cycle machinery can influence cell fate choices made by progenitor cells or differentiating cells.

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DISCUSSION

Leevers: If the ligand from the primary cell is what produces the secondary cell, why do you have to have that second layer of control in terms of which stage of the cell cycle the secondary cell comes from?

Ambros: Presumably you are inferring that there would already be a sequence built into the second one. One way to think about this would be in terms of commitment: the primary cell could be signalling before it is committing. So if you allow the secondary cell to commit in G1, it may be possible for it to become secondary before the other cell became primary.

Leevers: It is two ways of doing the same thing. Maybe it just has to be very robust.

Bryant: When is the LIN-12 receptor expressed?

Ambros: It begins in the early part of the L2 stage, more than a full stage prior to the time that LIN-12 acts.

Bryant: So that is not the limiting factor.

Ambros: No. However, the timing of expression of all the components of the signal transduction machinery has not yet been examined in these cells.

Nurse: I was wondering in more general terms about how this compares with Martin Raff's views about timing and differentiation. Here we have a real coupling to cell cycle progression.

Ambros: Here, the temporal regulator is the heterochronic gene pathway, which controls the time of G1/S in VPCs. I do not think that the heterochronic genes act by counting cell cycles. In particular, the down-regulation of *lin-14* — the regulatory event that really times the G1/S in VPCs — is probably not coupled to cell cycle. For example, our unpublished results, postembryonic cell cycles were inhibited by hyrdoxyurea treatment beginning in the L1, yet subsequent changes in the levels of heterochronic gene products occurred normally. However, after the heterochronic genes have acted and VPCs enter the cell cycle, then vulval cell fate commitment does seem to be coupled to cell cycle.

Raff: I seem to remember a story about a cyclin E mutant in this pathway. Could you tell us about this?

Ambros: This is work by David Fay and Min Han at the University of Colorado, Boulder. The homozygous cyclin E mutant larvae from heterozygous mothers seem to be OK until the next to last division of vulval development. They execute

only two rounds of vulval cell divisions instead of three, yet those final vulval cells exhibit the differentiated phenotype of what in the wild-type would have been expressed by their daughters. Since the vulval cell cycles are also significantly slowed in the mutant, Fay and Han propose that vulval differentiation is unlinked from the final cell cycle, and that vulval cells are programmed to differentiate after a set period of time, rather than after a specific number of cell cycles.

Nasmyth: The AC cell sends a signal. Can you read out this signal, and is it possible for you to do this *in situ* and ask when during this cell cycle, relative to other cell cycle events, this occurs? Being able to say when things happen usually gives even more information than looking at mutants. It would be useful if you had a direct read out of that and could look at when the receptor is firing, and you could relate all this to progression through G1.

Ambros: No one has looked at that directly. Ideally, one would like to monitor the phosphorylation state of different components of the signalling pathway and the status of key transcription factors, and so on. In principle this is feasible, and we would predict that we might find a cell cycle gating of one of those steps.

Nurse: How long is that G1 period?

Ambros: About 27 h.

Nurse: So there is a lot of time.

Nasmyth: Then in the *cki* mutant, when G1 is shorter, what would you predict would happen once these cells had initiated the cell cycle?

Ambros: In the *cki* mutant, where the cells divide in the middle of the second larval stage, they seem to divide without losing their tri-potent capacity. In the *cki* mutant we just double the number of cells.

Raff: What keeps cell division synchronous?

Ambros: That is a good question: vulval precursor cell divisions are exceedingly synchronous, although there are very slight, highly reproducible differences in the precise timing of mitosis among the various VPCs. There is probably an as yet unidentified mitogenic signal at work here. One possible timing signal for VPC division could be related to the moulting cycle. The heterochronic genes don't affect the pace of the moulting cycle, so these could represent parallel timers. It is striking that the rate of moulting and the rate of cell divisions throughout the animal are all coordinated. One way to coordinate them would be to have cross-talk between them. The heterochronic gene pathway and other developmental signals for cell cycle control may function in coordination with the moulting cycle.

Bryant: Is that moulting controlled by an ecdysone-like hormone?

Ambros: Nothing is known about the possible role of hormones in *C. elegans* moulting.

Bryant: Is there an endocrine organ that might be the source of a hormone that governs this?

Ambros: A likely candidate for such an organ has not been identified, to my knowledge.

Raff: Is the timing of *lin-14* decay understood?

Ambros: Reasonably well. The basic mechanism is translational regulation of *Lin-14* mRNA. *lin-4* is a small antisense RNA which accumulates towards the end of the first larval stage and binds to the 3′-UTR of *lin-14* mRNA, inhibiting LIN-14 translational elongation.

Raff: What is known about *cki-1*?

Ambros: Less is known about how *cki-1* is shut off in VPCs than about how *cki-1* is turned on in these cells. However, when we misexpress *lin-14* late in development, it will maintain *cki-1* expression later than in the normal vulval lineage. Therefore, extinction of *cki-1* expression in VPCs is influenced by some activity that is in turn dependent on *lin-14* down-regulation. All our *cki-1* expression constructs retain the 3′-UTR of *cki-1* (Hong et al 1998), and so the dynamic *cki-1* expression we observe could reflect post-transcriptional regulation in addition to transcription regulation.

Thomas: If you take your *cki* mutants and add hydroxyurea, what happens? Do they spend longer in G1?

Ambros: I have never done that, but it is worth doing. For that matter, it would be nice to be able to arrest them in an earlier stage than G1, and see whether we can delay the acquisition of the response to Ras.

Schmidt: If you are looking for a compound that is relatively specific for G1, there is a chemotherapeutic agent being introduced into clinical trials called flavupiridol, which has preferential effects on G1 and leads to degradation of cyclin D1 in breast cancer cells.

Nasmyth: What is its target?

Schmidt: It is thought to be Cdk4-specific, but it comes out of screens for general tyrosine kinase inhibitors. It seems to be the most specific, and it is the furthest along, so you could probably obtain it more easily.

Nurse: I would like to generalize the discussion a little, by addressing the whole issue of cell cycle phase or progression, and the ability of a cell either to generate or respond to developmental signals. At first sight there seems no particularly good reason why these things should couple. A general question is, what are the differences between these different phases and types of cells that might make them respond differently to developmental signals or might make them more prone to generate signals. The obvious question is whether something is happening during S phase, or is there something going on in mitosis which means that the cell surface is altered, for example?

Edgar: I find that idea surprising on the basis of what we see in *Drosophila*. Have you analysed any other cell lineages where there is a requirement for progression through S phase to get a cell committed?

Ambros: No, I haven't looked at other cases.

What about the synchronization of the cell cycle in the morphogenetic furrow in the *Drosophila* eye? Is there any sense that this stage in the cell cycle is important with respect to signalling events in that context?

Edgar: The cycles can certainly be deregulated quite a bit and you still get differentiation of all the cell types.

Lehner: If you specify the fate of single cells, you have to synchronize specification with proliferation of that cell. If you don't do this, there are multiple cells specified rather than just one, and the pattern is irregular. A good example is the *Drosophila* eye disc. So far, however, there is no evidence that cells need to be in G1 as opposed to G2 in order to be specified in the right way.

Nasmyth: What sort of experiment would address that question?

Lehner: It would be somewhat complicated. I think it could be done in *Drosophila*, but it hasn't been yet.

Nasmyth: Let's say that there is a series of lineages where cells spend most of their time in G1, and they are clearly responding to signals and are undergoing some kind of programmed change. If you were to go in there and arrest them in G2, has anyone done that experiment and then measured that readout to see whether anything has altered? This is quite different from saying let us alter the number of divisions and ask whether we still get patterning, because that doesn't really address the question. That is just saying it is not related to numbers of divisions. It doesn't address whether the timing of the cell cycle is important for how cells respond.

Edgar: I don't think that this experiment has been done cleanly for the eye, but in the fly embryo there is a G2-arrested situation and differentiation is very good.

Lehner: In *string* mutants, not everything works. We have heard from Bill Chia that the first neuroblast divisions follow a very stereotyped program that is also realized after transplantation or *in vitro*. If you do not allow divisions, the program is not realized and late neuron types are never specified.

Edgar: But if you allow the divisions and force the cells to differentiate in G2 instead of G1, then what would you get? Perhaps these kinds of defects can be attributed to just not having enough cells to get you the right juxtaposition of cells to signal to each other to set up the fates.

Nasmyth: You could arrest the cells at a certain stage in G1 to see whether there is a difference to having arrested them in G2.

Schmidt: I don't know whether I can answer the 'why?' question, but the 'how?' question has a conceptual framework to build it around. What you are looking for is some kind of phosphorylation target of cyclin B that is a non-cell cycle functioning receptor. You are looking for some cell cycle receptor sitting in the membrane that cyclin B goes over to, puts the phosphate on, activates it and says 'now I am receptive'.

Nurse: Does the worm, with its precisely determined development differ from the fly? Would this make a difference?

Ambros: In the case of the LIN-12 phenomenon that I described, one of the experiments involved commitment in response to the activity of truncated LIN-12 protein lacking a membrane spanning domain. This form of LIN-12 likely acts in the nucleus. This constrains our thinking concerning the possible phosphorylation targets to nuclear components.

Schmidt: The screen that might give it to you is actually a biochemical screen for phosphorylation targets on cell cycle regulators.

Gautier: Can you do the experiment to check whether this difference in responsiveness between G1 and G2 is dependent on transcription? Another explanation would be that the passage into S phase changes the transcriptional competence of the cell.

Ambros: That sounds like it might be a complicated experiment, but a good idea. In the case of LIN-12 our readout is the final fate of the cell. Iva Greenwald and others have shown that in response to signalling, the LIN-12 receptor is up-regulated, and in non-receiving cells it is down-regulated (Greenwald 1998). Our experiments suggest that the changes in LIN-12 level depend on the completion of S phase (Ambros 1999). S phase might bring about a change in the dependency of LIN-12 expression from VPC-specific factors to signalling-dependent factors. This change in dependency could be part of the commitment event.

Nurse: Periodically S phase is invoked in this sort of way. What do we think about it generally though?

Raff: I remember Harris & Hartenstein (1991) did an experiment in which they used hydroxyurea to block cell proliferation in the *Xenopus* retina. They showed that all the different cell types still developed. This same type of result has been seen in other systems.

Ambros: It could be a peculiarity of *C. elegans*. The animal is relatively simple and develops rapidly, so it must employ strategies to optimize developmental fidelity given its small number of cells and short time to develop. Cell cycle dependent cell fate commitment could be one strategy to achieve precision with limited cells to work with and limited developmental time.

Nurse: I think that makes some sense.

Bryant: I have always been struck by these nuclear 'elevator' movements (Fujita 1962). In *Drosophila* epithelium, during M phase the nucleus goes all the way to the apical side of the cell layer, the cell rounds up, the nuclear envelope breaks down, and at this point the nuclear contents are in contact with the cell junctions where all the signalling is happening (Woods & Bryant 1993). There is the opportunity for important signalling events to occur right at that point. Then when the nucleus goes back down for S phase, signalling must be a lot more complicated because the nucleus is so far away from the cell junctions. I don't think it happens this

way in the worm. Perhaps this is something that only happens in columnar epithelia.

Nurse: But the general principle is important. There is increasing evidence that cytoskeletal elements are involved in growth factor signalling. You could easily imagine that when a cell undergoes certain cell cycle transitions, involving reassembly of the cytoskeleton, this may profoundly influence signalling. One example is the interaction of Rho and Rac with the actin cytoskeleton.

Maller: In *Xenopus*, at the mid-blastula transition (MBT) where cell fate is first determined, the first change is not G1/G2 but instead a longer S phase, and this is when transcription turns on. Is the S phase between the primary/secondary and secondary/tertiary divisions longer than the previous S phases?

Ambros: I don't know.

Schmidt: It sounds to me like your problem is that since all cells have to go through all of these cell cycle phases, the tendency is that if you allow cell fate to be regulated in G1, you will get unbalanced organ development. There has to be a place at which organ development would give you a selective advantage in response to particular signals going through the cell cycle. Since G1 is there to monitor nutrients in some sense, is there any reason that you could go looking for a G1-specific regulator of building a bigger intestine where there might be a selective advantage to being in a nutrient rich environment and giving a disproportionate advantage to one organ over another? Is this the kind of thing that you are looking for with this kind of mechanism? There has to be some reason to use the cell cycle as the monitor, otherwise you will get imbalance in the organism between the brain and intestine. Since balanced growth of the organism is what will eventually make it a success, there has to be a counterbalancing even more positive selective value to using the cell cycle to monitor some element of the environment to allow it to determine cell fate. The only kind of paradigm that occurs instantly is nutrients being filtered through G1, and G1 therefore wanting to monitor some cell fate that would give an advantage in finding nutrients. It could also be the olfaction system in *C. elegans* in hunting down more food.

Ambros: If I understand what you are saying, it seems that this might work well in terms of mitogenic signals, where the cells are competing for relative growth. In this case you could imagine a corollary or analogous scenario where the cells are competing for signals that are determining their fate. The idea there would be perhaps more to coordinate their access to these signals.

Schmidt: It is going to require more plasticity in the decision about cell fate than is seen in this system.

Thomas: A basic cell fate decision made by tissue culture is restriction late in G1. I don't know how this applies, but the cell decides whether to progress through he call cycle or return to G1 arrest. I would think that some time late in G1 would be the time the cell makes such a decision.

Ambros: If we knew what the mitogenic signal was we could ask whether the beginning of commitment to the secondary fate corresponds to this time.

Schmidt: The only place that I can imagine that would be happening is in a pathological state in a mammalian organism, where the organism is in an insulin replete state and it hypertrophies its liver in response, where there is more plasticity.

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Responses to DNA damage in *Xenopus*: cell death or cell cycle arrest

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Abstract. *Xenopus* embryos divide rapidly following fertilization. During this rapid period of cleavage, cell divisions are not sensitive to DNA replication or spindle assembly inhibition. Here, we have investigated the consequences of eliciting DNA damage in these embryos. We show that the rapid cell divisions are not affected by DNA damage. However, as the embryos reach the onset of gastrulation, they undergo rapid and synchronous apoptosis. We have investigated the regulation of this delayed apoptotic response to DNA damage. Next, we have reconstituted a DNA damage cell cycle checkpoint *in vitro*, demonstrating that all the checkpoint signalling components are present in the embryos but are not activated under the experimental conditions used to generate DNA damage in the embryo.

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DNA damage events that can produce transmissible modifications in the genome are under surveillance in order to prevent their propagation. These surveillance mechanisms work through a network of signal transduction pathways that can either prevent cell cycle progression or alternatively result in the induction of cell death (Elledge 1996, Hensey & Gautier 1995).

During the cell cycle genomic DNA is replicated during S phase and then distributed to the daughter cells as chromosomes during mitosis. Replication should faithfully duplicate the genetic material, take place only once per cell cycle and always occur between intervening mitoses to maintain the characteristic ploidy of the genome. Replication should use undamaged templates, so that the information transmitted to the daughter cells does not get modified throughout cell generations. In a similar manner, mitosis should also segregate identical pools of undamaged chromosomes. Therefore, the decisions to start DNA

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replication or to start mitosis, the G1/S and G2/M transitions, are under tight regulation and surveillance.

The surveillance mechanisms that prevent cell cycle progression following DNA damage operate throughout the cell cycle, as it has been shown that damage can be sensed in G1, S or G2 phases. It has been proposed that cell cycle delay allows for the coordinated repair of damaged DNA. Alternatively, cells that have undergone DNA damage can be eliminated by apoptotic mechanisms. It appears that cell cycle arrest could also be a first step towards the expression of a cell death program as some of the critical regulators of cell cycle arrest such as p53 are also essential for the occurrence of cell death.

We have started to investigate these two types of responses to DNA damage in *Xenopus* eggs and embryos, and cell-free extracts derived from them. Early work suggested that *Xenopus* embryos lacked some of the surveillance mechanisms or cell cycle checkpoints that are normally operating in somatic cells. For example, experimental interference with DNA replication using aphidicolin (an inhibitor of DNA polymerase), or with mitotic spindle formation using microtubule poisons, did not prevent cell cycle progression in *Xenopus* embryos as seen by the continuing oscillations of the mitotic kinase Cdc2/cyclin B in treated embryos (Kimelman et al 1987, Newport & Kirschner 1982). Since then, it has been accepted that *Xenopus* embryos lacked all types of cell cycle checkpoints. In this work, we have more carefully looked at the consequences of inducing DNA damage on the early cell cycles.

Results and discussion

Induction of DNA damage to the cleaving Xenopus egg does not block cytokinesis

We used two different experimental means for inducing DNA damage in *Xenopus* eggs during cleavage. Fertilized eggs were injected at the one or two cell embryo stage with restriction enzymes, or embryos were subjected to γ-irradiation at various stages following fertilization. In either case, we did not observe a delay in cell cycle progression when compared to control uninjected embryos (Hensey & Gautier 1997).

Cell cycle progression in treated eggs was monitored by live video microscopy following both the cleavage of the egg into blastomeres and the cortical contraction waves preceding cytokinesis. One example of such an experiment is presented in Fig. 1A as still shots of a time-lapse video recording of a control untreated egg alongside an egg irradiated with 40 Gy one hour following fertilization. No difference in the timing of cell divisions was observed up to the 12th cleavage (Fig. 1A), the time of the midblastula transition (MBT). At the MBT, cell divisions normally become asynchronous in *Xenopus* embryos (Newport &

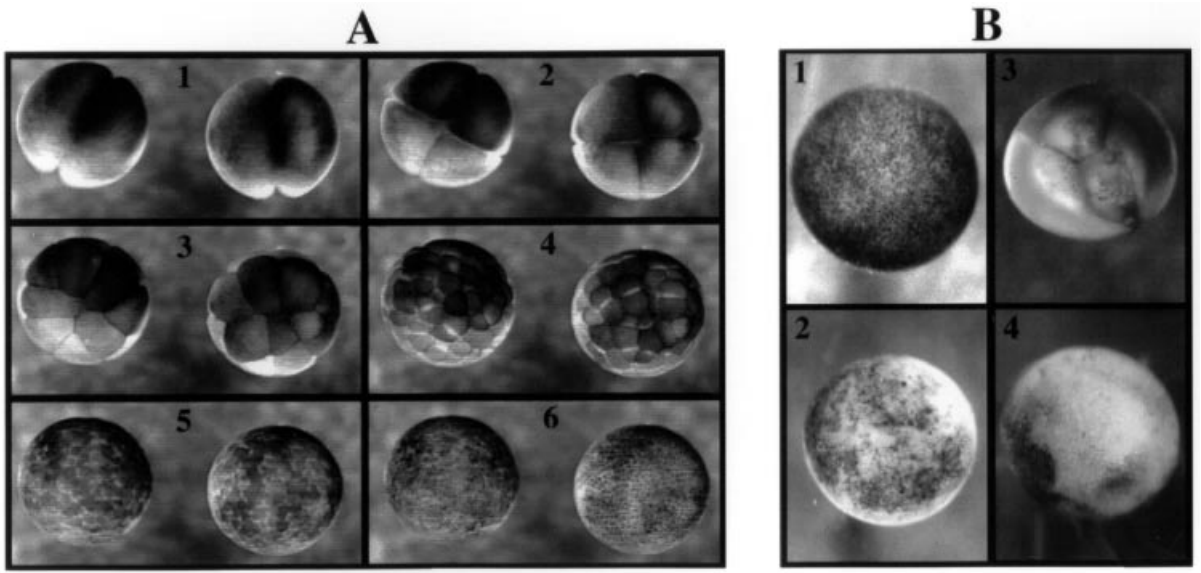

FIG. 1. Induction of synchronous cell death in embryos. Early cell divisions were monitored by time-lapse video recordings. (A) Panels 1–6 compare a control (right), and irradiated (left) embryo. Panel 1, St. 2; panel 2, St. 3; panel 3, St. 5; panel 4, St. 7; panel 5, St. 8; panel 6, St. 9. (B) Panel 1, control embryo, St. 10.5; panel 2, dying irradiated embryo, St. 10.5; panel 3, dying cycloheximide-treated embryo. This embryo was treated with cycloheximide at the 2-cell stage, at a concentration of 0.1 mg/ml which inhibited protein synthesis by 97%, cell division arrested at the 4-cell stage, and death occurred at a time equivalent to St. 10.5; panel 4, dying α-amanitin-treated embryo, St. 10.5. Death is defined as the rapid disintegration of the embryo characterized by a white mottled appearance.

Kirschner 1982). We observed this developmentally regulated cell cycle transition in untreated embryos. Surprisingly, irradiated embryos underwent another two synchronous cleavages as seen by both the progression of cytokinesis and by the contraction waves in time-lapse video microscopy. In addition, we followed cell cycle progression biochemically in irradiated and control eggs by monitoring the oscillation of the mitotic kinase Cdc2/cyclin B. We found no difference in the timing or the extent of the oscillation of Cdc2 protein kinase during the two cell cycles following irradiation as compared to unirradiated controls. Upon examination of the total genomic DNA content of irradiated embryos, we found that embryos that were irradiated at the one cell stage contained 80% of the DNA content of untreated embryos by the time they reached the 12th division. This established that although DNA replication was slightly impaired, the bulk of it took place in irradiated embryos. We propose that this 20% reduction in genomic DNA might be due to the early loss of broken chromosomes following the generation of double-strand breaks (DSBs) in the DNA that were not repaired before the first mitosis. A 20% decrease in genomic DNA at the time of the MBT would result in a 20% decrease in the nuclear–cytoplasmic ratio which probably accounts for the two extra synchronous divisions, i.e. for the delayed MBT observed in these embryos. Indeed, it has been proposed that the timing of the MBT is directly regulated by the nuclear:cytoplasmic ratio. Doses of γ-irradiation ranging from 20 to 200 Gy resulted in a similar phenotype. Similarly, restriction enzyme-injected embryos divided at the same speed as control uninjected embryos.

These experiments clearly demonstrated that despite the occurrence of extensive DNA damage, particularly DSBs, the embryonic cells kept progressing throughout the cell cycle unaffected, demonstrating that the checkpoint pathway that normally prevents cell progression following DNA damage was not operational in these cells.

DNA damage to the early embryos promotes a synchronous but delayed apoptotic response

When embryos treated as described above were left to develop, they reached the onset of gastrulation with no visible changes in morphology compared to untreated embryos. However, as they reached stage 10.5, the embryos underwent rapid and synchronous apoptosis. This rapid cell death had striking morphological characteristics: individual cells from the embryo lost their cohesion and adhesiveness and rapidly lysed so that few or no intact cells were found in each embryo 20 minutes following the onset of apoptosis (Fig. 1B).

This delayed apoptotic response is developmentally regulated and exhibits some striking features. Induced apoptosis could never be elicited prior to the MBT,

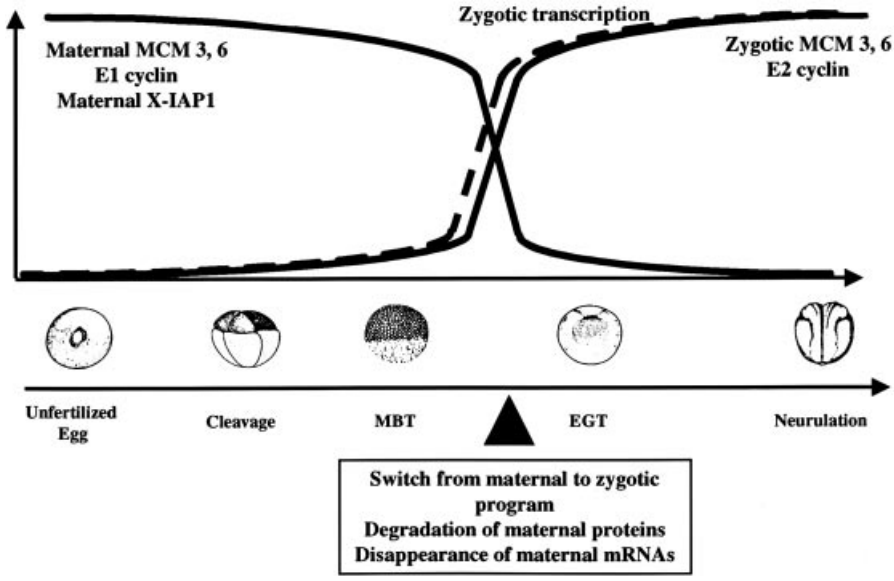

FIG. 2. Schematic representation of the switch from a maternal to a zygotic programme taking place in *Xenopus* between the MBT and the onset of gastrulation. This switch has been shown to operate for cell cycle regulators such as cyclin E and MCM proteins. We propose that maternal regulator(s) of apoptosis such as X-IAP 1 are also expressed in this kind of pattern and could explain the apoptotic program we have uncovered.

regardless of the nature of the apoptotic inducer and of the dose, provided it was above a threshold. However, the components of the apoptotic cascade are present as early as the unfertilized eggs since extracts from these eggs can undergo genuine apoptosis *in vitro* (Evans & Kornbluth 1998). The apoptotic stimulus could only elicit the response when applied prior to the MBT (Hensey & Gautier 1997, Sible et al 1997). Finally, the apoptotic response did not require *de novo* transcription or protein synthesis. A similar programme has been identified by others in *Xenopus* and zebrafish (Hensey & Gautier 1997, Ikegami et al 1999, Sible et al 1997, Stack & Newport 1997).

These observations lead us to propose the hypothesis shown in Fig. 2, in which the cleaving eggs contains maternal stockpile of stable protein(s) that inhibit apoptosis. Following the MBT and prior to the onset of gastrulation, these proteins become highly unstable leading to apoptotic death of the embryo unless a zygotic form of the inhibitor is synthesized. When this synthesis is prevented experimentally by inducing DNA damage, blocking transcription or blocking protein synthesis, apoptosis takes place.

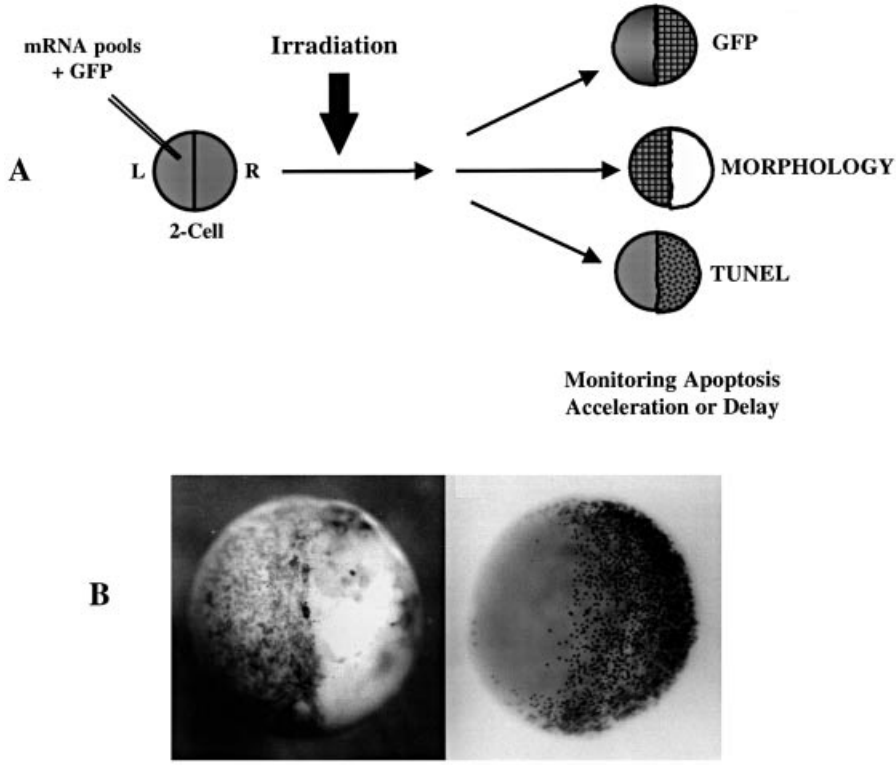

FIG. 3. (A) Screening scheme for maternal genes that can modulate apoptosis when expressed in cleaving embryos. One cell of a two-cell stage embryo is injected with GFP and the expression library (see text). The injected embryos are irradiated and score for delay or acceleration of apoptosis in the injected half. (B) The example of *BCL2* injection in this screen is shown. Left panel shows embryonic morphology, right panel shows TUNEL stained embryos. Both panels show a St. 10.5 embryo, cells in the left half of the embryo, i.e. those arising from the blastomere injected with *BCL2* RNA, look normal while the right half of the embryo is dying.

Search for maternal factors that can modulate the apoptotic response

In order to identify maternally inherited factors that regulate this apoptotic response, we used a screening strategy depicted in Fig. 3. We expressed pools of mRNAs, transcribed from a stage 4 maternal library (Lagna et al 1999). Each pool contained 100 clones. Each pool was tested for its ability to delay or accelerate apoptosis when injected in once cell of a two cell stage embryo. The recipient embryo is induced to undergo apoptosis at stage 10–10.5 by γ-irradiation at the two cell stage. The embryos were screened for morphology as well as processed for wholemount TUNEL staining. The injected side was marked by co-injecting

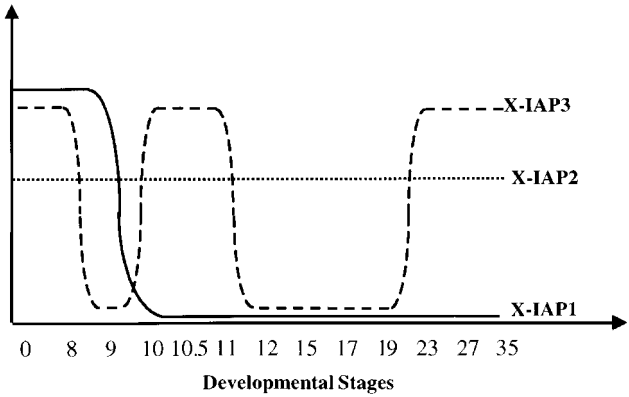

FIG. 4. Temporal expression pattern of three *Xenopus* IAP family members. We have cloned three IAPs designated X-IAP1, 2 and 3. This graph shows their developmental profile as assessed by RT-PCR.

mRNAs encoding for green fluorescent protein (GFP) (Fig. 3A). In such a screen, an activity will be uncovered when the injected half undergoes apoptosis either late or early, as compared to the uninjected side. A visual example of the assay is given in Fig. 3B using *BCL2* mRNA to delay apoptosis in the injected half. When a pool of mRNAs displayed activity, the cDNA responsible for the effect observed was isolated by sib-selection (Smith & Harland 1992). To date, we have not isolated a clone that accelerated apoptosis, supporting the hypothesis that maternal factors are preventing cell death in the early embryos. However, we have isolated two clones that delay apoptosis to a similar extent as human *BCL2* mRNA does. Interestingly, one clone delays apoptosis induced by either irradiation or transcription inhibition while the other delays apoptosis induced by irradiation only. The expression and characterization of the role of these molecules in regulating apoptosis is under current investigation.

In a second approach, we cloned *Xenopus* homologues of the inhibitor of apoptosis gene family (IAPs), a conserved family of potent inhibitors that can block cell death at different steps of the apoptotic cascade (Deveraux & Reed 1999). In *Drosophila*, a maternal IAP has been recently implicated in regulating cell death early during development (Wang et al 1999). So far, we have isolated three different *Xenopus* genes encoding for IAP family members. All three are maternally inherited, and two of them are also transcribed later during development as shown on Fig. 4. Interestingly, the clone designated X-IAP1 is exclusively maternal and is rapidly degraded between the MBT and the onset of gastrulation, fulfilling the criteria for being a bona fide maternal regulator of the apoptotic programme.

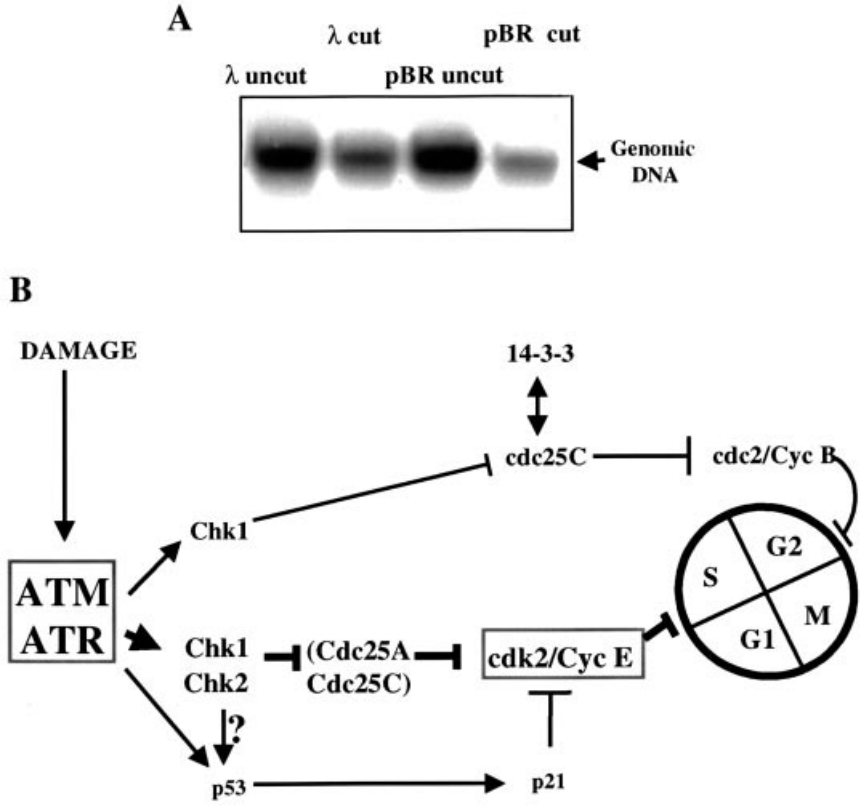

FIG. 5. DNA damage cell cycle checkpoint in *Xenopus* extracts. (A) Genomic DNA replication was measured using intact chromosomal templates in different extracts. Extracts were incubated with either plasmid or X, DNA, intact or digested, as indicated. In the presence of digested DNA that contained double strand ends, genomic replication was inhibited. (B) A model for the ATM-dependent inhibition of DNA replication initiation following DNA damage. Following the detection of double strand breaks in G1, S or G2 phases, ATM protein is activated. Prior to S-phase, activation of ATM promotes a cascade of events leading ultimately to the inhibition of Cdk2/cyclin E kinase activity. By analogy with the ATM-dependent signalling taking place in G2, we propose that ATM could activate a Chk protein (possibly Chk2/Cdc1/Rad53), which in turn would inhibit the activity of Cdc25. This would lead to the accumulation of inactive Cdk2/cyclin E complexes in which Cdk2 is phosphorylated on tyrosine 15.

Reconstitution of a DNA damage checkpoint in extracts derived from Xenopus eggs

DNA damage in the context of the embryo does not inhibit cell division as seen by the synchronous and timely occurrence of cytokinesis. In a similar fashion as a cell cycle checkpoint was uncovered in extracts following inhibition of DNA

replication (Dasso & Newport 1990), we investigated whether it was possible to elicit a cell cycle response following DNA damage *in vitro*. We decided to use initiation of DNA replication as a readout for monitoring DNA damage checkpoint activation. It is not really understood how DNA damage is recognized when a cell cycle checkpoint is activated and what type of DNA structures can elicit such responses. It has been proposed that DNA breaks themselves could be recognized (single or double strand breaks), or a single strand region of DNA. In either case, molecules with affinity for breaks or single strand DNA could be involved and these molecules could also be essential for DNA replication. To circumvent this problem we established an *in vitro* system in which we monitored the activation of a DNA damage checkpoint *in vitro*. DNA molecules containing double-strand breaks (DSBs) were introduced into a *Xenopus* interphase extract and signalling was allowed to take place for 15 min. The extract was then fractionated (Chong et al 1997) and the fractions were reassembled into a cell-free system in which DNA replication initiation of undamaged chromosomal DNA was monitored. In this system, the DNA used to elicit the signalling is eliminated during the fractionation procedure.

Using this system we showed that DSBs-containing DNA can elicit a checkpoint as seen by the inhibition of DNA replication of intact genomic templates (Fig. 5A). Undamaged DNA (circular plasmid or undigested lambda DNA) did not elicit the checkpoint. Using specific drugs that inhibit ATM (the product of the gene mutated in ataxia-telangiectasia) and the ATM family of protein kinases (Jeggo et al 1998) as well as antibodies specific for ATM (Robertson et al 1999) we showed that the checkpoint is ATM dependent (Fig. 5B). Finally, we showed that the checkpoint leads to the inactivation of Cdk2/cyclin E protein kinase which is essential for DNA replication (Fig. 5B).

In summary, we showed that DNA damage can elicit a variety of responses in *Xenopus* early embryos. While γ-irradiation induces apoptosis in the embryos, DSB-containing DNA prevents initiation of DNA replication in cell-free extracts derived from eggs.

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DISCUSSION

Maller: We find a Bcl2 band in the pre-MBT embryo that doesn't change in abundance after the MBT. If we put in Bax, which promotes apoptosis, we can pull out that Bcl2. In addition to the maternal IAPs that you got, there is maternal Bcl2 preventing apoptosis before the MBT.

Gautier: That is why we have this biased approach with IAPs, because there are data in *Drosophila* showing that there is a maternally inherited inhibitor of apoptosis. We are also screening for a maternally inherited molecule that can affect the onset of apoptosis. These could be regulating apoptosis directly. Also, as John Newport mentioned, I tend to believe that it might be some sort of more general mechanism linking all these maternal zygotic switches through degradation of mRNA.

Maller: I was surprised that you could remove the damaged DNA and retain the signal for all these manipulations. You would have thought that removing the DNA would be analogous to having repaired all the damage, and everything should come back to equilibrium.

Gautier: It looks like the modification of the signalling is dominant and stable.

Maller: This almost implies that the repaired DNA would have to send a signal back saying that now everything is OK.

Gautier: It is a very stable signal.

Nurse: In relation to that, after having sent the signal with broken DNA, if you now put the good DNA back, would you reverse it?

Gautier: There is genomic DNA in the assay and such reversal doesn't take place. It is clear that a circular plasmid doesn't do that, even at a tenfold higher level. So checkpoint signalling is dependent on DNA ends.

Nurse: Does this mean that it is the repair process itself that is removing the signal?

Maller: Yes, it suggests this. More than that, it says that the repaired product must send a signal back. You have removed the damaged signal by centrifugation.

Gautier: That is clear. We cannot see any DNA in any of the B or M fractions. There is a stable modification that is induced by DNA breaks in the B fraction.

Nasmyth: Is that in addition to undamaged B? Is it dominant?

Gautier: It is dominant. If we mix B, M and B* there is a checkpoint. This is another way of showing that this is real signalling.

Harper: Can you bypass the checkpoint by using a Cdk2AF mutant? The simplest hypothesis is that you are phosphorylating Cdc25A, it is not active and you can accumulate phosphorylated Cdk2, which is just blocking activity.

Gautier: I don't know.

Harper: Steve Jackson's lab has reported that ATM binds to DNA ends as visualized by SEM. This would be consistent with you bringing down a certain amount of ATM.

Edgar: I am still puzzled by the relationship between what you found *in vivo* and what you did in the extracts. How can you reconcile having a checkpoint in the extracts but not in the animal?

Gautier: There are two possible explanations. The trivial one is that it is a problem of dose, in that in the embryo the right number of breaks is not reached, because it is a fairly large amount of cytoplasm and the signalling is too weak. The other option is that there is no replication checkpoint. Blocking replication in the embryo will therefore not affect the G2/M transition. The embryo will still divide even though it doesn't replicate.

Edgar: That should be obvious if you just stain those irradiated MBT embryos with DAPI.

Nasmyth: They go on dividing not just for the next division but for multiple divisions. This is inconceivable.

Gautier: There is replication. We have looked at the amount of DNA, and there is replication. I suspect it is more likely to be a problem of dose. We need to reach a threshold of DNA ends in order for activation to occur.

Nasmyth: What dose of radiation do you use?

Gautier: In the embryo we get a uniform cell death at about 20 Gy.

Maller: We have gone as high as 50 and don't see any difference.

Gautier: We have been to 200 Gy and still the cells die at the same time.

Raff: Is it known what the zygotic inhibitor might be that takes over from the maternal one? Is there a Bcl2 family member that could explain the zygotic switch?

Gautier: There is a Bcl-like zygotic member that Tata originally cloned.

Raff: Jim Maller, I know you found some Bcl2 family members after MBT. Could they be the answer?

Maller: Tata reported that Bcl2 mRNA only came up after the MBT. Our default model is that the cell will apoptose unless it produces a zygotic inhibitor. We thought this was Bcl2, but we have now made an antibody against it and there is lots of Bcl2 in the pre-MBT embryo. It is functional in the sense that you can get effects on apoptosis by chelating it with things like Bax. This means that there will be multiple inhibitors in the embryo. We haven't been able to clone a maternal form of Bcl2, but this does not mean that it isn't there.

Raff: Which Bcl2 family members turn on transcriptionally at MBT?

Maller: There is no change in the blottable abundance of Bcl2.

Raff: Is most of that maternal?

Maller: The maternal and zygotic gene products are immunologically indistinguishable. We see the message increase with zygotic transcription but no change in the protein. This also occurs for Cdc2, for example. You degrade maternal Cdc2 mRNA at the MBT without changing Cdc2 protein levels on blots.

Raff: So we don't know what the putative zygotic inhibitor is?

Gautier: No. There could be more than one. The only thing we need as a hypothesis is that there is a switch from the maternal to the zygotic whatever it is, and then at some point, if you just block the zygotic transcription, the default pathway is cell death.

Edgar: Has anyone done an experiment that results in apoptosis earlier than the MBT?

Gautier: That is what we would like to do. I mentioned that when you increase the dose of protein synthesis inhibitor or irradiation up to 200 Gy, the onset is not moved. One of the experiments to do is to attempt to deplete the maternal store of IAPs, to see whether the cells enter apoptosis more rapidly.

Maller: You can do the same kind of experiment with Bax and functionally remove all maternal Bcl2 in the embryo. Apoptosis is still not initiated until after the MBT.

Gautier: This doesn't mean necessarily that there is no inhibitor present.

Maller: Yes, there could be another inhibitor, but Bcl2 is pretty powerful as far as controlling apoptosis goes. You might expect the removal of Bcl2 to have changed the timing of apoptosis if that was possible.

Nurse: If you inject DNA at the early stage, do they then undergo apoptosis?

Gautier: We have never done the experiment, but if you modulate the content of genomic DNA, you change the onset of the MBT. We don't know what happens as far as apoptosis is concerned. If the timing mechanism that controls apoptosis is similar to the one that controls the destruction of cyclin A, we would predict that this wouldn't change because the onset of cyclin A degradation doesn't seem to be dependent on when the MBT is taking place.

Lehner: What I understood so far about timers in the early frog embryos is that most people think that they involve an mRNA-based mechanism. Maternal mRNAs have a limited stability and disappear suddenly.

Gautier: The mRNA disappears, but one idea would be that there is a protein that inhibits the degradation of this maternal RNA. This is why it is degraded at this time.

Lehner: This suggestion is not sufficient to explain your results with cycloheximide. If cycloheximide is added, the inhibitor should already have been made. In this case the timing can only work if a protein degradation system is switched on on time in the absence of protein synthesis.

Gautier: I agree.

Raff: That is the real mystery. Specific RNA and protein degradations are turned on at precise times, even though there is no RNA or protein synthesis.

Nasmyth: The whole cell cycle can be done without protein synthesis.

Richardson: I wanted to clarify that the apoptosis machinery is present in the early embryo.

Gautier: Yes. One would argue that everything required for apoptosis is present in the egg. If you dissociate the egg and make cell-free extract from the one cell stage embryo, you can in certain conditions have a system to follow apoptosis.

Nurse: Why are checkpoints present in the early frog embryo?

Maller: In some cases I think it is a matter of thresholds. If you add enough DNA to an egg extract, you have a signal sent and heard. If it is a DNA-based signal, you need a certain amount of DNA to send the signal. The amount of DNA required is different for each checkpoint. Only 4N of irradiated DNA (4 nuclei/μl) is sufficient for inducing apoptosis at the MBT. Andrew Murray showed that the spindle checkpoint requires a much higher amount of DNA (10 000 nuclei/μl) in the system to respond to nocodazole than does the replication checkpoint with aphidicolin (1000 nuclei/μl).

Nurse: So you would say there is no biological significance to it; it is simply that because you have so much cytoplasm to DNA, the thing just can't work.

Maller: You need a nuclear:cytoplasmic ratio that can send an adequate signal to be heard. I would speculate that the signal is sent, even when there is only one nucleus, but it may not be heard by the cell cycle or apoptotic machinery that is present in great abundance.

Nasmyth: There is a similar situation in *Drosophila*, where you also have a high cytoplasm:nucleus ratio. The only problem in *Xenopus* is that the whole cell cycle clock is occurring throughout the entire cytoplasm, whereas *Drosophila* has a certain degree of nuclear autonomy. Even there, these controls are missing to some extent.

Lehner: The spindle checkpoint works well and the DNA replication checkpoint is compromised but present in early *Drosophila* embryos. In addition, Sullivan has proposed that there is a back-up checkpoint mechanism during the syncytial stages (Fogarty et al 1997). If nuclei continue to go through the cell cycle in the presence of unreplicated DNA, they get disposed. The nuclei lose the connection with the centrosome, they fall into the interior of the egg and fail to become cellularized.

Maller: An analogous event occurs in *Xenopus*. Low-level irradiation results in a few damaged cells being removed into the blastocoel, and development proceeds normally without apoptosis.

Nurse: Is there any evidence that checkpoint signals are changing during development in other ways in other systems?

Kubiak: In mouse, there is a weak checkpoint in meiosis for spindle formation. There are some responses where if the spindle is destroyed, the passage from metaphase I to metaphase II is blocked. There is other evidence that there is no checkpoint. I think there is a special checkpoint in meiosis. Starting from the first mitotic division there is no spindle checkpoint.

Nurse: What about replication DNA damage?

Harper: Embryonic stem (ES) cells used to be thought of as not having checkpoints, but recent work has shown that there are γ-irradiation inducible checkpoints in ES cells.

Nurse: Are there changes in checkpoints in other circumstances in development?

Edgar: Imaginal disc cells arrest in G2 when you irradiate them, not G1.

Nurse: I assume that the cells are mainly in G1 when you irradiate them.

Edgar: A lot of them are.

Nasmyth: In budding yeast in meiosis there is G2 arrest, which never occurs in mitotic cells.

Reference

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Control of cell fate in plant meristems

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Abstract. In contrast to animals, plants do not stop to initiate organs with the end of embryogenesis. Instead, most of the growth and development of higher plants will take place during later phases following germination of the seed. Plant development depends on the activity of two meristems, the root meristem and the shoot apical meristem (SAM), that are located at opposite ends of the plant embryo. These meristems serve as a source of pluripotent stem cells and, in case of the SAM, provide a centre for repetitive organ initiation. In order to maintain the SAM throughout plant life, the cells that are lost from the meristem through organ initiation and differentiation have to be replaced from the stem cell population. In this paper, we will discuss recent results indicating that the fate of stem cells in plant meristems is controlled by directional signalling systems.

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Structure of the shoot apical meristem

The angiosperm shoot apical meristem (SAM) is initiated during embryogenesis and subsequently produces the basic elements of the plant shoot structure, leaves and stems. In *Arabidopsis* and many other dicotyledonous plants, the SAM consists of a small dome of cells that are organised into regions with different functions and fates (Steeves & Sussex 1989, Vaughan 1952). At a first level, cells are organized into the outer tunica layers and the inner corpus layer (Fig. 1). In many species, the tunica consists of only two clonally distinct cell layers called L1 and L2. Cell divisions within these layers are exclusively anticlinal, so that all new cell walls are formed perpendicular to the surface of the meristem. The progeny of a cell in the L1 will therefore remain in this layer and eventually differentiate as an epidermal cell. Cells in the underlying L2 will form a sub-epidermal cell layer and the gametes. Below the tunica, cell divisions are not oriented, and this corpus or L3 will give rise to the pith and the vascular system. This early allocation of cells in the

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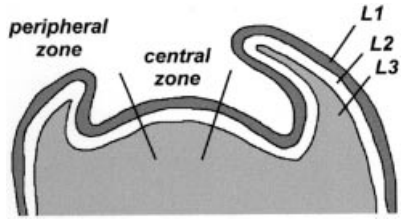

FIG. 1. Shoot apical meristem structure. The shoot apical meristem consists of three clonally distinct cell layers (L1, L2, L3). L1 and L2 represent tunica layers, L3 represents the corpus. Cell divisions in L1 and L2 are exclusively anticlinal, cell divisions in the L3 occur in all planes. The central zone harbours the stem cells and is surrounded by the peripheral zone where organ primordia are initiated.

meristem into separate clonal cell layers may suggest cell-lineage dependent mechanisms of development. However, studies using genetic mosaics have shown that the position of a cell, and not its clonal origin, determines its fate (Poethig 1989, Irish 1991). All three layers contribute to organ formation and growth of the stem, indicating that cell proliferation and cell fate specification during development are coordinated. One of the mechanisms that enables the necessary intercellular communication appears to involve transfer of informational molecules (proteins and RNA) through intercellular organelles, the plasmodesmata, that establish a cytoplasmic continuity between neighbouring cells (Lucas 1995).

At a second level of organization, the shoot meristem becomes divided into a central zone at the summit of the meristem, and a surrounding flanking region, or peripheral zone during embryogenesis (Steeves & Sussex 1989). These zones that consist of cells from all layers differ in their cell division rates: cell divisions are infrequent in the central zone, but frequent in the periphery. The central zone harbours a reservoir of pluripotent stem cells that will serve as a source for new cells. All appendages like leaves, but also new meristems that will give rise to flowers, are formed exclusively from cells in the peripheral zone, and are inhibited from developing from the central zone.

Maintenance of the SAM

During organ formation, cells are continuously lost from the SAM, and these cells have to be replaced through cell divisions in the stem cell zone in order to maintain a functional SAM. The rate of cell divisions and the size of the stem cell population has to be coordinated with the rate of organ formation, which may vary during different developmental phases. How is this coordination achieved, and how can cells in the SAM communicate their position and their fate to each other? An

elegant study used periclinal chimeras of tomato differing in the genetic makeup of meristem cell layers to address this question (Szymkowiak & Sussex 1992). In one experiment, such chimeras were generated by grafting between wild-type tomato plants and the tomato mutant *fasciated*, which has larger floral meristems and produces more carpels. When the outer two meristem layers were composed of wild-type cells, and only the L3 layer carried the *fasciated* mutation, the whole floral meristem increased in size and developed extra carpels, indicating that the cells in the L1 and L2 perceived and responded to a size increasing signal from the L3. The molecular basis of this signalling process has not been identified in tomato, but mutants similar to *fasciated* have been analysed in other species, like *Arabidopsis thaliana*.

The *CLV* signalling pathway

The recessive *clavata*-mutants (*clv1*, *clv2* and *clv3*) of *Arabidopsis* accumulate too many cells in the central zone of the SAM and floral meristems, resulting in fasciation of the shoot and the initiation of supernumerous floral organs (Clark et al 1993, 1995, Kayes & Clark 1998). Most obviously, additional carpels are formed that fuse to form a club-shaped silique (hence the name *clavata*, derived from the latin word *clava*, meaning club). The shoot and floral meristems of *clv*-mutants enlarge and tend to fasciate while the initiating organs grow out normally, indicating that development of the peripheral zone is not affected. A study using confocal microscopy to compare cell division patterns in wild-type and *clv*-mutant meristems revealed that this increase in meristem size is not due to an increased rate of cell divisions in the central zone (Laufs et al 1998). One interpretation is that cells in the central zone are not recruited in time to the peripheral zone, leading to a continuous build-up of cells in the centre of the meristem and a displacement of the surrounding peripheral zone. The additional organs produced by *clv*-mutant meristems could thus be explained by the size increase of the peripheral zone where organs are initiated.

Most mutations in *clv1* or *clv3* are fully recessive, and double mutants are mutually epistatic. This genetic evidence indicates that these two genes may act together in the same pathway to control cell proliferation in the central zone (Clark et al 1995). The shoot and floral meristems of the third mutant in this group, *clv2*, resemble those of the other *clv*-mutants, and both *clv1* and *clv3* mutants are epistatic to *clv2* (Kayes & Clark 1998). However, some aspects of the *clv2*-mutant phenotype are not affected in the other *clv* mutants, so that *CLV2* seems to function not only together with *CLV1* and *CLV3*, but also independently in the development of several floral organ types. Both the *CLV1* and *CLV2* genes encode leucine-rich repeat (LRR) transmembrane proteins (Fig. 2) (Clark et al 1997, Jeong et al 1999). The CLV2 protein carries only a

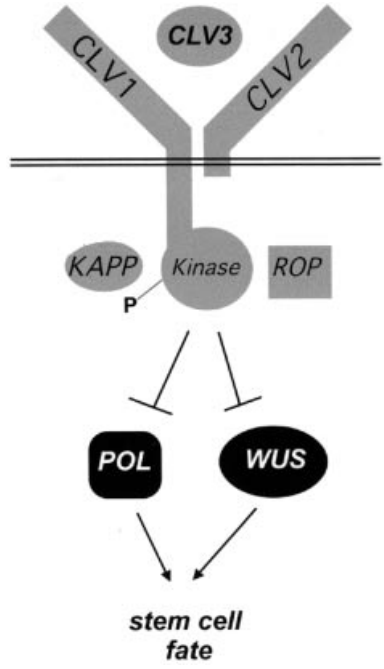

FIG. 2. The CLAVATA signal transduction cascade. The *CLV1* and *CLV2* genes encode leucine-rich repeat (LRR) transmembrane proteins that probably form a heterodimeric receptor complex that is localized in the plasma membrane (double line). The CLV1 protein carries an intracellular C-terminal serine/threonine kinase domain that can autophosphorylate (P). The *CLV3* gene encodes a small, probably secreted protein that may interact with the extracellular domains of CLV1 and CLV2. The active form of CLV1 is present in a 450 kDa protein complex that also includes a kinase-associated protein phosphatase (*KAPP*) and a member of a plant Rho GTPase-related protein family (ROP). The *KAPP* protein has been shown to interact with the CLV1 kinase domain and is able to down-regulate CLV signalling, possibly by dephosphorylating activated CLV1. *POLTERGEIST (POL)* and the homeodomain transcription factor *WUSCHEL (WUS)* appear to act downstream of the CLV signalling pathway and to promote stem cell fate in the meristem. These two genes are likely targets for repression through the CLV signal transduction cascade.

short C-terminal, and probably cytoplasmic tail region, whereas CLV1 carries a C-terminal serine/threonine kinase domain. More than 50 related LRR-transmembrane receptor kinases, and about the same number of LRR-transmembrane receptor proteins that lack a kinase domain have been identified so far in the *Arabidopsis* genome. In a number of cases, e.g. the response of plants to pathogens, such LRR-carrying receptor proteins have been implicated in signal transduction cascades (Staskawicz et al 1995). It is likely that the CLV1 and CLV2 proteins form a heterodimeric receptor molecule that is localised in the plasma

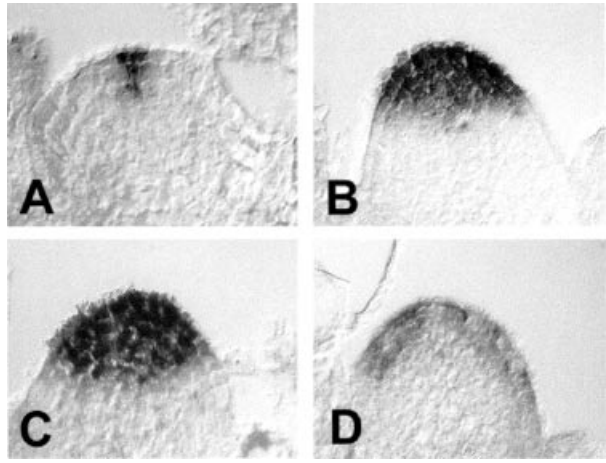

FIG. 3. *CLV3* expression. *In situ* hybridization with digoxigenin-labelled *CLV3* antisense probe of longitudinal sections through *Arabidopsis* shoot apical meristems of wild-type (A), *clv1-4* (B), *clv2-1* (C) or *clv3-2* (D). In the wild-type, only a few cells at the tip of the meristem (the putative stem cells) express *CLV3* RNA. In all *clv* mutants, stem cells accumulate in the meristems, and the *CLV3* expression domain is enlarged.

membrane. CLV3 is the best candidate for a ligand that binds and activates the CLV1/CLV2 receptor so far (Fletcher et al 1999, Brand et al 2000). *CLV3* encodes a relatively small protein with no conspicuous amino acid motifs. However, it carries an N-terminal signal peptide, making it likely that the CLV3 protein can be secreted and interact with the extracellular domains of the CLV1 and CLV2 receptors. There is no direct evidence yet that CLV3 acts as a secreted ligand. Periclinal chimeras derived from the unstable, transposon induced *clv3-7* loss-of-function mutant were used to investigate the cell-autonomy of CLV3 function (Fletcher et al 1999). Secondary shoots that showed a wild-type phenotype were isolated on *clv3-7* mutant plants, and seeds were collected from the revertant sectors after self-pollination. A wild-type (revertant) *clv3* allele would segregate among the progeny of these somatic revertants only when the reversion occurred in the L2 layer of the meristem, which gives rise to the gametes. All reversions that occurred in the L1 or L3 layer should not be transmitted to the next generation. We found that many revertant sectors segregated only the mutant allele, indicating that *CLV3* function was restored somatically in the L1 or L3 cell layer, but not in the L2. Restoration of *CLV3* activity in a single cell layer is therefore sufficient to control the proliferation and differentiation of the meristem, and CLV3 can therefore function non-cell-autonomously.

CLV3 mRNA is found primarily in the L1 and L2 layer of the central zone of shoot and floral meristems (Fig. 3), while the RNA of *CLV1* is found mostly in an

underlying domain in the L3 (Clark et al 1997, Fletcher et al 1999). The expression pattern of *CLV2* has not yet been analysed in great detail, but RNA can be detected in all shoot tissues of the plant (Jeong et al 1999). So the stage is set for CLV3 as a signal that is secreted from cells in the outer layers of the meristems and binds to a heterodimeric receptor, consisting of CLV1 and CLV2, in deeper layers. A combination of biochemistry and genetics provided further evidence that CLV1 function depends on *CLV3* (Trotochaud et al 1999). In the wild-type, the CLV1 protein is found primarily in a 450 kDa protein complex, which is assumed to be the active complex (Fig. 2). Formation and stability of this complex depends on the presence of functional CLV3 and CLV2 protein. Two more proteins were identified as members of this complex: One of them is KAPP, a kinase associated protein type 2C phosphatase, which has been shown to down-regulate *CLV* signalling, possibly by inhibiting the auto- or transphosphorylating activity of CLV1 (Williams et al 1997). The second protein, ROP, is a small GTPase related to the Rho protein family. Other members of this family control cytoskeleton reorganization during pollen tube growth (Li et al 1998), thus raising the possibility that the cytoskeleton itself is a target for *CLV* signalling.

Since the central zone of the meristem expands in *clv* mutants, *CLV* signalling would act to restrict expansion of the central zone and promote the timely exit of cells into the surrounding peripheral zone. The studies on the tomato *fasciated* mutant have shown that expansion of the stem cell zone is promoted from L3. In *Arabidopsis*, the *WUSCHEL* (*WUS*) gene encoding a homeodomain transcription factor promotes stem cell fate, since *wus* mutants fail to initiate or maintain an active stem cell population in meristems, resulting in a premature differentiation of cells in the central zone (Laux et al 1996). *WUS* RNA is found only in the L3 layer of shoot and floral meristems, indicating that this gene may act non-cell autonomously (Fig. 4, Mayer et al 1998). However, *wus* mutants can still produce axillary inflorescences and occasionally abnormal flowers that lack most organs in the inner whorls, so *WUS* is not required for meristem initiation *per se*. Mutations in *WUS* or the *CLV* genes have essentially opposite phenotypes, but *wus* mutants are epistatic to *clv* mutants. Therefore, *WUS* could be a target gene for repression by the CLV proteins. Alternatively, *WUS* could be required to establish the cells upon which the CLV proteins can act. In *clv* mutants, *WUS* RNA is no longer confined to a few cells in deeper layers of the meristem (Fig. 4, Schoof et al 2000, Brand et al 2000), indicating that one consequence of *CLV* signalling is to down-regulate *WUS*, or to limit expansion of the *WUS* expression domain. The gradual enlargement of the shoot meristem in *clv* mutants could then be due to the deregulation of *WUS*. The increased *WUS* activity would specify more stem cells, resulting in an expanded central zone and eventually fasciation of the shoot meristem. Support for this model of *CLV*|*WUS* interaction came from two types of ectopic expression experiments:

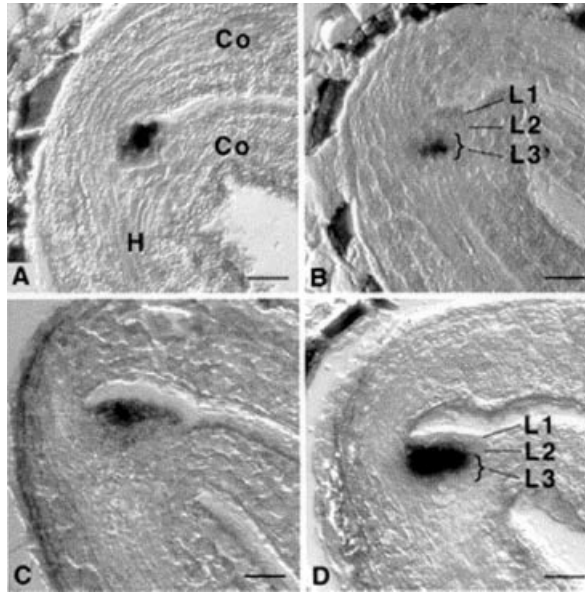

FIG. 4. *CLV3* and *WUS* expression in wild-type and *clv1* mutant embryos. (A and C). *In situ* hybridization with *CLV3* digoxigenin-labelled antisense probe (A) Longitudinal section through a wild-type embryo, bent-cotyledon stage. (C) In a *clv1* mutant embryo, the *CLV3* expression domain expands, indicating that stem cells accumulate. (B and D) *In situ* hybridization with *WUS* digoxigenin-labelled antisense probe.(B) Wild-type embryo. *WUS* is expressed in deeper cell layers underlying the stem cells. (D) *clv1* mutant embryo. *WUS* is now expressed in a larger domain in the L2 and L3 layers. Bars, 20 m. Abbreviations: L1, L2, L3, meristem layers; H, hypocotyl; Co, cotyledon.

Transgenic *Arabidopsis* plants that express *CLV3* constitutively resemble wild-type at early stages of development, but soon after emergence of the first leaves, the meristem arrests and ceases to produce further organs (Brand et al 2000). In some transgenic plants, the meristem resumes activity and will even produce an inflorescence with flowers that lack the inner organs. The opposite phenotype is observed in *clv3* loss-of-function mutants, where stem cells accumulate in the centre of shoot and floral meristems, and additional organs, whorls of organs and/or undifferentiated tissue are formed. Thus, *CLV3* loss-of-function causes excessive accumulation of stem cells, while *CLV3* gain-of-function causes premature differentiation and loss of stem cells, possibly by causing a down-regulation of *WUS*.

Transgenic plants expressing *WUS* in a broader domain of the meristem resembled *clv*-mutants with large and fasciated meristems (Schoof et al 2000). When *WUS* was expressed from the *ANT* promoter in organ primordia, these primordia were converted into shoot meristems that expressed *CLV3*,

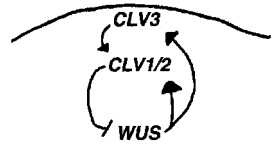

FIG. 5. Model for the feedback regulation of stem cell fate. The surface of the shoot apical meristem is indicated by the black line, gene names are roughly positioned where the genes are expressed. CLV3 acts from the tip of the meristem and activates the CLV1/CLV2 receptor complex. Activity of the CLV pathway represses transcription and/or function of WUS, and possibly other genes. WUS promotes non-cell autonomously stem cell fate in the overlying cells, and also promotes expression of the *CLV* genes in their respective domains. Together, these gene functions establish a feedback loop with negative and positive interactions for the control of stem cell fate in meristems.

suggesting that *WUS* is indeed sufficient to specify stem cell identity. However, *WUS* function is not required for *CLV3* expression (Brand et al 2000). It is likely that there additional factors that function together with *WUS* in a stem cell-promoting pathway. Mutations in the *POLTERGEIST (POL)* gene have been identified as partial suppressors of mutations in the *CLV* genes (Yu et al 2000). When the *CLV* genes are functional, *pol* mutants are nearly indistinguishable from wild-type plants. *POL* appears to act downstream of the CLV signalling pathway and redundantly with *WUS*, indicating that this gene could be a target for repression by the CLV signal transduction pathway.

A possible model consistent with these observations is that *WUS*, together with other genes like *POL*, acts as an organizer and promotes stem cell fate in the overlying cells (Fig. 5). These stem cells express the *CLV* genes, which in turn act to restrict the expression or function of *WUS*, so that these two antagonistic activities can constantly readjust the size of the central zone. During organ formation, cells in the peripheral zone need to be replaced, which are ultimately derived from the central zone. One attractive hypothesis is that the central zone is influenced by signals emanating from organ primordia, which could act via the CLV pathway to control cell division rates or fate in the central zone.

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DISCUSSION

Nasmyth: I'm confused by what you mean by a stem cell. What is your operational definition?

Simon: That is a difficult question. The cells in the meristem zone don't have any special qualities. Their properties — what will become of the cells — are justified by their position. If you find a cell at the top of the meristem, this is a stem cell. It is a stem cell because if you do clonal analysis, marking this cell and all its progeny, you find that it gives rise to a huge sector of the plant.

Nasmyth: So you are saying that operationally it is just a cell that is capable of getting into many lineages.

Simon: Plants don't really have lineages. It is a cell that gives rise to a large part of the plant and it stays in the meristem and divides for a long time. We can't be much more specific than that.

Nurse: Just to amplify that, you have files of cells going down from the meristem. How much lateral division is there away from the stem cell to generate more files?

Simon: Of course there is lateral division. There is also circumferential growth of the plant later on. I don't know how much. When I said that there were three or four stem cells in each individual layer, this was done by classical clone analysis, marking cells during development and then looking for the maximum sized sector that can be found. The maximum sector comprised one-third to one-quarter of the entire circumference of the plant, indicating that there are three or four stem cells.

Goodwin: Since the mutants you described are affecting stem cell fate, what is it that restricts the effects to the carpels? Why don't we see effects on other whorls?

Simon: It is just because in the species that I have discussed the carpels are the last organs to develop in the centre of the flower. There are a few exceptions in other plants where the carpels are not so central. We can introduce homeotic mutants that replace carpels with sepals or petals, and the same effects are seen.

Goodwin: So the carpels are just a marker for the centre of the floral meristem.

Simon: That is correct.

Bryant: CLV3 is produced by some cells and affects others. Is there some special mechanism for getting a protein like this through the cell wall to its target?

Simon: We don't know how it goes through the cell wall. We know that the first 17 amino acids of the protein are required for it to be secreted. In addition, plant cells can exchange molecules via plasmodesmata, which seem to have a size exclusion limit of about 1 kDa. Others have studied the movement of proteins and viruses and have shown that a plant virus, which is bigger than 1 kDa, can move from one cell to another. It has movement proteins which can enlarge the plasmodesmata. There was a study by Mezzit & Lucas (1996) showing that plant cells in different layers communicate and exchange not only small molecules, but also RNAs and transcription factors.

Bryant: These are presumably proteins that don't have signal sequences.

Simon: That is right. On the other hand, when you inject tracer dyes you can find that there are certain areas within the meristem which have symplastic connections. If you inject the dye into one cell, it spreads into a set of adjacent cells. This spread has a sharp outer boundary that corresponds with the outer boundary of the central zone. This delimination of symplastic connectivity is regulated and seems to depend on the developmental stage of the plant. Long or short day treatments can change the connectivity.

Bryant: You want the CLV3 protein to interact with the surface of the target cells, not to get into their cytoplasm.

Simon: Yes, it just needs to be secreted to contact the next cell. We don't know any details about whether secretion is regulated.

Goodwin: Presumably there is another relationship between the cell division cycle and differentiation: the delimitation of successive whorls of organs in the flower. Is there anything known about how this is regulated?

Simon: Samach et al (1999) have studied a gene called *UFO* that controls cell division patterns at organ boundaries. The *UFO* gene encodes an F-box protein. In yeast two-hybrid assays they found partners such as a SKP1-like protein from *Arabidopsis*. The idea is that there is probably an SCF complex incorporating the UFO protein, and that this restricts cell division in the regions between individual organs. However, there is an additional complication in that this gene serves a double function. It restricts cell division and may also be involved in the degradation of a repressor of a set of transcription factors.

Goodwin: Does *Superman* have any role?

Simon: Superman's role is to establish a boundary between stamen and carpel whorls (Sakai et al 1995).

Chia: What constraints allow this system to make the correct number of stem cells?

Simon: I think it is self-regulatory.

Chia: What turns on WUS?

Simon: If you knock out *CLV3* or *CLV1*, there is an increase in WUS activity, which in turn leads to an increase in *CLV3* expression. It is self-regulatory. In a wild-type background, any increase in CLV3 activity will in turn lead to the repression of WUS. You could imagine, therefore that the size of the stem cell population is constantly fluctuating.

Reik: It is sort of the wrong way round. If stem cells proliferate and CLV3 is a short range factor, they move away from this signal and lose it. This should then activate WUS. If you say that this is some kind of feedback system to regulate the size of the stem cell pool, how does it work?

Simon: There is a factor in deeper cell layers that promotes stem cell fate in overlying cells: WUS. And there are several factors expressed in stem cells, the *CLV* genes. They act together to repress WUS. This is a simple feedback system.

Nasmyth: In the *wus* mutant, is there an increased number of *CLV3*-expressing cells?

Simon: No, there is a defect already in embryogenesis. At the early stage you don't see these cells at all which would normally express *CLV3*. We can't identify any stem cell at an early stage of development.

Nasmyth: Is it possible to address whether *CLV3*-expressing cells are indeed stem cells?

Simon: That is what we are planning to do. We need to show that all stem cells express *CLV3*, and that all *CLV3*-expressing cells are stem cells. We are designing an experiment to test this.

Richardson: You say that the stem cells are dividing much more slowly than the peripheral cells. Why is this?

Simon: I don't think it is known. Some years ago researchers rounded up the usual suspects from yeast, complemented yeast mutants and checked for the expression patterns of factors such as the cyclins and Cdcs in plant meristems. They saw some spotty expression patterns in the meristems, but there were no clear domains of down-regulation of cell divisions, for example.

Nurse: I have a general question about the contribution of cell division to overall plant form, given that cells have invariant positions in the plant. One could imagine that the overall form of the plant is likely to be influenced by cell divisions more obviously in an animal. The plane of division and its control is very important for organism form. Would this be a reasonable view?

Simon: Yes, because there is no cell migration. If you want to construct a lobed leaf, for example, you have to define areas where cell divisions are still going on while in the adjacent areas they have stopped. We don't know how this is done. Apoptosis is a factor that is only occurring in a very few cases in plant development. It is controlled division that is important.

Bryant: The size of the whole plant must be a result of how long the meristem continues to produce cells. Are there mutants that alter plant size by changing the lifetime of the meristem?

Simon: You can make an *Arabidopsis* plant grow much longer and much bigger by just cutting off the siliques. There is a fruit ripening signal which induces senescence of the whole plant, and that is one reason why it stops developing after a while. The easiest way of growing monster *Arabidopsis* plants is by using a mutant that doesn't produce any flowers.

Edgar: Jim Murray in Cambridge has done some interesting work with D cyclins in plants. By overexpressing one of the D cyclins they got tobacco plants that grew quite a bit faster but ended up the same size. The rate of proliferation was altered, but the final size was not.

Nasmyth: In the *clv* mutant, the overall rate of division was lower. This suggests that it is having effects on cells other than the stem cells. Or is this a secondary effect of the effect on stem cells?

Simon: In the *clv* mutant the cell division rates in the central region of the meristem are affected. But we think it is only the stem cells that are affected.

Bryant: I am a little concerned about going straight from mitotic index to proliferation rate. Is that a valid assumption to make for these cells? Really, the mitotic index only tells you what fraction of the cells are in mitosis, and it could be the same for a fast-growing cell population as a slow one.

Simon: That is a valid argument.

Nasmyth: The mitotic index of the first cleavage mouse is very high — they are just stuck there.

Raff: Also, if there are more stem cells, and these are dividing more slowly, this alone could explain the decrease in the mitotic index in the mutant.

Nasmyth: Peter Bryant's point is that they could just be going through mitosis more quickly.

Raff: They could, but an increase in slowly dividing stem cells is another possible explanation.

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Final discussion

Nurse: In this final session, I wonder if it would be useful for us to identify what we think are the most important problems that ought to be addressed in the area of the cell cycle and development.

Edgar: Two questions that we have talked about a lot are what causes cells to exit the cycle and stop proliferating, and how is cell growth coupled to the cell cycle control apparatus? Does a coupling mechanism exist and does it vary in different developmental situations?

Raff: One of the questions we haven't really addressed is what is actually being controlled in the control of organ or organism size? Is it total cell mass, or total cell number, or both?

Bryant: It would be nice to understand the relationship between central controls on growth and peripheral controls. What is the role of endocrine control of growth and how does it interact with the local controls that are operating? How are extrinsic factors such as insulin-like growth factor (IGF) and growth hormone (GH) operating on the intrinsic growth control mechanisms within organs?

Nasmyth: When cells exit from the cell cycle, how often is there a close coupling between changes in growth and exit from the cell cycle? You can imagine situations where both of them shut down and the cell becomes a non-growing differentiated cell. We heard about heart cells, which exit from the cycle but growth goes on and cells get bigger. What we need is a natural history of those two events, and to know how often they are uncoupled. And when they are uncoupled, how often do you find situations where the cell cycle rolls on when the growth has been stopped?

Schmidt: A really useful experiment would be to show where things really don't work the same at all, so that we don't keep trying to extrapolate lessons from one organ or system to another. For example, if we showed that liver is profoundly coupling cell division to cell growth in a totally different manner than a neuron, then you could focus your efforts on answering the question in a more developmentally specific manner.

Nurse: That is an interesting point and leads on to the question of how general these mechanisms will be.

Schmidt: The danger of this kind of symposium is that we have all had an implicit, hidden assumption that we are all talking about the same thing, and each person throws a totally different system up which looks vaguely similar, yet

there are enough nuances in each that it becomes dangerous to make those assumptions.

Edgar: I think we will find lots of universals when we look at the functions of individual molecules, but there won't be many universal answers when we start comparing different cell types and different organisms.

Goodwin: It is clear that the same genes can function in quite different ways. Having the same genes operating during development doesn't mean that you are going through the same type of morphogenetic process.

Nurse: If we are going to look for universals at all, do we look for them through common molecules or common systems?

Goodwin: I would say systems.

Raff: Another big question is how cell-cycle withdrawal and terminal differentiation are coupled.

Nurse: One thing we haven't talked much about is how cell cycle genes are developmentally patterned, as worked out by promoter bashing. This is something that could be informative: has it been?

Edgar: Very much so. There are few genes that are clearly either cell-cycle regulated or cell-growth regulated when this sort of dissection has been done.

Nasmyth: The question is, will their regulation be any different from that of things that aren't cell cycle regulators? At the end of the day the String promoter is the promoter of a cell cycle gene, but would you learn anything new about transcription?

Edgar: Not about transcriptional mechanisms in general.

Nasmyth: The String promoter action probably won't be anything very different from the Hairy promoter, for example.

Nurse: Gene chips will probably answer this for us.

Maller: But Bruce Edgar told us that if he puts in a simple *string* gene with no upstream regulation, it still works.

Edgar: A lot of the regulation is not going to be transcriptional at all.

Lehner: I think it would be really interesting to look at some of the cell cycle regulatory genes in the mouse and repeat the analysis that Bruce Edgar has done with *string* in flies. If you only do this in tissue culture, you will never see different regulatory elements specific for different developmental stages or different cell types. You can only find these by analysis in real organisms. Most people think that the genes driving the cell cycle in either a liver cell or a prospective neuron use the same control elements and are regulated by the same pathways. Accordingly, a lot of generalizations from one cell type to another are being made. I am not sure that this is justified. In order to find out, we need more analysis in real organisms.

Nurse: It is clear that there is a lot of context dependency.

Nasmyth: This boils down, for example, to how complex the Cdc25 promoter will be in the mouse.

Edgar: The competing model will be that fancy promoters are not used to control cell cycle genes, but instead there is some kind of growth coupling mechanism.

Leevers: Are there complex cell division patterns during the development of mice? If you look at a specific organ, are there patterns?

Raff: There must be in order to get structures that are a particular shape, such as various bones.

McMahon: Quite a lot is known about the basic control of growth and differentiation, for example in the skeleton. Basically, the growth modulator seems to be a Hedgehog signal called Indian hedgehog. Through a feedback system which uses a second signal, the position at which cells can differentiate is set and a growing zone is maintained within the skeleton over a long period. Most of this work has been done during embryonic stages. It is also known that the plane of cell division is highly polarized. How this is controlled is not clear.

Raff: I recall that there is complex signalling going on between these cells as they get towards the end of their proliferation period.

McMahon: We can play around with the IGF signalling system and get a smaller skeleton that way. We can also get a small skeleton by manipulating the Hedgehog signalling system. What isn't known is how those two systems talk to one another. I think it is an excellent system to study. If you want to pick a mammalian system, you want to have it such that the arrangement is easy to determine. It is a very stereotypical arrangement of growth and cell movements.

Raff: This raises again the interesting issue of size. Why do bones grow so much bigger in a big animal than in a much smaller one?

McMahon: The subject hasn't arisen in our discussion, but we should mention homeobox (Hox) genes here. The current model of the profound role that these play is that they regulate sizes and structures. It is a powerful argument that the reason that our radius and ulna are a different size from our humerus is because of the Hox genes that somehow intrinsically control that system. In the absence of the Hox genes, what you get is the formation of the initial anlages but a failure of growth of those structures.

Raff: Is the connection between the Hox genes and the cell cycle still a mystery?

McMahon: Actually, I think it is even worse than that. The connection between the Hox genes and what is going on in those primordia in any sense is unknown. I can't understand why this is the case: the model, formulated by Denis Dubole, has been out there for some time. It is a persuasive model, but we don't even know whether the Hox genes are actually expressed within the cartilage cells and have cell autonomous effects, or whether they are outside the cartilage.

Nasmyth: Is this relevant to the fly?

Lehner: In *bithorax* mutants the haltere disc grows just like a wing disc: it is an obvious example where a Hox gene determines organ size. The mechanism is not understood.

Bryant: But I don't think that in *Drosophila* we have the equivalent condition that Andy McMahon is talking about, where in the absence of genes there is no growth. The situations you mention just involve change of the overall fate.

Reik: Is it useful to ask whether and how nutritional influences in the environment in general can have long-term effects on the cell cycle and development at much later stages? For example, smaller babies are more prone to have heart disease and diabetes.

Nurse: There is obviously a connection, it is just mysterious.

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