CONSTRUCTION AND EXAMINATION OF THE CHIMERIC GENES CONTAINING DELETIONS IN THE 5'-PROMOTER REGION OF THE L-PK GENE

By

WENJUN HUANG

Bachelor of Science

Shanghai Medical University

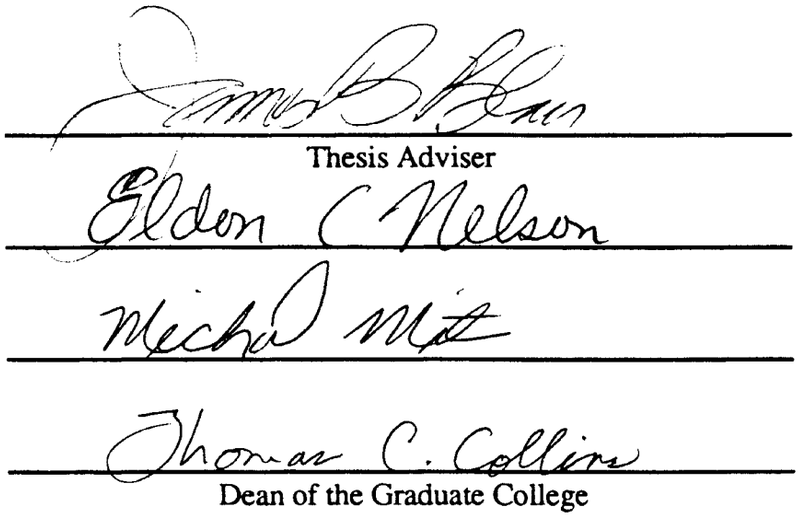
Shanghai, China

1986

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
MASTER OF SCIENCE
December, 1994

CONSTRUCTION AND EXAMINATION OF THE CHIMERIC GENES CONTAINING DELETIONS IN THE 5'-PROMOTER REGION OF THE L-PK GENE

Thesis Approved:



Thesis Adviser

Dean of the Graduate College

ACKNOWLEDGMENT

I wish to express my sincere appreciation to my major advisor, Dr. James B. Blair for his financial support, constructive guidance and inspiration throughout the course of this work. I would like thank my committe members: Dr. Eldon C. Nelson and Dr. Michael Mitas. Their suggestions and friendship were very helpful to my work.

I would like to express my gratitude to my colleagues Aron Fenton and Troy D. Long for their work with cell preparation and transfection.

Moreover, I would like to thank those who provided suggestions and assistance for this study: Dr. Franklin Leach's group, Dr. Sharon Ford, Dr. Ulrich Melcher, Dr. Steven Hartson, Ms. Ann Williams and Ms. Janet Rogers.

A special appreciation to my husband, Feng Qiu, and my parents for their understanding and help.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ATP	Adenosine 5'-triphosphate
ATPase	Adenosine 5'-triphosphatase
cAMP	Adenosine 3', 5'- cyclic monophosphate
DNA	Deoxyribonucleic acid
HEPES	N-[2-hydroxyethl] piperazine-N'-[2ethanesulfonic acid]
L-PK	Liver pyruvate kinase
kb	Kilo-base pair(s)
KRB	Krebs-Ringer Bicarbonate
mRNA	Messenger RNA
mol. wt.	Molecular weight
PCR	Polymerase chain reaction
PK	Pyruvate kinase
RNA	Ribonucleic acid
UV	Ultra violet

CHAPTER I

INTRODUCTION

More than 5 million Americans suffer from diabetic disease. Type I or insulin dependent diabetes can be controlled by continuous administration of insulin, but Type II or non-insulin dependent diabetes can not. Noticeably, vanadium, a widely distributed trace element, was found to normalize blood glucose levels in streptozotocin-treated rats (STZ-rats) which exhibit symptoms of both Type I and Type II diabetes (1). Drinking water containing ~ 0.7 mg/ml of vanadate or 0.2 mg/ml of vanadyl (vanadium in reduced form) decreased blood glucose levels of the streptozotocin-treated rats without affecting that of normal rats. Various laboratories documented that vanadate has insulin-like effects *in vivo* and *in vitro* as well as inhibiting (Na^+-K^+)-ATPase. However, the concentrations of vanadate required to inhibit glucose synthesis in isolated cells were much higher (mM vs. μM) than those observed in the intact rats. Thus, specific actions of vanadate to lower blood glucose levels in diabetic animals might be the consequence of long-term adaptation in gene expression. A chimeric gene construct containing the 1000 bp 5'-promoter region of the rat pyruvate kinase gene with firefly luciferase was made and transfected into primary culture of rat hepatocytes (2). The results of preliminary studies showed that both insulin and vanadate regulate the gene expression of L-type pyruvate kinase (L-PK). However, it is not known whether insulin and vanadate regulate the gene expression through the same region(s) in the L-PK promoter. The present research is to address the effects of glucose, insulin and vanadate on gene expression of L-type pyruvate kinase by constructing a series of chimeric genes with different deletions in the region of the L-type pyruvate kinase promoter.

CHAPTER II

LITERATURE REVIEW

Vanadium was discovered by the Swedish scientist N.G.Sefström in 1830 and was named after the Scandinavian goddess "Freya Vanadis". Gamyee and Larmuth were perhaps the first workers to study the biological properties of this element in frog heart in 1876 (3). Although vanadium is a trace element, it is widely distributed in nature and exists in almost all living things. The concentration of vanadium in sea water is about 5 x 10^{-8} M. The amount of dietary vanadium required for normal growth in rats is about 100 ppb or 2 nM and normal tissue concentrations are about 0.1-0.7 nmol/g wet weight. Most tissues of higher animals contain intracellular vanadium at a concentration of 0.1-1.0 μM except that within the blood cells of the tunicate *Ascidia nigra,* the concentration of vanadium is of the order 1 M. No other known organisms have so efficient a mechanism for vanadium accumulation (1, 4).

Vanadium has also been recognized as an essential nutrient in higher animals. Most available studies support the view that vanadium in the diet is essential for growth of various organisms. Even though vanadium compounds could be toxic to man and animals (actually, vanadium is relatively non-toxic to human, severely toxic to chicks), removal of the minute quantities normally present in the diet leads to deficiency disorders in experimental animals. Rats given a low vanadium diet exhibited reduced body growth and increased iron in blood and bone. Vanadium deficiency in chicks could lead to abnormalities in bone and increased plasma cholesterol levels, impaired reproductive performance and severe disorganization of the cells of the epiphysis with subsequent bone marrow formation. Cholesterol synthesis and feather growth in chicks are influenced by

vanadium. Lipid metabolism and red cell concentration in blood are also influenced by the ion in rats (1, 3, 5-7). These studies show that vanadium may play an role in developmental physiological states, but the biological basis for its role is still unknown.

Vanadium Chemistry

Vanadium (V), mol. wt. 50.9, is an element of the transition series. Its oxidation states of biological interest are +3, +4, and +5. The valence states change readily with environmental conditions. V^{+3} and V^{+4} is stable only in acidic solution (pH < 2) and in the absence of oxygen. They will be rapidly oxidized by dissolved oxygen at physiological pH. V^{+3} and V^{+4} interconvert readily under physiological conditions. Below pH 3, V^{+4} exists as the blue vanadyl cation VO^{2+} which has an unpaired electron and is paramagnetic and hence can be detected by electron spin resonance spectroscopy (ESR). V^{+5} is a powerful oxidant in acid solution or even at pH 7. V^{+4} is the major form present in mammals, but tunicates have also been found to contain vanadium V^{+3}. Vanadium can form polynuclear species with phosphate. The close similarity of vanadate to phosphate gives rise to much of its biological interest (6, 8).

Vanadate — Inhibitor of ATPase

In the last two decades, intensively research on the biological actions of vanadium was due to the fact that vanadate inhibited (Na^+-K^+)-ATPase. This property was first found from "Sigma Grade" ATP. In 1977 Cantley and co-workers (9) successfully purified from "Sigma Grade" ATP a potent inhibitor of (Na^+-K^+)-ATPase which was found to be identical with sodium orthovanadate (Na_3VO_4, vanadate is vanadium in +5 oxidation state, V^{+5}) and then vanadate was confirmed to be present in commercially available ATP prepared from equine and rabbit muscle but not in ATP isolated from yeast (10). 40 nM Na_3VO_4 caused 50% inhibition of (Na^+-K^+)-ATPase . The inhibition could

be completely reversed by norepinephrine and was unaffected by EDTA or dithiothreitol at millimolar concentrations (9).

After vanadium inhibition was found as an impurity of ATP, studies on both the physiological and biochemical effects of vanadate were carried out and have revealed a wide range of its actions. Particular interest of vanadium is its apparently selective inhibition on some ATPases, which has led to its use as a selective probe. It inhibits several enzymes, which hydrolyze phosphate ester bonds, such as alkaline phosphatase (11), phosphofructokinase (12) and adenylate kinase (13). Enzymes known to be either inhibited or activated by vanadium are listed in Table 2-1 (6), their mechanisms of actions, where known, frequently include covalent phosphoenzyme intermediates.

In order to understand the ability of ATPase inhibition of vanadium, it is helpful to know the (Na^+-K^+)-ATPase system. The (Na^+-K^+)-ATPase of plasma membrane is a active transport protein of cells. It consists of two types of subunits: A 110-kD nonglycosylated α subunit that contains the enzyme's catalytic activity and ion-binding sites, and an 55-kD glycoprotein β subunit of unknown function (Fig. 2-1) (14):

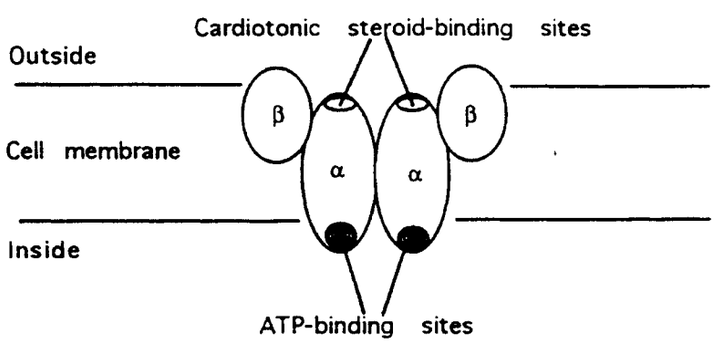


Fig. 2-1
The putative dimeric structure of the (Na^+-K^+)-ATPase (p494 in reference 14).

It pumps Na^+ out of and K^+ into the cell with the concomitant hydrolysis of intracellular ATP to maintain water osmotic pressure of animal cells. The ATP hydrolysis drives the endogenous transport of Na^+ and K^+ against electrochemical gradients (Fig. 2-2) (14):

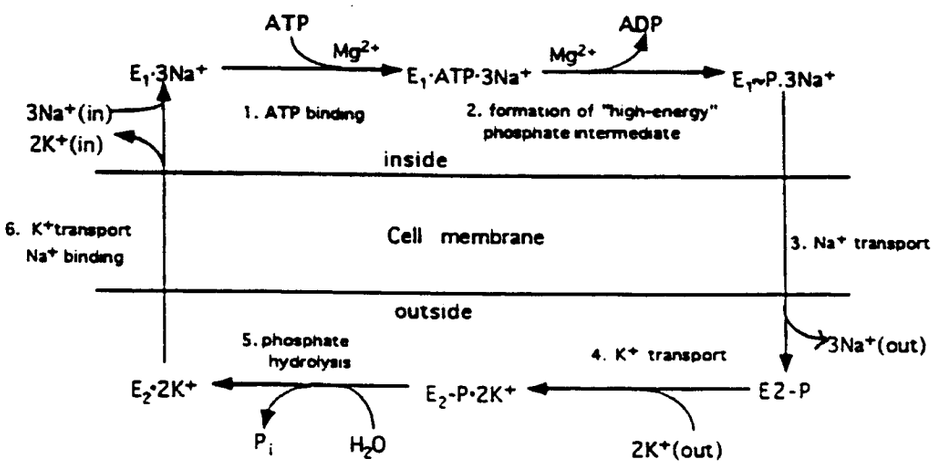


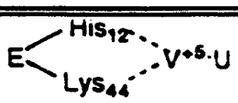
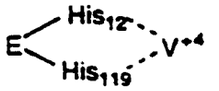
Fig. 2-2
A scheme for the transport of Na^+ and K^+ by (Na^+-K^+)-ATPase (p495 in reference 13).

The protein is phosphorylated by ATP on an Asp residue to form a highly reactive aspartyl phosphate intermediate. The enzyme has two conformational states, E_1 and E_2, which have different tertiary structures, catalytic activities and ligand specificities. E_1 has an inward-facing high affinity Na^+-binding site and reacts with ATP to form the activated product E_1~P only when Na^+ is bound. E_2-P has an outward-facing high affinity K^+-binding site and hydrolyzes to form P_i + E_2 only when K^+ is bound.

Inhibition of (Na^+-K^+)-ATPase by vanadium was identified as vanadate (+5 oxidation state of vanadium) (9). Vanadate (VO_4^{+3}, an analog of phosphate) competes with phosphate by binding to the site where phosphate is released during turnover (15). It is possible that vanadate may combine with a phosphate site to cause inhibition of the pump. High effective inhibition of vanadate is thought to occur in that (11) pentavalent

Table 2-1

Enzymes known to be inhibited or activated by vanadium (from reference 6)

Enzyme	Postulated form of E·V complex	Apparent inhibition or dissociation constants (M)
Ribonuclease		1 X 10^5
		6 X 10^5
Acid phosphatase	E-His·V^{+5}	2 X 10^7
Alkaline phosphatase	E-His·V^{+5}	2 X 10^6
	E-His·V^{+4}	4 X 10^7
(Na^+-K^+)-ATPase	K·E_2-Asp-V^{+5}·Mg	4 X 10^9
Dynein 1 ATPase	?	0.5-1.0 X 10^6
Myosin ATPase	E_2·ADP·V^{+5}	→ 0 ?
Ca^{2+}-ATPase (sarcoplasmic reticulum)	E_2-V^{+5}·Mg ?	~ 7 X 10^6
Ca^{2+}.Mg^{2+}-ATPase (ascites and human red blood cell plasma membranes)	?	> 1 X 10^5
Mg^{2+}-ATPase (*N. crassa*, *S. cerevisiae*, ascites plasma membranes)	?	4.5-10 X 10^7 (*N. crassa*)
K^+-ATPase	?	~ 1 X 10^5
Adenylate kinase	E·$V_{10}O_{28}H^5$?	< 1 X 10^6
Phosphofructokinase	?	~ 3 X 10^6
Squalene synthetase	?	?
Glyceraldehyde-3-phosphate dehydrogenase	E-G·V → E +G-V^{+5}	—
Adenyl cyclase	E_2·GDP·V^{+5} ?	Activated

U=uridine; G=glyceraldehyde 3-phosphate; E-V indicates acovalent link between enzyme and vanadium. Mitochondrial H^+-ATPase and ascites plasma membrane Ca^{2+}-ATPase are not inhibited by vanadate, presumably because they do not generate E-P intermediates.

vanadium can easily adopt a stable trigonal bipyramidal structure. This structure resembles the transition state of phosphate during reaction. Vanadate binds to the phosphorylation site of the (Na^+-K^+)-ATPase during reactions from the cytoplasmic side of the plasma membrane. It should be noted that maximal ouabain binding was achieved with μM concentration of vanadate, whereas mM concentrations of P_i (inorganic phosphate) were needed for the same effect (16). Several reports indicated that vanadate binding is facilitated by internal Mg^{2+}, and it appears to lock the enzyme in the E_2 state which binds K^+. Similar inhibition of Ca-ATPase by vanadate has been demonstrated in various tissues (17-20). The dissociation rates of vanadate from ATPases differ considerably (17, 20). Ethylenediamine tetraacetic acid (EDTA) rather than ethylene glycol-bis (β-aminoethyl ether) N, N, N', N',-tetraacetic acid (EGTA) and trans-1, 2-diaminocyclohexane-N, N, N', N'-tetraacetic acid (CDTA) can reverse the inhibition. Inhibition by vanadate is dependent on pH.

Cantley *et al* (14, 21) have demonstrated that vanadate inhibits the sodium pump from the cytoplasmic site. Thus, vanadate has to pass through the cell membrane to get into these sites. In certain cells, such as erythrocytes or in bull frog corneas vanadate is transported into the cells via the anion carrier system. The anion flux inhibitors SITS (4-acetoamido-4'-isothiocyanatostilben-2-2-disulfonate) or DIDS (4, 4'- diisothio-cyanostilbene-2-2-disulfornic acid) prevent vanadate penetration of the cell membrane. However, in cardiac cells vanadate is apparently transported through the cell membrane by a different non-energy-dependent carrier system. Vanadate often seems to be reduced to vanadyl ions (VO^{2+}) when it binds to proteins inside cells. "Vanadyl complex" (vanadium in reduced form) appeared to be the weakest of all analogs in either inhibiting the (Na^+-K^+)-ATPase or producing pharmacological actions (22), Vanadyl ions have different effects from vanadate. VO^{2+} is a powerful inhibitor of alkaline phosphatase, but has no effect on the (Na^+-K^+)-ATPase, whereas other vanadate compounds were approximately

equal in potency. This may partially explained why vanadate is not a more potent inhibitor of transport in whole cells. The question of whether cation transport may be regulated *in vivo* by an oxidation-reduction change in vanadium is still obscure. In the plasma, normal oxygen pressure keeps it in an oxidized state as vanadate, which is a more potent inhibitor of the pump (2). Vanadium regulates the (Na^+-K^+)-ATPase associated with oxidation-reduction states (21).

Not only vanadium oxidation states but its monoanion and polynuclear complexes can also influence biological functions. For instance, adenylate kinase is inhibited more strongly by oligomeric vanadate than by the monoanion (12). X-ray diffraction studies suggest that the oligomer sits across the phosphate-binding portions of both the ATP and the AMP sites on the enzyme (23). When the concentration of HVO_4^{2-} is above 10^{-4} M, it will form polynuclear complexes (24).

Curiously, in addition to vanadate as a potent ATPase inhibitor, opposite effects of vanadate are also documented. For example, vanadate activates rather than inhibits adenylate cyclase. Vanadate induced vasoconstriction is not associated with attenuation of sodium pump activity (3). On the other hand, vanadium compounds have been shown to inhibit cholesterol synthesis, enhance phospholipid oxidation, inhibit liver ATP production (4) and lower hepatic coenzyme A levels both necessary for sterol production. Furthermore, vanadate inhibits multifunctional glucose-6-phosphatase as well as several other enzyme systems involved in carbohydrate metabolism, which suggests that vanadium might well affect the metabolism of another energy source, glucose (2). Vanadium in the form of sodium orthovanadate, sodium metavanadate and/or ammonium vanadate has been employed in studies of these effects.

Insulin-like effects of vanadate

reduced intracellularly to vanadyl (VO_2^+) ions (+4 oxidation state) and vanadyl is a relatively ineffective inhibitor of this enzyme.

Insulin-like effects of vanadate influence glucose metabolism in various tissues — adipocytes, hepatocytes, diaphragm and intestine. Both $VOSO_4 \cdot 2H_2O$ and Na_3VO_3, which stimulate the oxidation of glucose -^{14}C to $^{14}CO_2$, is concentration dependent and is additive (4). Like insulin, vanadate also augments ^{125}I-IGF-II (type II insulin-like growth factor) binding on the cell surface of rat adipocytes in a dose-dependent fashion (32). It would appear that changes in (Na^+-K^+)-ATPase are not decisive for effecting change in IGF-II binding since ouabain and amiloride, other inhibitors of this enzyme (33), did not influence ^{125}I-IGF-II binding. Morever, vanadate increases fructose-2,6-biphosphatase levels in isolated hepatocytes from non-diabetic rats and counteracts the effects of glucagon (opposite effector of insulin) on cAMP levels and cAMP-dependent protein kinase activity (35). The ion affects fructose-2,6-biphosphatase concentration in a time- and dose-dependent manner (34). Therefore, vanadate has insulin-like effects on the glucose metabolism pathway in rat hepatocytes. Incubation of isolated rat adipocytes with vanadate elicited an insulin-like stimulation of the rate of glucose oxidation and 2-deoxyglucose transport. The maximal 6- to 12-fold stimulation of the transport observed in vanadate-treated adipocytes was equivalent to that produced by a maximally activating concentration of insulin (36). Vanadate and insulin have additive properties at their submaximal concentrations in these cases.

These observations suggest that vanadate acts via a pathway similar to that of insulin. Cellular insulin action is initiated by the interaction of the hormone with its specific cell surface receptors. Insulin receptor is embraced in the plasma membrane and consists of two α-subunits (MW ~135,00 each) and two β-subunits (MW ~95,000 each). The major portion of α-subunit faces to the extracellular space and β-subunit is exposed to intracellular space. It is now known that the β-subunit of the insulin receptor is a tyrosine

kinase whose activity is stimulated by insulin (37, 38). Insulin stimulates autophosphorylation of the β-subunit and thereby activates its tyrosine kinase activity (39, 40). Upon insulin binding, tyrosine residue will be phosphorylated. This phosphorylation initiates a cascade of cellular phosphorylation reactions which result in multiple enzymatic processes. Vanadate may enhanced the degree of phosphorylation of the β-subunit selectively on tyrosine residues (41). This may be brought about by activation of a specific tyrosyl-protein kinase or inhibition of a phosphatase. The effect of vanadate, like that of insulin, was reversed by incubation with epinephrine (41, 42).

Studies (81) showed that the concentrations of vanadate needed to inhibit glucose synthesis were 10 to 100 -fold higher than those observed in the intact rat responding to the ion, and that the specificity for diabetic animals was not explained by studies with isolated cells. Thus, it was proposed that specific actions of vanadate to lower glucose concentrations in diabetic animals was also the consequence of long-term actions of vanadate on those enzyme gene expressions. Studies documented that insulin regulates gene expressions of albumin, aldolase B, phosphoenolpyruvate carboxykinase (PEPCK) and L-type pyruvate kinase (L-PK), and that vanadate, like insulin, inhibits gene expression of phosphoenolpyruvate carboxykinase and induces mRNA level of L-type pyruvate kinase (43, 44). Phosphoenolpyruvate carboxykinase gene was contrary regulated by insulin and cAMP (second messenger of glucagon) (45).

However, in isolated hepatocytes from healthy (46) or diabetic (47) rats, vanadate produces the inactivation and phosphorylation of glycogen synthase and the activation of glycogen phosphorylase, effects opposite to those of insulin and similar to the effects of the glucagon. Second, the effect of vanadate on glucose transport is independent of the insulin receptor (48). Induction of a post-receptor defect (which may be a decrease in the total number of cellular glucose transporters) by prolonged expose to insulin decreases the potency of a maximally effective concentration of vanadate. The findings demonstrate that

vanadate stimulates glucose transport by an effect at a level distal to the insulin receptor (48). Third, the insulin-like effect of vanadate on lipolysis in rat adipocytes is not accompanied by an insulin-like effect on tyrosine phosphorylation (49). Bosch (50) reported that in the hepatocytes vanadate exerts opposite effects of that in the adipocytes and skeletal muscle, where vanadate had an insulin-like action. In the study of PEPCK (43), vanadate inhibition of gene expression was similar to that required for the vanadate-mediated activation of the insulin receptor tyrosine kinase, but a comparison of the inhibitory effects of insulin and vanadate in this system indicated a major difference in the site of action of these two compounds. Finally, the diabetes disease has two clinical manifestation: insulin dependent (type I which is irreversible insulin defect) and non-insulin dependent (type II which is insulin resistant). The molecular basis of type II diabetes is still poorly understood despite intensive research. In an animal model, STZ-rats have both type I and type II diabetes symptoms, but vanadate can normalized their glucose levels to control healthy value.

The discrete insulin-like actions of vanadate on carbohydrate metabolism challenge us to further explore the insulin-like mechanism of vanadate by employing L-type pyruvate kinase which has been studied for a long time.

L-type Pyruvate Kinase (L-PK)

The concentration of blood glucose in mammals is tightly regulated both by production of glucose by liver and uptake by muscle and adipose. The pathways of glycolysis and gluconeogenesis share common enzymes except some key enzymes which are unique to each physiologically irreversible steps. The key enzymes include: pyruvate carboxylase, phosphoenolpyruvate carboxykinase (PEPCK), phosphofructokinase (PFK), fructose-1,6-biphosphatase (FBP), and glucose-6-phosphatase (G6P), etc. Pyruvate kinase (PK) is one of the key enzymes. It catalyzes the following reaction:

$$\text{Phosphoenolpyruvate} + \text{MgADP} \xrightarrow[Mg^{2+},\ K^{+}]{\text{Pyruvate kinase}} \text{Pyruvate} + \text{MgATP}$$

Insulin and insulin counter-regulatory hormones such as glucagon and epinephrine regulate hepatic glucose production. Glucagon and epinephrine enhance glucose production by increasing the rate of glycogen breakdown and *de novo* glucose synthesis (gluconeogenesis). Insulin, which lowers blood sugar and decreases glucose output by the liver, counteracts the actions of glucagon. Genes for PK, PEPCK and GK (glucose kinase) have been isolated and studied. Administering insulin to diabetic animal models causes rapid changes in the phosphorylation state of several these enzymes and restores their gene expression to normal. The enzyme pyruvate kinase exemplifies all levels of regulation by hormones known to influence hepatic carbohydrate metabolism and reflect the insulin-like response of vanadate (51).

Pyruvate kinase has four isoenzymes which are named M_1, M_2, L and R (52, 53). M_1-type is found in adult skeletal muscle and is a major isozyme in heart and brain. M_2-type appears to be a primordial form of the enzyme and is predominant in the internal organs (such as spleen and lung). R-type is found in erythrocytes. L-type is the major isozyme of adult liver and the only isozyme found in the parenchymal cells of this tissue in adults (54, 55). The organizations and expressions of pyruvate kinase isozyme genes are interesting. Genetic studies showed that the M_1- and M_2-types of rat pyruvate kinase are derived from a signal gene, so do L- and R-types . M_1- and M_2-types are produced from the same gene by alternative RNA splicing (56). L- and R-type isozymes of rat pyruvate kinase are generated from a single gene by use of different promoters (53).

A rat haploid genome contains a single L-PK gene and the gene is composed of 12 exons and 11 introns with a length of about 9.3 kilobase pairs. The first (exon R) and second (exon L) exons encode the 5'-terminal sequences specific for the R- and L-type

respectively. The remaining downstream exons encode a sequence common to both of them (53):

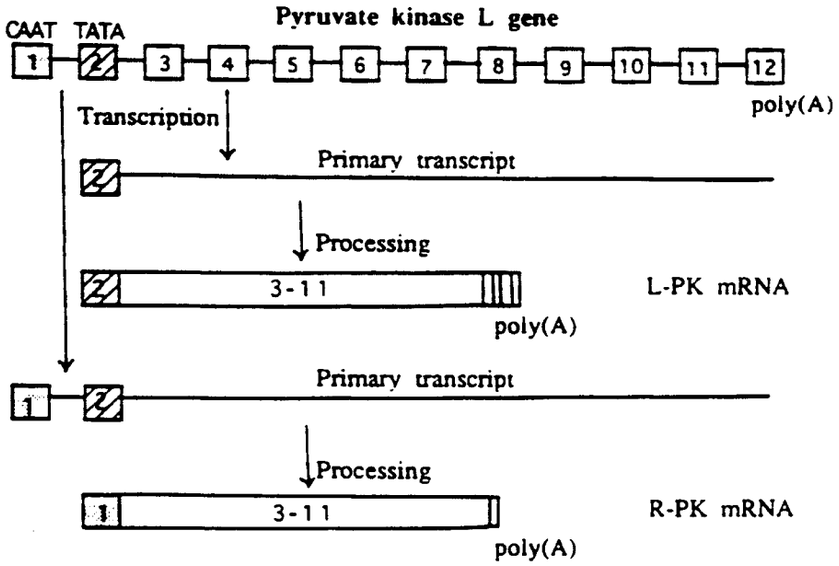


Fig. 2-3
A scheme of expression of the pyruvate kinase L gene. Exons are numbered. *CAAT*, CAT box; *TATA*, TATA box; poly(A), polyadenylation signal; L-PK, L-type pyruvate kinase; R-PK, R-type pyruvate kinase. (scheme presented by Villar-Palasi, C. in reference 53)

The last exon contains the entire 3'-untranslated region which has several putative polyadenylation signals. Sequences downstream from the poly(A)$^+$ signal are essential for the formation of its mature mRNA (57). Multiple mRNA species for the L-type might be generated by alternative use of these signals. The R-type uses only the first poly(A)$^+$ signal. There is 16-nucleotide mRNA cap site for the L-type within the PK gene. The R-type might have multiple cap sites within the PK gene. The L-type has a canonical TATA box in the upstream sequence of exon L, whereas one (or several) CAT box(S) for the R-type (53).

A total sequence of a 13,021 base-pair (bp) genomic fragment containing the rat L-PK was sequenced by Unite (58). This fragment includes 8360 bp of the L-PK gene, plus

3193 bp of the 5'-flanking and 1468 bp of the 3'-flanking regions. L-PK gene exhibits a fully conserved exon-intron structure. At position -776 and +7821, a TGTTCT sequence that is present in the glucocorticoid receptor binding site for DNA was found (59). The sequence, 5'-CTTACCTCAGT G-3' at position -2338, and 5'-CTTACGTCTGAG-3' at position +5788 were found homologous to the cAMP regulatory elements described in the phosphoenolpyruvate carboxykinase gene (60).

Studies by Dynan and Maniatis (61, 62) indicated that the control of gene expressions are achieved through the interactions between cis-acting DNA elements and trans-acting proteins that bind to these DNA sequences. 5'-flanking region contains cis-acting DNA elements responsible for tissue-specific expression (63-65) and hormonal regulation (60, 66).

Transient DNA transfer experiments with L-PK/CAT (chloramphenicol acetyltransferase) fusion genes performed by Yamada (67) showed that three positive regulatory regions are necessary for expression of L-PK in adult rat hepatocytes by functional analyses of a 5'- and internal deletion constructs of the fusion genes. These regions are: -76 to -94, -126 to -149, and -150 to -170. Vaulont (68) reported that region -96 to -66 (with respect to the cap site) binds a liver-specific protein.

Decans (69) developed a model of adult rat hepatocytes in primary culture, whose behavior is identical to that *in vivo*, to study the action of different effectors on the expression of L-PK gene. In these cells, the genes expressed only when glucose and insulin are present together, each of them being inactive by itself. *In vivo* studies have shown that the expression of L-PK gene is regulated by hormones and carbohydrates at both transcriptional and post-transcriptional levels. The major negative effector is glucagon and its second messenger cAMP, the major positive effectors are carbohydrates in the presence of insulin, thyroid hormones and glucocorticoids which appear to play a permissive role (70, 71). Like insulin in primary culture of adult rat hepatocytes, vanadate

stimulates L-PK gene expression in the presence of glucose. Similar results were obtained on glucokinase gene expression (44). However, Decaux (72) indicated that transcription of the albumin gene is active in the presence of insulin alone, with or without glucose, whereas transcription of the aldolase B is stimulated by glucose and insulin together, but not by insulin or glucose alone.

Recently, Towle *et al* (73) constructed a series of chimeric genes containing different deletion of 5'-flanking sequence of rat L-PK fused to CAT gene and transfected these genes into primary hepatocytes. Sequence from -4300 to +12 of L-PK gene directed an increase in CAT activity when hepatocytes were switched from media containing 10 mM lactate to 25 mM glucose. Carbohydrate regulation of the L-PK promoter was retained with 5'-deletion to -197, but constructs deleted to -96 were completely unresponsive. Therefore, a carbohydrate response element (CRE) is located between -197 to -96 of the L-PK gene, and it has the enhancer-like properties of functioning in concert with a heterologous promoter and functioning in an orientation-independent manner. The transfected gene was also regulated by insulin and glucagon in a pattern similar to that seen for the endogenous L-PK gene, suggesting that control of L-PK promoter activity was responsible for carbohydrate-mediated changes in L-PK mRNA transcription.

These observations might suggest that insulin and/or vanadate (and/or glucagon) may affect gene expression indirectly by altering rates of carbohydrate metabolism and thus influencing the activity of the same nuclear factors affected by glucose. Alternatively, insulin and/or vanadate (and/or glucagon) affect nuclear events more directly, possibly via phosphorylation schemes resulting in altered transcription factor activity to what had been previously descried for glucagon action on the somatostatin gene by Yamada (67).

Apparently, vanadate mimicking insulin effects is not always exactly the same as that elicited by insulin. Since a gene construct (pGL-Reg5) containing a 1000 bp 5'-region (Reg5) of the L-PK promoter with the firefly luciferase gene was responsive to glucose,

insulin and vanadate by yet unknown mechanism, in which insulin and vanadate seemed to act directly on the gene (81) rather than playing a "permissive" role. The present research involves constructing chimeric genes, which contain a series of L-PK promoters with deletions, to detect the sequences responsive to insulin and vanadate. If vanadate indeed exactly mimics insulin on L-PK gene, it should activate the gene by using the same promoter region(s).

CHAPTER III

EXPERIMENTAL METHODS

Plasmid Construction and Isolation

Seven fragments (designated E, F, G, H, I, J and K) represented on a serial deletions of the complete promoter end of the L-PK gene were obtained by polymerase chain reaction (PCR) using synthetic oligonucleotides which were about 28-base long and have appropriate restriction enzyme sequences flanking the promoter regions of interest as PCR primers. K was made by directly priming the corresponding primer to rat genomic DNA, and E through J were from Reg5, which was studied by Mr. Chris Otiko (2), by PCR using their corresponding prime respectively. Thus, each fragment has *Hind III* restriction site at 5'-end and *Bgl II* restriction site at 3'-end.

The above work was done by Raul Espinosa-Nava (unpublished). The fragments from PCR were respectively inserted into the *Hind III* and *Bgl II* sites of pGL2-Basic (Promega) vector, containing a luciferase marker, by first digesting the vector with *Hind* III and *Bgl* II restriction enzymes followed by ligation with T_4 ligase to construct a series of chimeric genes. The inserted DNA fragments were oriented in pGL2-Basic vector such that the luciferase gene is under control of the L-PK promoter and regulatory elements. The plasmids were transformed into *Escherichia* coli JM109 and screened by ampicillin resistence and agarose gel electrophoresis. The plasmids containing the desired promoter region were finally confirmed by sequencing with a Applied Biosystems 373A DNA Sequencer in two directions from both ends of the desired promoter region using the same primers as that for PCR.

The transformed JM109 cells, which propagate plasmids at high copy numbers, grew in LB medium for about 18 hours. The plasmid DNAs were isolated originally by alkaline lysis followed by PEG (polyethylene glycol) precipitation (74) to remove trace amount of protein, but we found plasmids isolated in this way gave no expression at all. Therefore we changed the purification method by banding the plasmids twice in CsCl-ethidium bromide isopycnic gradients (75) to obtain the super coiled plasmid DNA. After the banding, ethidium bromide was removed by extraction with butyl alcohol (saturated with a equal volume of TE buffer containing 4M NaCl) 7-8 times. CsCl was removed by precipitation using 7.5 M ammonium acetate and ethanol 5 times. The purified plasmid DNAs were stored in H_2O. The DNA concentrations were determined by measuring their absorbences at 260 nm. The plasmid purity was determined by their ratio of A_{260}/A_{280}, spectrum scanning from 200 nm to 320 nm, and restriction digestion.

Isolation and Culture of Primary Hepatocytes

Primary hepatocytes were isolated from male Wistar rats (weight 200-250 g) liver which was first perfused with collagenase (40 mg of Worthington type II Lot. #F3B517) in Krebs-Ringer Bicarbonate media without calcium (KRB without calcium, 200 ml of 0.15 M NaCl, 8 ml of 0.15 M KCl, 1.5 ml of 0.15 M KH_2PO_4, 1.5 ml of 0.15 M $MgSO_4 \cdot 7H_2O$, 42 ml of 0.15 M $NaHCO_3$, 0.1 ml of 400 mg/ml streptomycin sulfate, pH 7.4) approximately 20 minutes under 5% CO_2, 95% O_2 at 37 °C followed by KRB wash and centrifugation to remove dead cells and other debris. Cell viability was determined by cell appearances under microscope. The isolated cells were resuspended in incubation media and plated at a initial density of 7.5 x 10^5 cell/ml in 60-mm tissue culture dishes (Falcon) coated with rat-tail collagen (Table 3-1). After a 4-hour attachment period in incubation medium (Table 3-2), the medium in the plates were replaced with Waymouth's medium and incubated for another 18-24 hours, then transfection was started in SUM

medium (Table 3-2) without antibiotics and incubated for 16-18 hours. Cells were then washed with KRB (Table 3-1) once and maintained for the remainder of the culture time in KRB media. Experimental compounds were added after cell wash, and 24 hours later, the experiment was terminated by removing the media from the plates and putting them in the freezer.

Transfection

For each 60-mm culture dish, 1.9-2.0 μg of DNA sample was added into 100 μl of SUM without antibiotics, and 12 μl of lipofectin (Gibco) was added into another 100 μl of SUM without antibiotics, then the DNA/SUM was put into the lipofectin/SUM and mixed. After 20 min. setting at room temperature the mixture was added dropwise into a 60 mm dish of cell culture.

Luciferase Assay

To prepare the Luciferase Assay Reagent, 10 ml of Luciferase Assay Buffer (Promega) were added to the vial containing the lyophilized Luciferase Assay Substrate. Cell Culture Lysis Buffer (promega) was prepared by adding 4 volumes of water were added to 1 volume of Cell Culture Lysis 5X Reagent to produce a 1X solution. Both the diluted Lysis 1X Reagent and an aliquot of Luciferase Assay Reagent were equilibrated to room temperature.

Four hundred μl of Lysis 1X reagent was added into each of the frozen cell dishes to cover the cells and incubated at room temperature for 15 minutes. The lysed cells were scraped off the dish and the cell lysate were transferred to a microcentrifuge tube and spun briefly to pellet large debris.

The relative light units were recorded and integrated on a M3000 luminometer after mixing 40 μl of cell extract with 100 μl of Luciferase Assay Reagent at room temperature.

Table 3-1

Compositions of coating solution and experimental media.

Stock solution	**KRB medium** (1 liter)	**Collagen coating solution** (500 ml)
0.9 % NaCl	750 ml	411.2 ml
1.3 % $NaHCO_3$	157.5 ml	78.8 ml
1.15 % KCl	30 ml	---
1.62 % $CaCl_2*2H_2O$	11.25 ml	---
2.11 % KH_2PO_4	7.5 ml	---
3.82 % $MgSO_4*7H_2O$	7.5 ml	---
Streptomycin (400 mg/ml)	250 µl	625 µl
Amino Acid solution (Gibco)	20 ml	---
Ampicillin	60 mg	---
BSA	2000 mg	---
Rat-tail collagen stock	---	7.5 ml

Adjust pH to 7.0-7.1 and filter sterile.

Table 3-2

Compositions of cell culture and transfection media

Stock solution	**Incubation medium** (1 liter)	**SUM medium** (1 liter)
Minimum Essential Medium (Sigma)	750 ml	750 ml
Waymouth (Gibco)	250 ml	250 ml
Ampicillin	60 mg	---
Streptomycin (400 mg/ml)	250 µl	---
Amphotericin B	250 µl	---
Glutamine	292 mg	---
BSA	2000 mg	---
Alanine	40 mg	40 mg
Serine	52.6 mg	52.6 mg
$NaHCO_3$	2240 mg	2240 mg
Selenium (3×10^{-5} M)	---	1 ml
3,5,3' triiodo-L-thyronine (2.50 mg/ml)	---	673 µl
Dexamethasone	---	0.788 mg

Adjust pH to 7.1-7.2 and filter sterile.

Other Reagents

Insulin Solution

Stock solutions were prepared by dissolving 1 mg of crystalline porcine zinc insulin (Lilly Research Laboratories) in 1 ml of 1 mM HCl, and the concentration were determined spectrophotometrically at 276 nm using $A_{0.1\ \%} = 1.05$. Stock solutions were stored at 4 °C. Aliquots of the stock were further diluted as required in media containing bovine serum albumin.

Vanadate Solution

Purified grade Na_3VO_4 (Fisher Scientific) was used in all experiments. A 0.1 M stock solution was prepared by dissolving powder in 0.1 M HEPES buffer (pH 7.2). The pH of the solution was adjusted to 10 with 5 N NaOH, which gave a colorless solution. The final concentration of vanadate solution was verified by measurement of absorbance at 260 nm using an extinction coefficient of $3.55 \times 10^3\ M^{-1}cm^{-1}$ and a 0.1 N of NaOH solution as blank. It was reported that at this pH vanadate is predominantly monomeric HVO_4^{-2} (29). Immediately before each experiment an aliquot of a freshly prepared stock was diluted as required in media containing bovine serum albumin.

CHAPTER IV

RESULTS AND DISCUSSION

Construction of Chimeric Genes

To test the response regions to glucose, insulin and vanadate, the chimeric genes containing a series of L-PK promoter deletion regions were made. The vector DNA was pGL2-Basic plasmid (commercially from Promega) with luciferase marker, which is promoterless and enhancerless. A 5'-flanking segment E (-70 to +10 bp relative to the L-PK cap site) (Fig. 4-1) of the PK gene containing TATA box for L-type was inserted upstream of the luciferase gene to create pGL-E (Fig. 4-2). Similarly, pGL-F, pGL-G, pGL-H, pGL-I, pGL-J and pGL-K were created by inserting these fragments upstream of the luciferase gene, respectively. pGL-R was obtained by multiplying and purifying pGL-Reg5, which was prepared by Raul Espinosa-Nava (unpublished), therefore pGL-R and pGL-Reg5 are structurally identical. Fragment F (-101 to +10 bp) consisted of fragment E and one of the three liver-specific expression elements (67, 68). Fragment G and F were from -121 to +10 bp, -145 to +10 bp, respectively. Fragment I (-171 to +10) was composed of fragment E through H and all the three liver-specific expression elements. Fragment J (-503 to +10) contained the first exon of R-type isozyme and fragment I. Fragment R (-1028 to +10) included CAT box for R-PK as well as fragment J. Fragment K (-3113 to +10) had almost the whole promoter region of L- and R-type pyruvate kinase including a putative cAMP regulatory element. These plasmids were propagated in *E.* coli JM 109 and purified by alkaline denaturing and CsCl-ethidium bromide gradient centrifuge.

The DNA concentration was quantitated after resuspension in sterile H_2O using a Shimadzu UV 160 spectrophotometer. The purified plasmid DNAs were subjected to UV

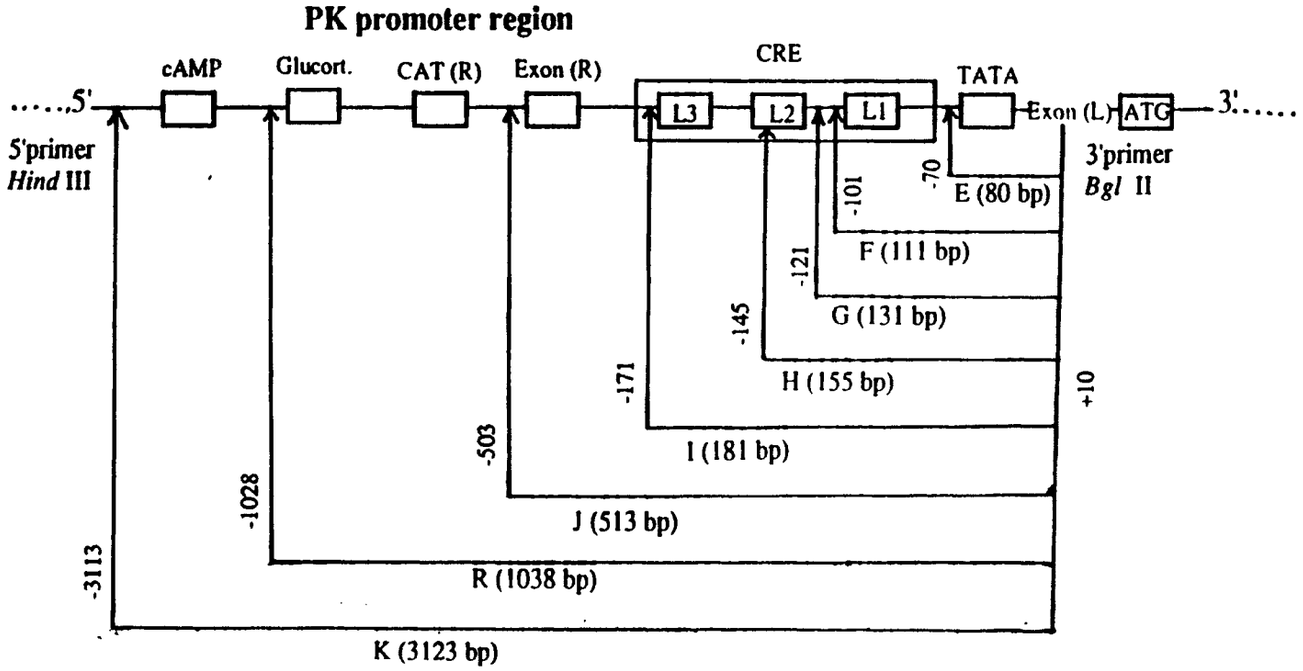


Fig. 4-1

Locations of a series of deletion fragment in the promoter region of L-type pyruvate kinase. E, F, G, H, I, J, R and K are the fragments with different length from L-PK promoter region. These fragments were used for the construction of chimeric genes. Each of the fragments has *Hind* III restriction site at 5' end and *Bgl* II restriction site at 3' end. cAMP, regulatory element for adenosine 3', 5'-cyclic monophosphate found in the phosphoenolpyruvate carboxykinase gene; Glucort., the sequence homologous to the glucorticoid receptor binding site for DNA ; CAT (R), CAT box for R-PK; Exon (R), the first exon of R-PK in the pyruvate kinase gene; CRE, carbohydrate response elements; L1, L2 and L3, liver-specific expression elements; TATA (L), tata box for L-PK; Exon (L), the first exon of L-PK in the gene; ATG, start codon for L-PK. The plot is not in scale. The references are in the text.

Fig. 4-2

Construction of chimeric genes with a series of deletion region of L-PK. Panel A: Vector DNAs of plasmid pGL2-Basic (from Promega) were first digested with restriction enzymes of *Hind III* and *Bgl II*. After treatment of calf intestinal alkaline phosphatase, inserted fragments were ligated respectively into the vector by T_4 ligase. Panel B is chimeric gene. The numbered positions are relative to the cap site of L-PK. Panel C is a positive control plasmid of pGL-SV40 (pGL-2 promoter, commercially from Promega). ori, origin of plasmid replication in *E. coli*; f1 ori, origin of replication derived from filamentous phage; SV40 promoter, region of DNA from SV40 virus early promoter; Amp', gene conferring ampicillin resistance in *E. coli*; Luc, cDNA coding firefly luciferase (74).

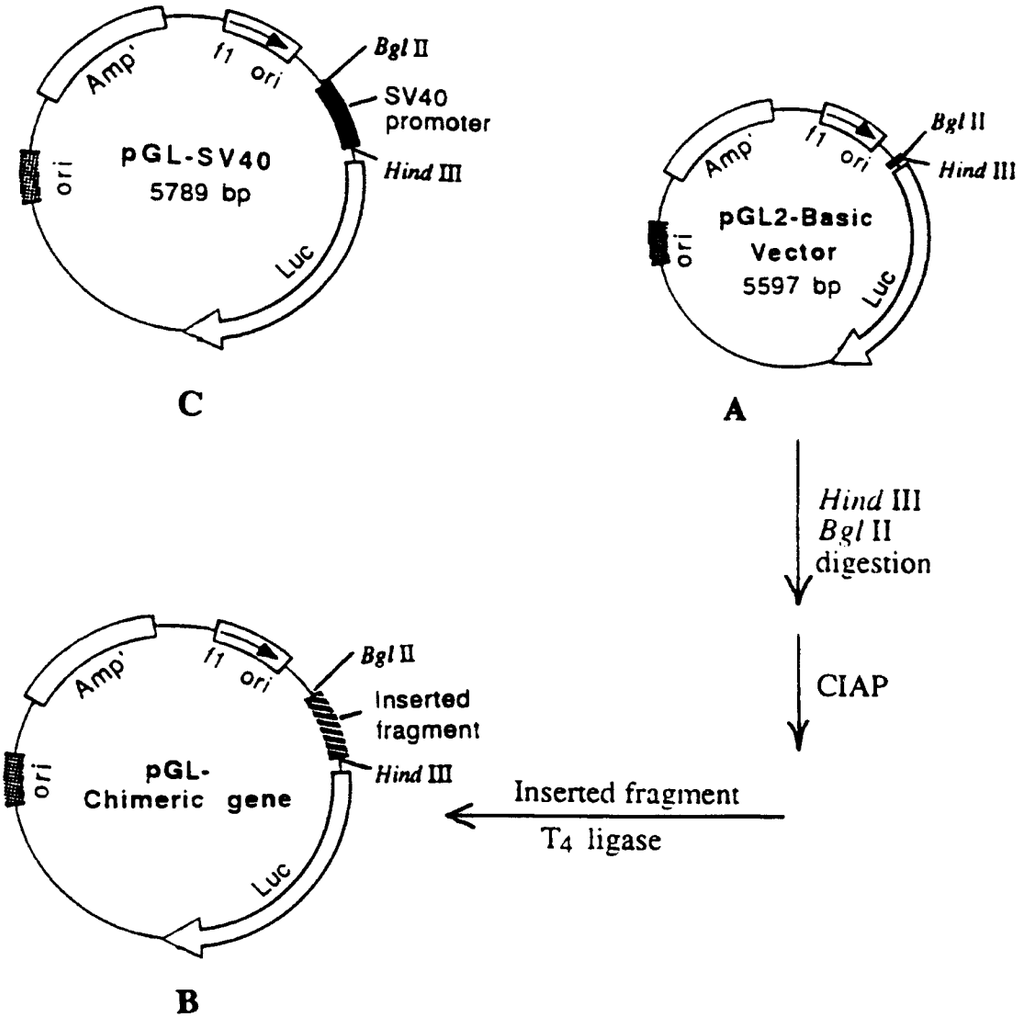


Fig. 4-2
Construction of chimeric genes with a series of deletion region of L-PK.

scanning (Fig. 4-3), restriction digestion and agarose gel electrophoresis to test their qualities (Fig. 4-4). The ratio of A_{260}/A_{280} (absorbances at 260 nm and 280 nm) of these samples were greater than 1.85. The scanning spectrums from 200 to 320 nm indicated no impurities in the samples. Gel electrophoresis showed that the major amount of the sample DNAs were supercoiled plasmids and they were very sensitive to restriction digestions.

Finally, each of these samples were partly sequenced using previous PCR primer from both 3'- and 5'-end, respectively. The sequences were aligned to the published sequence of pyruvate kinase in GeneBank database (58). The sequences agreed within 98 % of the published sequence (Fig. 4-5). As predicted by restriction sites, orientation of the promoter regions were reversed at the site of transcription initiation from that projected for the native PK gene promoter.

Optimization of Transfection

Since transfection efficiency is very important to gene expression experiments, we tested two transfection protocols and a series of mass ratios of lipofectin (transfection reagent) : DNA for selective samples. In protocol 1 which was employed by Mr.Chris Otiko (81), the cultured cells were washed twice with phosphate buffered saline after 4-hour attachment phase and then were transfected in SUM medium. In protocol 2, the cell culture medium was replaced with Waymouth's medium and incubated for 18-24 hours after 4-hour attachment period, and then the cells were washed twice with SUM medium and the transfection was carried out in SUM medium. The difference between Protocol 1 and Protocol 2 is an additional 18- to 24-hour incubation in Waymouth's medium after 4-hour cell attachment, which might partially recover the cell functions after isolation. The expression units (light units in 60 seconds) dramatically increased under Protocol 2 (Fig.4-6). Fig.4-7 shows the luciferase activities after different amount of DNA while keeping lipofectin constant at 12 μg. At about 2 μg of DNA pGL-F, pGL-H, pGL-J and pGL-K

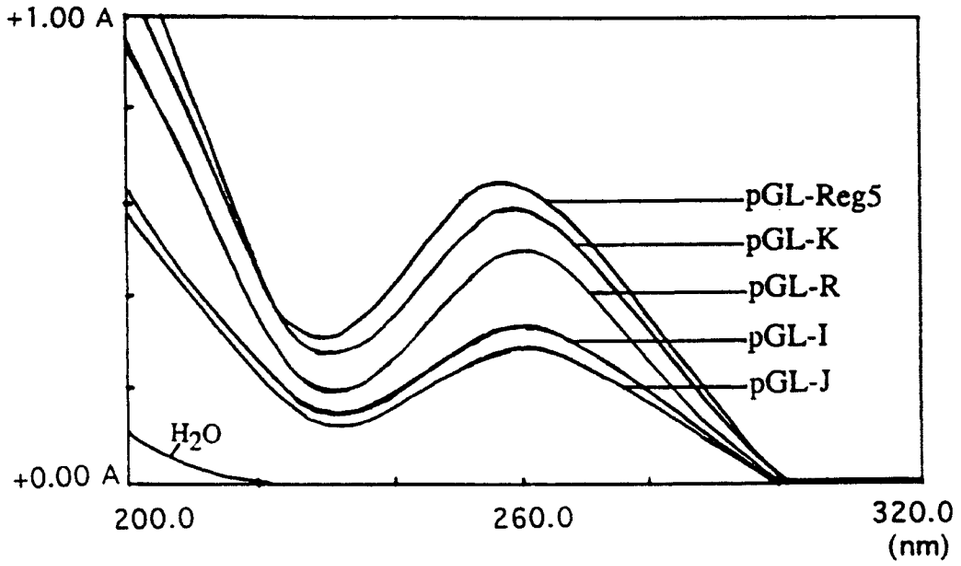
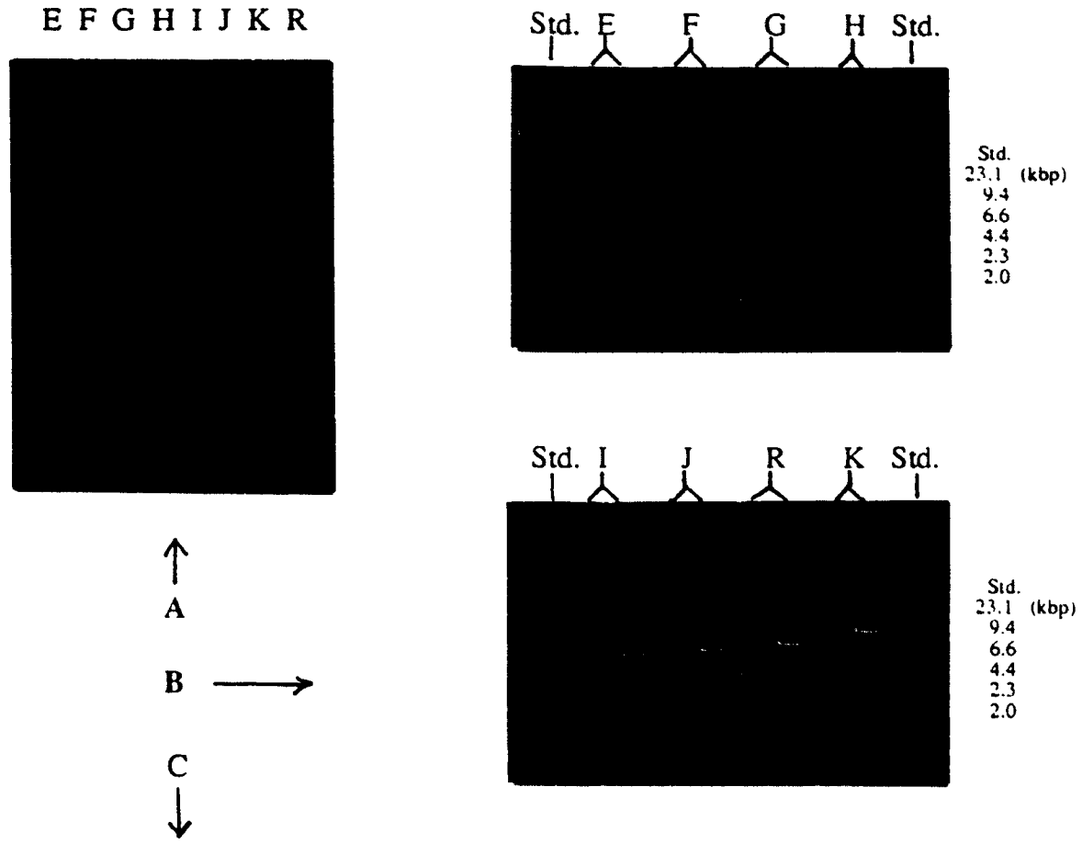


Fig. 4-3

UV light scanning spectra of chimeric gene pGL-I, pGL-J, pGL-K, pGL-R and pGL-Reg5. The DNAs were dissolved in H_2O. The peak of pGL-Reg5 prepared by Raul Espinosa-Nava was slightly less than that of the others.

Fig. 4-4

Electrophoresis analyses of plasmid pGL-E through pGL-K on agarose gel. Panel A: E, F, G, H, I, J, K and R represented plasmid pGL-E to pGL-R. These plasmids were obtained by Alkaline denature followed by PEG precipitation and used for sequencing. The amount of DNA loaded in each well of 0.75 % agarose was about 40 ng. The samples were run in 1x TAE buffer, 100 volts for 1 hour at 4 °C. Panel B: E, F, G, H, I, J, R, K and Std. represented plasmid pGL-E through pGL-K and standard of high molecular weight DNA. These plasmids were purified by CsCl-ethidium bromide binding and used for the remaining experiments. Each plasmid in the right lane was digested by restriction enzyme of *Hind III* (~1 U/μg DNA) for 2.5 hours, the left was not digested. Other conditions were the same in Panel A. Panel C: The left lanes were the plasmids, which were the same as those described in Panel B, digested by restriction enzymes of *Hind III* and *Bgl II* (~1 U/μg DNA for each enzyme). The samples in the right lanes were the corresponding fragment products directly from PCR. The conditions of electrophoresis were described in Panel A.



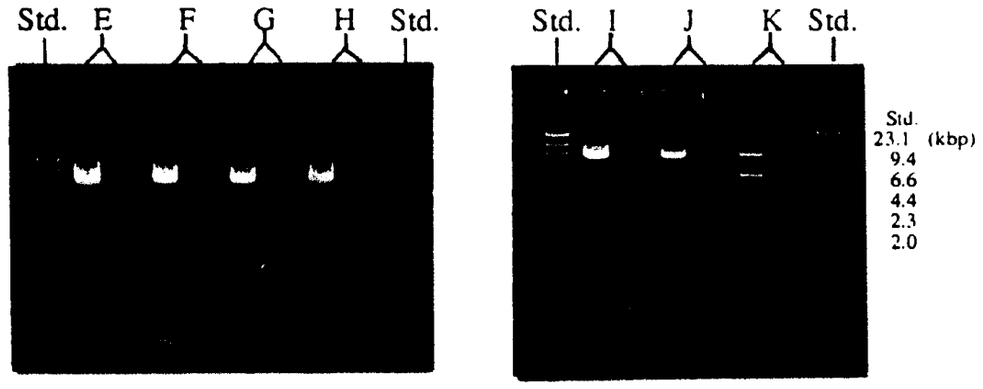


Fig. 4-4

Electrophoresis analyses of plasmid pGL-E through pGL-K on agarose gel.

Fig. 4-5

Sequences of Fragment E, F, G, H, I, J and partial sequences of Fragment R and K. Panel A are the sequences from both the 3'- and 5'-end, except K and R, using the previous PCR primer. Panel B are the sequences of K and R from the 5'-end using the previous PCR primer containing *Hind* III site. These fragment sequences were aligned to the published L-PK sequences. The published sequences are underlined. The errors of bases are boxed. N , unidentified bases; *Luc*, luciferase gene; *Hind* III, *Hind* III site. The numbers in the sequences are relative to the L-PK mRNA cap site.

```
          * -499                                                                                                  -404 *
5' ... ACTCTC  CTCCCAGTCT  GCCGTTCTTG  CAGACACAGT  TCCACGCTTT  GGAAGCATGT  CTGTCCAGGA  GAACACTCTA  CCCCAGCAGC  TCTGGCCGTG
                                                   K:          GGAAGCATGT  CTGTCCAGGA  GAACACTCTA  CCCCAGCAGC  TCTGGCCGTG
                                        R:    ACAGT  TCCANGCTTT  GGAAGCATGT  CTGTCCAGGAG GAAGACTCTA  CCCCA CAGC  TCTGGCCGTG
                                  J:    CCGTTCTTG  CAGACACAGT  TCCACGCTTT  GGAAGCATGT  CTGTCCAGGA  GAACACTCTA  CCCCAGCAGC  TCTGGCCGTG

     * -403                                                                                                      -304 *
     GATCTTTAGG  TCCCAAAAAG  ACTTGGCAAA  GTCTGCCTTA  AGTGGGGCTC  CCGGAGGTAA  GAAGAGGAAG  GGAAGCCACT  GAAGAGAGAG  GAGAATTAGG
K:   GATCTTTAGG  TCCCAAAAAG  ACTTGGCAAA  GTCTGCCTTA  AAGTGGGNCTC  CCGGAGGTAA  GAAGAGGAAG  GGAANCCACT  GAAGAGAGAG  GAGAATTAGG
R:   GATCTTTAGG  TCCCAAAAAG  ACTTGGCAAA  GTCTTGCCTTA  AGTGGGGCTC  CCGGAGGTAA  GAAGAGGAAG  GGAAGCCACT  GGAGAGAGAG  GAGAATTAGG
J:   GATCTTTAGG  TCCCAAAAAG  ACTTGGCAAA  GTCTGCCTTA  AGTGGGGCTC  CCGGAGGTAA  GAAGAGGAAG  GGAAGCCACT  GAAGAGAGAG  GAGAATTAGG

     * -303                                                                                                      -204 *
     GTAGAATCAG  CGTTGAGAGA  TGGAGGCCTT  GTGGGGTAGG  ATGCCCAATA  TAGCCTCACC  TCGGCTAAAT  ACAGACCTGA  TCTGAGCCTT  TGATCCAGGC
K:   GTAGAATCAG  CGTTGAGAGA  TGGAGGCCTT  GTGGGGTAGG  ATGCCCAATA  TAGCCTCACC  TCGGCTAAAT  ACAGACCTGA  TCTGAGCCTT  TGATCCAGGC
R:   GTAGAATCANG CGTTGAGAGA  TGGAGGCCTT  GTGGGGTAGA  ATGCCCAATA  TAGCCTCACC  TCGGCTAAAT  ACANGACCTGA TCTGAGCCTT  TGATCCAGGG
J:   GTAGAATCAG  CGTTGAGAGA  TGGAGGCCTT  GTGGGGTAGA  ATGCCCAATA  TAGCCTCACC  TCGGCTAAAT  ACAGACCTGA  TCTGAGCCTT  TGATCCAGGG

     * -203                                                                                                      -104 *
     TCTGCAGACA  GGCCAAAGGG  GATCCAGCAG  CATGGGCGCA  CGGGGCACTC  CCGTGGTTCC  TGGACTCTGG  CCCCCAGTGT  ACAAGGCTTC  CGTTGGCAAG
K:   TCTGCAGACA  GGCCAAAGGG  GATCCAGCAG  CATGGGCGCNA CGGGGCACTC  CCGTGGTTCC  TGGACTCTGG  CCCCCAGTGT  ACAAGGCTTC  CGTTGGCAAG
R:   TCTGCAGACA  GGCCAAAGGG  GATCCAGCAG  CATGGGCGCNA CGGGGCACTC  CCGTGGTTCC  TGGACTCTGG  CCCCCAGTGT  ACAAGGCTTC  CGTTGGCAAG
J:   TCTGCAGACA  GGCCAAAGGG  GATCCAGCAG  CATGGGCGCNA CGGGGCACTC  CCGTGGTTCC  TGGACTCTGG  CCCCCAGTGT  ACAAGGCTTC  CGTTGGCAAG
     <== LUC  ... Hind III ...  I:      TGGGCGCA  CGGGGCACTC  CCGTGGTTCC  TGGACTCTGG  CCCCCAGTGT  ACAAGGCTTC  CGTTGGCAAG
                                <== LUC  ... Hind III ...  H:      C  TGGACTCTGG  CCCCCAGTGT  ACAAGGCTTC  CGTTGGCAAG
                                                           <== LUC  ... Hind III ...  G:      AGGCTTC  CGTTGGCAAG

          * -103                                                                                          * -11
     AGAGATGCTA  GCTGGTTATA  CTTTAACCAG  GACTCATCTC  ATCTGAGCCA  GGCCCCATCC  CACTGACAAA  GGCGCAGTAT  AAAGCAGACC  CAC ... 3'
K:   AGAGATGCTA  GCTGGTTATA  CTTTAACCAG  GACTCATCTC  ATCTGAGCCA  GGCCCCATCC  CACTGACAAA  GGCGCAGTAT  AAAGCAGACC  CAC      K
R:   AGAGATGCTA  GCTGGTTATA  CTTTAACCAG  GACTCATCTC  ATCTGAGCCA  GGCCCCATCC  CACTGACAAA  GGCGCAGTAT  AAAGCAGACC  CAC      R
J:   AGAGATGCTA  GCTGGTTATA  CTTTAACCAG  GACTCATCTC  ATCTGAGCCA  GGCCCCATCC  CACTGACAAA  GGCGCAGTAT  AAAGCAGACC  CACA     J
I:   AGAGATGCTA  GCTGGTTATA  CTTTAACCAG  GACTCATCTC  ATCTGAGCCA  GGCCCCATCC  CACTGACAAA  GGCGCAGTAT  AAAGCAGACC  CAC      I
H:   AGAGATGCTA  GCTGGTTATA  CTTTAACCAG  GACTCATCTC  ATCTGAGCCA  GGCCCCATCC  CACTGACAAA  GGCGCAGTAT  AAAGCAGACC  CAC      H
G:   AGAGATGCTA  GCTGGTTATA  CTTTAACCAG  GACTCATCTC  ATCTGAGCCA  GGCCCCATCC  CACTGACAAA  GGCGCAGTAT  AAAGCAGACC  CAC      G
F:     GATGCTA  GCTGGTTATA  CTTTAACCAG  GACTCATCTC  ATCTGAGCCA  GGCCCCATCC  CACTGACAAA  GGCGCAGTAT  AAAGCAGACC  CAC      F
       <== LUC  ... Hind III ...  E:      TCATCTC  ATCTGAGCCA  GGCCCCATCC  CACTGACAAA  GGCGCAGTAT  AAAGCAGACC  CAC      E
```

Panel A

Fig. 4-5

```
        * -3109                                                                                                -3014 *
5' ... ATAGAG  AGTGTCCTCT  GGTAGGAGCA  TGTGCTCAGC  TTACTCCTCA  CCCAGTCTCC  CACGGGTGCT  ATTCCCCACT  GACCAAACTC  TGTGGAAGCC
R:       ------Primer------  GGTAGGAGCA  TGTGATCAGC  TTACTCCTCA  CCCAGTCTCC  CACGGGTGCT  ATTCCCCACT  GACCAAACTC  TGTGGAAGCC

    * -3013                                                                                                    -2914 *
    CTGGGACAAA  GCCTTTGGCT  CCTTGCCCCA  AATCAGAGAA  GAAGGGGCCA  GAGACAGGGT  CTGGAGTGAC  CCCAGATGCA  TGGCACACTC  TCCTCAACTC
R:  CTGGGACAAA  GCCTTTGGCT  CCTTGCCCCA  AATCAGAGAA  GAAGGGGCCA  GAGACAGGGT  CTGGAGTGAC  CCCAGATGCA  TGGNACACTC  TCCTCAACTC

    * -2913                                                                                                    -2814 *
    CCTGGGGCCT  CCTGTCAGGA  CAGGAAAGCA  AAAGCATGCC  CAGCTGTCAG  TCCTGGATTT  TAATGATTAA  CTGGCACTTC  AAACACAGCC  TGTGCCAAAC
R:  CCTGGGGTCT  CCTGTCAGGA  CAGGAAAGCA  AAAGCATGCC  CAGCTGTCAG  TCCTGGATTT  TAATGATTAA  CTGGCACTTC  AAACACAGCC  TGTGCCAAAC

    * -2813                                                                                                    -2714 *
    AGGAGTCTTG  GATTCCACCA  TCTTTTTTCT  TTCTTTCTTT  TCTTTCTTTC  TTTTTTTTTT  TTGATATTTT  TATTTACATT  TCAAATGTTA  TTCCCTTCCC
R:  AGGAGTCTTG  GATTCCACCA  TCTTTTNNCT  TNCTTTCTTT  TCTTTCTTTC  TTNNGGTGGT  TTGATATTTT  TATTTACATT  TCAAATGTTA  TTCCCTTCCC

    * -2713                                                                                                    -2614 *
    CGGTTTCCCT  TTCTATGAGG  ATGTTCCCCC  TCCCCAACCA  CCCCCCATTC  CTGCCTCTCT  CTCTCTCTCT  CTCTCTCTCT  CACACACACA  CACACACACA
R:  CGGTTTCCCT  TTCTATGAGG  ATGTTCCCCC  TCCCCAACCA  CCCCCCATGC  CTGCCTCTCT  CTCTCTCTCT  CTCTCTCTCT  CACACACACA  CACACACACA

    CACACACACA  CACACACACA ... 3'
R:  CACACACACA  CACAC    * -2594

        * -1024                                                                                                -929 *
5' ... TATGAT  CTCCCTGGTG  AGATCGATGC  CCTTGTGATG  GCTGAGGAGA  TCCCCTGTAC  TCAGGTTTCC  ATGTGTTTAT  CCATACGGCT  GGTCAACAAC
R:       -------Primer-----  AGATCGATGC  CCTTGNGATG  GCTGAGGAGA  TCCCCTGTAC  TCAGNTTTCC  ATGTGTTTAT  CCATACGGCT  GGTCAACAAC

    * -928                                                                                                     -829 *
    AATACAAGTT  TATGAGTTAG  TGCCTAGGAA  TGTTCATGGT  TTCATCTCTG  GGTTCTCAGA  GGAACATGTG  CGAGAAACTG  AGAGACCCTC  TAACCTTTAA
R:  AATACAAGTT  TATGAGTTAG  TGCCTAGGAA  TGTTCATGGT  TTCATCTCTG  GGTTCTCAGA  GGAACATGTG  CGAGAAACTG  AGAGACCCTC  TAACCTTTAA

    * -828                                                                                                     -729 *
    CTACCAAAGG  GTATCCCTGT  TTTCCACAGC  CTAGTCCCCA  CCTTCAAGCT  CTTGTTCTGA  GAATACACCT  AAGATTCTCT  TCAATAAAAT  GCAAAGAGAA
R:  CTACCANAGG  GTATCCCTGT  TTTCCACAGC  CTAGTCCCCA  CCTTCAAGCT  CTTGTTCTGA  GAATACACCT  AAGATTCTCT  TCAATAAAAT  GCAAAGAGAA

    * -728                                                                                        -639 *
    AGGCATTGTA  CTGTTGGCAA  TCAACCAATC  TCTTCTCTAA  TGATGAGCCA  ACAATGTGAA  TGGACTAATG  ATTGATCAGG  AAAAAATAGA ... 3'
R:  AGGCATTGTA  CTGTTGGCAA  TCAACCAATC  TCTTCTCTAA  TGATGAGCCA  ACAATGTGAA  TGGACTAATG  ATNGATCAGG  AAAAAATAGA
```

Panel B

Fig.4-5 (continued)

gave reasonably high expression. Thus a mass ratio of lipofectin : DNA of 6 :1 was chosen for these preparations and no morphological toxicity was observed during the experimental period. For pGL-SV40 (pGL2-promoter, purchased from Promega) and pGL-Reg5, no peaks appeared on the curve, which meant that their optimal expressions were beyond the testing range. Considering the other plasmid samples, we chose 2 μg of each for the remaining experiments.

A linearity test was always carried out using purified luciferase protein in each experiment to determine if light units produced fell within the proportional range in the luciferase assay.

Expression of Chimeric genes

To test the expressions of the chimeric genes containing L-PK promoter sequences, the plasmid constructs were introduced into primary hepatocytes. After 18- to 24-h incubation in SUM medium without antibiotics, the transfected cells were washed twice and cultured in KRB medium, and the experimental compounds were added as indicated.

The expressions under desired conditions last 16-18 hours and then the cell extracts were assayed for luciferase activity by measuring the light units produced after addition of substrates of luciferin and ATP. The negative control was pGL2-Basic plasmid vector itself, which is promoterless and enhancerless. The positive control was pGL-SV40 (Promega) (Fig. 4-2) which has SV40 early promoter upstream of the luciferase reporter gene. The quality or internal control was pGL-Reg5, which allowed us to compare the current results to previous results in our laboratory.

Since a large number of plates were needed due to seven constructs and three control plasmids all in triplicate, we were unable to test all plasmids in one experiment. Table 4-1 showed two individual experiments data from triplicate samples and the average activities of replicate experiments are summarized in Fig. 4-8. The magnitude of induction

Fig. 4-6

Luciferase activities of pGL-Reg5 and pGL-SV40 in different transfection protocols and different mass ratios of DNA : lipofectin. In Protocol 1, the cultured cells were transfected with pGL-Reg5 and pGL-SV40 after 4-h cell attachment phase. In Protocol 2, the cultured cells were transfected with pGL-Reg5 and pGL-SV40 after 4-h cell attachment phase and a 18- to 24-h additional incubation in Waymouth's medium. All transfections were carried out at constant amount of 12 μg lipofectin with indicated amount of DNA. The luciferase activities were determined by light units produced in 60 second.

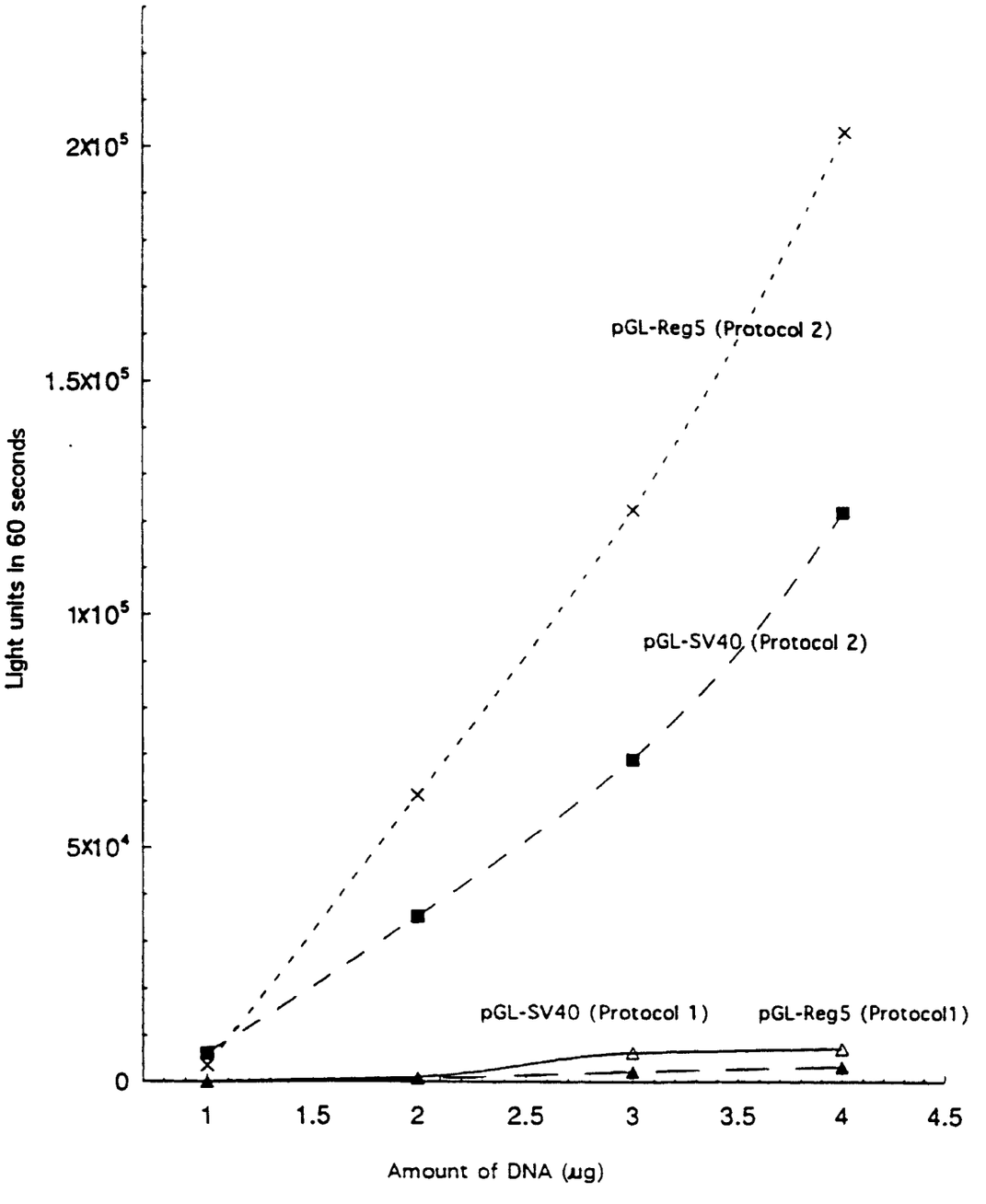

2X10^5
1.5X10^5
1X10^5
5X10^4
0
Light units in 60 seconds
pGL-Reg5 (Protocol 2)
pGL-SV40 (Protocol 2)
pGL-SV40 (Protocol 1)
pGL-Reg5 (Protocol1)
1
1.5
2
2.5
3
3.5
4
4.5
Amount of DNA (µg)

Fig. 4-6

Fig. 4-7

Luciferase activities of pGL-F, pGL-H, pGL-J and pGL-K under different mass ratios of DNA : lipofectin in Protocol 2. All transfections were carried out at constant amount of 12 μg lipofectin with indicated amount of DNA. The luciferase activities were determined by light units produced in 60 seconds.

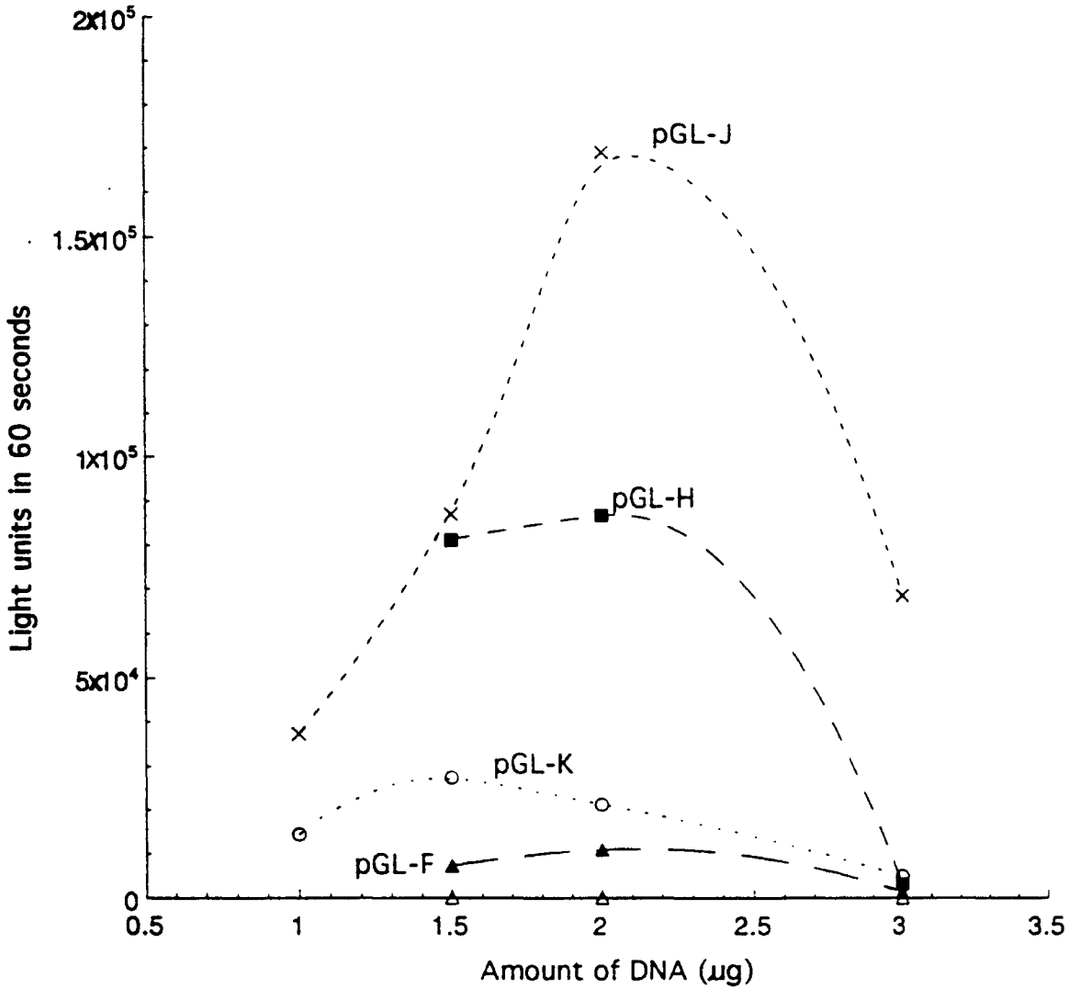

2x10^5
1.5x10^5
1x10^5
5x10^4
0
Light units in 60 seconds
pGL-J
pGL-H
pGL-K
pGL-F
0.5
1
1.5
2
2.5
3
3.5
Amount of DNA (µg)

Fig. 4-7

Table 4-1

Modulation of L-type pyruvate kinase gene expression in isolated hepatocytes by glucose, vanadate and insulin (luciferase assay) D represent mean ± S.E. (light units x 10^{-3}) of two separate experiments in triplicate. Numbers with the same letter are not significantly different. P-values for all experiments presented were < 0.01 except pGL-K which was 0.034. Numbers in parentheses represent percentage expression relative to each plasmid under condition of 5 mM glucose. S.E., standard error.

Table 4-1

Modulation of L-type pyruvate kinase gene expression in isolated rat hepatocytes by glucose, vanadate and insulin (luciferase assay) (LU x 10^{-3})

Plasmid	5 mM Glucose	20 mM Glucose	5 mM Glucose 10^{-7} M Insulin	5 mM Glucose 1.5 μM Vanadate
	(Experiment 1):			
pGL2-Basic	0.03±0.00	1.16±0.11	------	------
pGL-SV40	22.9^{c}±1.28 (100)	46.6^{a}±3.00 (203)	33.7^{b}±3.04 (147)	26.5^{bc}±0.30 (115)
pGL-E	0.40^{b}±0.06 (100)	0.82^{a}±0.06 (204)	0.48^{b}±0.04 (120)	0.35^{b}±0.03 (88)
pGL-F	19.7^{bc}±1.35 (100)	43.7^{a}±1.09 (147)	33.0^{b}±3.0 (111)	25.2^{c}±0.88 (85)
pGL-H	288^{b}±10.2 (100)	485^{a}±5.24 (169)	299^{b}±14.4 (104)	236^{c}±7.0 (82)
pGL-Reg5	20.4^{b}±1.31 (100)	51.3^{a}±3.37 (251)	25.0^{b}±1.91 (122)	25.2^{b}±0.94 (123)
	(Experiment 2):			
pGL2-Basic	0.00±0.00	0.00±0.00	------	------
pGL-SV40	0.31^{b}±0.14 (100)	1.56^{a}±0.23 (506)	0.58^{b}±0.07 (186)	0.20^{b}±0.07 (64)
pGL-G	13.6^{b}±0.69 (100)	24.3^{a}±1.31 (179)	15.4^{b}±2.52 (113)	12.8^{b}±0.19 (94)
pGL-I	28.3^{b}±0.69 (100)	46.1^{a}±1.96 (163)	31.1^{b}±1.84 (110)	20.6^{c}±3.67 (73)
pGL-J	29.8^{b}±2.41 (100)	58.0^{a}±3.78 (194)	21.3^{b}±1.16 (71)	27.4^{b}±3.47 (92)
pGL-K	6.20^{c}±0.69 (100)	10.9^{a}±0.51 (176)	10.7^{ab}±1.58 (172)	7.27^{bc}±1.23 (117)
pGL-R	91.4^{b}±9.51 (100)	190^{a}±10.8 (207)	88.0^{b}±9.24 (96)	82.9^{b}±15.9 (91)
pGL-Reg5	2.58^{b}±0.11 (100)	5.35^{a}±1.40 (208)	1.15^{b}±0.32 (45)	2.50^{b}±0.34 (97)

varied between hepatocyte preparations. All data were statistically subjected to "Analysis of Variance" on SAS/STAT™ program (SAS Institute Inc. Cary, NC, USA; 1985, Version 6).

From Table 4-1, the expression of pGL-E was very low and we did not repeat pGL-E any more. The other constructs all expressed very well and responded to glucose. These results agreed with the work of Towle *et al* (73). The data from Table 4-1 and Fig.4-8 suggest that although pGL-K contains all necessary elements for expression, its activity was the lowest perhaps suggesting the presence of a silencer element.

The luciferase activities from the constructs pGL-F, pGL-G, pGL-H, pGL-I, pGL-J, pGL-R and pGL-K increased markedly when concentrations of glucose were elevated from 5 mM to 20 mM. Expressions could be observed under only glucose alone without insulin or insulin-mimic reagent vanadate, which did not agree with Decaux (69) and Munnich (71) observations who showed glucose and insulin together needed for increased mRNA levels in pyruvate kinase in culture rat liver cells.

Statistical data indicated that there were no physiologically different effects from insulin and vanadate in this research.

Treatment with 10^{-7} M insulin and 5 mM glucose, or with 1.5 μM vanadate and 5 mM glucose did not significantly stimulate the gene expressions, which was not consistent with Mr.Chris Otiko's results (2) who got marked effects of vanadate or insulin on pGL-Reg5 expression. To test if the effects of insulin and vanadate on the gene expression are protocol-dependent, the gene expression of pGL-Reg5 was caried out in Mr. Christ Otiko's protocol (2) and the results are shown in Fig. 4-9. The results were obtained from one experiment in triplicate samples, which suggest that the expression of pGL-Reg5 in response to glucose, insulin and vanadate was influenced by manipulation of cell culture conditions.

Fig. 4-8

The effects of glucose, insulin and vanadate on gene expression of L-type pyruvate kinase. The rat hepatocytes were transfected with control plasmid pGL-SV40 and chimeric gene pGL-F through pGL-Reg5 which contained segments of the L-PK promoter deletion regions, respectively. These transfected cells were expressed under indicated conditions in KRB medium. The expression activities were measured by light units produced in 60 seconds. The results are presented as a percentage relative to their corresponding construct under control condition of 5 mM glucose. These data were determined from averaging the results of at least two experiments with triplicate samples. The bars with * indicate the data significantly different from their relevant control at 5 mM glucose (statistical analysis was carried out individually in each single experiment). Numbers in parentheses represent the numbers of repeated experiments.

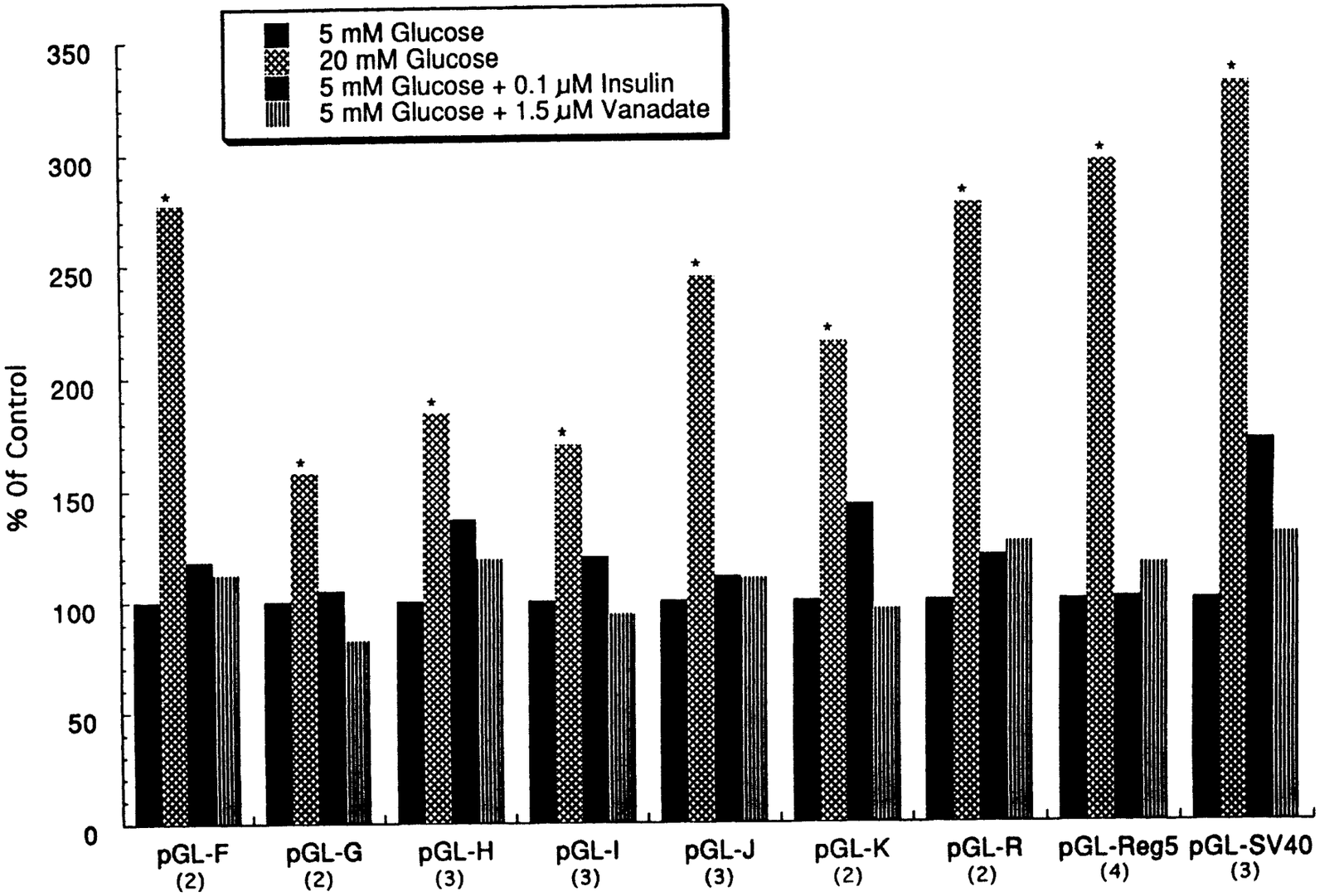

5 mM Glucose
20 mM Glucose
5 mM Glucose + 0.1 μM Insulin
5 mM Glucose + 1.5 μM Vanadate
% Of Control
350
300
250
200
150
100
50
0
pGL-F (2)
pGL-G (2)
pGL-H (3)
pGL-I (3)
pGL-J (3)
pGL-K (2)
pGL-R (2)
pGL-Reg5 (4)
pGL-SV40 (3)

Fig. 4-8

Fig. 4-9

The transfection and expression of chimeric gene in different protocols. The transfection and expression of pGL-Reg5 were carried out by using different protocols. The data of pGL-Reg5 with standard error bars in the figure represent the results obtained from the present protocol, in which the transfection was carried out under protocol 2 described in the text, and the transfected cells were expressed under indicated conditions in KRB medium, by duplicate samples. The data of pGL-Reg5* with standard error bars in the figure represent the results obtained from Chris Otiko's protocol, in which the transfection of pGL-Reg5 was carried out under protocol 1 described in the text, and the transfected cells were expressed under indicated conditions in SUM medium, by triplicate samples. The expression activities were measured by light units produced in 60 seconds. The results are presented as a percentage of the corresponding construct under control condition of 5 mM glucose and none respectively. These data were determined from a single experiment. ⊤, standard error bar. The bars with * are significantly different from their corresponding controls.

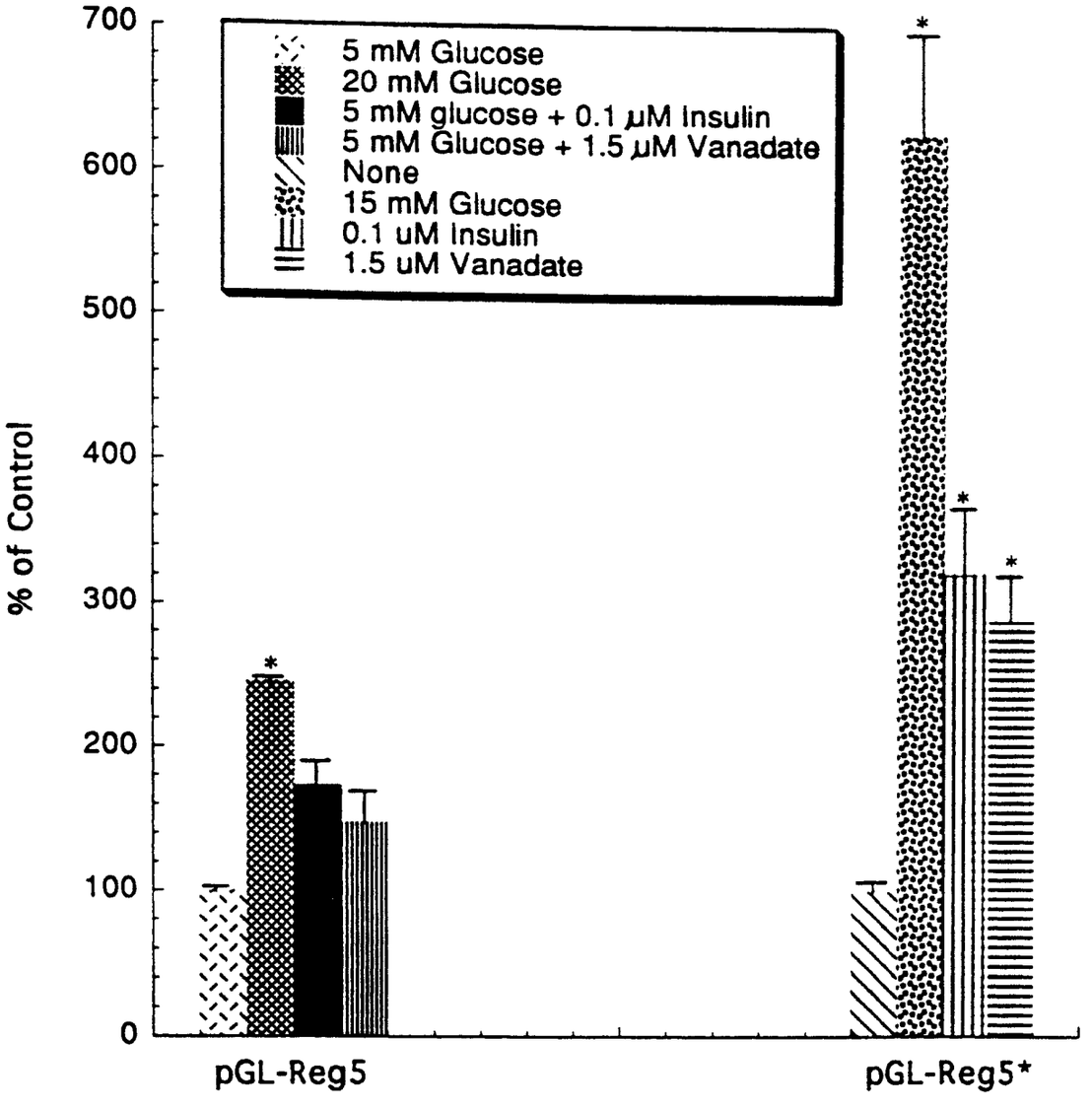

700
600
500
400
300
200
100
0
% of Control
5 mM Glucose
20 mM Glucose
5 mM glucose + 0.1 μM Insulin
5 mM Glucose + 1.5 μM Vanadate
None
15 mM Glucose
0.1 uM Insulin
1.5 uM Vanadate
*
*
*
*
pGL-Reg5
pGL-Reg5*

Fig. 4-9

Discussion

Lipofectin-mediated DNA transfection is a simple and effective technique for studying gene expression in a variety of cell lines. This method would facilitate studies to directly compare the relative strengths of different promoters. It was reported to be from 5- to 100-fold more effective than either the calcium phosphate or the DEAE-dextran technique (76, 77). Constructs in which albumin, PEPCK and L-PK promoters were linked to a CAT gene all can be expressed after lipofection into primary hepatocytes, and the PEPCK promoter exhibits appropriate hormonal control (73, 78).This indicates that liver-specific transcription is not disrupted by this method. However, a ratio of lipofectin : DNA is very important in this technique. Optimization of the ratio is necessary for every batch preparation and individual samples. In this research, although the purification method for pGL-E through pGL-K was changed from PEG precipitation to CsCl-ethidium bromide gradient binding, these chimeric genes did not express any more at the ratio of 4 :1 at which pGL-Reg5 expressed very well in Mr. Chris Otiko's studies (2), but the same samples of pGL-E to pGL-K highly expressed at the mass ratio of lipofectin : DNA of about 6 : 1 that is closed to 6.67 : 1 reported by Katherine (78) and Jacoby (79).

Lipofectin is a liposome comprised of a positively charged lipid. The postulated mechanism of action for the liposomes is that sonicated lipid vesicles in water form spontaneously DNA-lipofectin complexes by interaction between positive and negative charge after mixing an aliquot of the reagent with an aqueous solution of DNA, trapping nearly all of the polynucleotide in the complex interior (80) and then these complexes fused to cell membrane and delivered the polynucleotides into the cells. If the ratio of liposome : polynucleotide is not appropriate, it might affect the liposomes, completely trapping DNA (if less) or blocked the wrapped DNA delivery (if more). This would bring about large variability and even result in no expression ultimately.

Since complexes of DNA-lipofectin are formed by charge-charge interaction, some chemicals involved in DNA preparations may interfere with transfection. The most known interfering compound is EDTA. The chimeric genes purified by PEG definitely did not express even though these samples behaved very well in agarose gel electrophoresis, restriction digestion, sequencing and etc. perhaps some impurity existied in the chemicals . Of course we should have optimized the ratio for this batch preparation to confirm if no expression from this preparation resulted from the inappropriate ratio of 4:1 or PEG interference. At the same conditions, pGL-R expressed always higher than pGL-Reg5 possibly due to the reason that pGL-Reg5 was not in optimal ratio which are indicated in Fig.4-6. The same was true for pGL-SV40. This might be why they have relatively large standard errors (data not shown).

Luciferase as a reporter gene is sensitive, rapid and quantitative. It does not require the use of radioactivity and allows many samples to be assayed in short time period. However, variability likely arose in luciferase assay since luciferase and its substrates may not be mixed well in a very small assay volume and very short assay period. It is reported that (78) only a total of 5-10 % of the cells can be transfected, then the cells in the dish seems to be excess. Therefore, the cell numbers in each culture plate were not critical for the experiments. In fact, the expression units were not proportional to total number of cells in the plate. Lower number of cells in the plate could also gave higher light units when other conditions were the same in one experiment (data not shown). Of course, some unknown and random factors can cause variability, for instance, if the transfected cells were lost during several washes, these culture plates would give lower expression units.

As mentioned above, the data variability was mainly due to three things. First, the mass ratio of lipofectin : DNA strongly affects transfection efficiency, but careful optimizing the ratio would not be practical for multiple samples since it is time-wasting and costly job. Second, luciferase assay may cause variations in light units produced by assay

mixture. Finally, some unknown factors randomly influence the reproducability. These limited us to parallely compare the expression strength of the regulatory region(s) to carbohydrate, insulin and vanadate simultaneously. Of course, the standard errors from each triplicate samples were under reasonable range (~ 10 % of the mean)

We chose adult rat hepatocytes in primary culture rather than hepatoma cell lines for the studies because rat hepatocytes conserve a correct tissue-specific expression and hormonal response (69). Why could we not repeat out Chris Otiko's results (2), in which insulin and vanadate seemed to more directly stimulate pGL-Reg5 expression rather than a "permissive" effect, with pGL-R and even pGL-Reg5 itself. One of the possible explanations is that the insulin receptors on the cell surface were damaged during collagenase perfusions. We did find that the collagenase from different lot numbers greatly affected cell viability and morphology. In Fig.4-9, the gene expression of pGL-Reg5 was markedly stimulated by vanadate and insulin, which suggests that the effects of vanadate and insulin on the gene expression of pGL-Reg5 or other L-PK promoter constructs might be protocol- or medium- dependent although I have not enough data to confirm this. Because in Chris Otiko's procedure (2), transfections were carried out after 4-hour cell attachment period when high concentration (10^{-7} M) of insulin was maintained, and the transfected cells were expressed in SUM medium under indicated conditions, whereas we performed transfections after 4-hour cell attachment phase when the cells were under 10^{-9} M insulin. and a 24-h additional incubation in Waymouth's medium, and the expression of luciferase activities was going on in KRB medium. However, with careful analysis of the compositions of SUM medium which was used for gene expression by Chris Otiko, I found that SUM medium contains high basal level of glucose at about 10 mM, the increased expression of pGL-Reg5 stimulated by vanadate and insulin alone might come from the high basal level of glucose in the medium. In addition, the concentrations of insulin and vanadate might not be high enough to significantly stimulatie these gene

expression during my experiments because Bosch *et al* (43) claimed that the inhibitory effect of vanadate was observed at concentrations of 0.5 mM or greater, the concentration approximates the minimal levels of vanadate which activate the tyrosine kinase of the insulin receptor. Based on our present results, it is hard to tell if the effects of vanadate and insulin on the gene expression are protocol-dependent or that vanadate and insulin do not directly affect the gene expression.

Although pGL-K contains all necessary elements for expression, its expressions were lowest. It was possible that in reverse direction, the TATA box in the longest promoter sequence was farthest from the report gene, which had the weakest actions on the transcription of downstream luciferase. On the other hand, sequence at position -2338 to -2328 is a cAMP regulatory element found in the phosphenolpyruvate carboxykinase gene promoter region. Dexamethasone in SUM medium might antagonize stimulation of glucose since Dexamethasone was reported to regulate gene expression perhaps via secondary signal cAMP (81).

Table 4-1 showed that the activities of pGL-I and pGL-J were higher than pGL- G. It could be explained that both pGL-I and pGL-J contain all three liver-specific expression elements (Fig. 4-1) but pGL-G has only one of them, and these elements had synergistic effect (67).

Surprisingly, the expressions of the positive control plasmid pGL-SV40 were apparently stimulated by glucose and also slightly ncreased under addition of insulin or vanadate in a similar way to the others. This suggests that glucose might universally influence transcription of a certain kind of genes, or there might not be carbohydrate response elements in specific region of L-PK promoter. Alternatively, glucose responses may be present in the SV40 promoter.

CHAPTER V

SUMMARY AND CONCLUSION

1. The chimeric genes of pGL-F, pGL-G, pGL-H, pGL-I, pGL-J pGL-R and pGL-K were highly expressed under our experimental conditions. They well responded to glucose although their promoters were inserted in the reversed direction. This indicated that the carbohydrate regulatory elements in pyruvate kinase promoter are independent of orientation.

2. Insulin and vanadate neither significantly stimulated the gene expressions nor apparently exhibited discrete effects on those chimeric genes based on this research. The stimulation of vanadate and insulin on the expression of these chimeric genes might be protocol-dependent. That vanadate and insulin directly affect the gene expression or that they only play a "permissive role" in glucose for the gene expression remains unknown.

3. The variability of lipofectin transfection limits its use on quantitative comparison of expression strength from multiple gene constructs.

4 The positive control plasmid of pGL-SV40 responded very well to glucose. The fashion of the response to glucose, insulin and vanadate was similar to that of the other chimeric genes, which suggests that either there might not be carbohydrate-specific element(s) (CRE) in the L-PK promoter or SV40 promoter contains the CRE.

5. We need to reverse the promoter direction in each deletion construct and further locate the regulatory regions responded to insulin, vanadate and glucagon in future.

REFERENCES

1. Shechter, Y.; Amir, S. and Meyerovitch, J. The insulin-like metabolic effects of vanadate: possible implications to the future care of diabetes. Diab. Nutr. Metab. **1**: 1-4; 1988.
2. Otiko, C. Vanadate regulation of carbohydrate metabolism and modulation of rat liver pyruvate kinase gene expression (Thesis); 1993.
3. Jandhyala, B. S. and Hom, G. J. Physiological and pharmacological properties of vanadium. Life Science **33**: 1325-1340; 1983.
4. Macara, I. G. Vanadium - an element in search of a role. TIBS April: 92-94; 1980.
5. Tolman, E. L.; Barris, E.; Burns, M.; Pansini, A. and Partridge, R. Effects of vanadate on glucose metabolism *in vitro*. Life Science **25**: 1159-1164; 1979.
6. Hopkins Jr., L.L. and Mohr, H. E. Vanadium as an essential nutrient. Fed. Proc. **33**: 1773-1775; 1974.
7. Nielsen, F. H. and Ollerich, D. A. Studies on a vanadium deficiency in chicks. Fed. Proc. **32**: 927; 1973.
8. Erdmann,E.; Werdan, K.; Krawietz, W.; Schmitz, W. and Scholz, H. Vanadate and its significance in biochemistry and pharmacology. Biochemical Pharmacology **33** (7): 945-950; 1984.
9. Cantley Jr., L. C.; Josephson, L.; Warner, R.; Yanagisawa, M.; Lechene, C. and Guidotti, G. Vanadate is a potent (Na,K)-ATPase inhibitor found in ATP derived from muscle. J. Biol. Chem. **252**: 7421-7423; 1977.
10. Josephson, L. and Cantley Jr., L. C. Isolation of a potent (Na-K)ATPase inhibition from striated muscle. Biochemistry **16**: 4572-4578; 1977.

11. Lopez, V.; Stevens, T. and Lindquist, R. N. Vanadium ion inhibition of alkaline phosphatase-catalyzed phosphate ester hydrolysis. Archs. Biochem. Biophys. **175**: 31; 1976.

12. Choata, G. L. and Mansour, T. E. Inhibition of sheep heart phosphofructokinose by orthovanadate. Fed. Proc. **37**: 1433; 1978.

13. DeMaster, E.G. and Mitchell, R. A. A comparison of arsenate and vanadate as inhibitors or uncouples of mitochondrial and glycolytic energy metabolism. Biochemistry **12**: 3616; 1973.

14. Voet, D. and Voet, J. G. Biochemistry. New York: John Wiley & Sons, Inc. 1990.

15. Cantley Jr., L. C.; Resch, M. D. and Guidotti, G. Vanadate inhibits the red cell (Na+,K+)ATPase from the cytoplasmid side. Nature, Lond. **272**: 552-554; 1978.

16. Hansen, O. Facilitation of ouabain binding to (Na^+-K^+)ATPase by vanadate at *in vivo* concentions. Biochim. Biophys. Acta. **568**: 265-269; 1979.

17. Cantley Jr., L.C.; Cantley, L.G. and Joseph, L. A characterization of vanadate interactions with the (Na,K)-ATPase. J. Biol. Chem. **253**: 7361-7368; 1978.

18. Bond, G. H. and Hudgins, M. P. Inhibition of red cell Ca^{2+}-ATPase by vanadate. Biochim. Biophys. Acta. **600**: 781-790; 1980.

19. Baraabin, H.; Garrahan, P. J. and Rega, A. F. Vanadate inhibition of the Ca^{2+}-ATPase from human red cell membranes. Biochim. Biophys. Acta. **600**: 796-804; 1980.

20. Goodno, C. C. Inhibition of myosin ATPase by vanadate ion. Pron. Natl. Acad. Sci. U.S.A. **76**: 2620-2624; 1979.

21. Cantley, L. C. and Aisen, P. The fate of cytoplasmic vanadium. J. Biol. Chem. **254**: 1781-1784; 1979.

22. Grantham, J. J. The renal sodium pump and vanadate. Am. J. Physiol. **239**: F97-F106; 1980.

23. Pai, E. F.; Sachsenheimer, W.; Schimer, R. H. and Schalz, G. E. Substrate positions and induced-fit in crystalline adenylate. J. Mol. Biol. **114**: 37-45; 1977.

24. Schwabe, U.; Puchstein, C.; Hannemann, H. and Söchtig, E. Activation of adenylate cyclase by vanadate. Nature (London) **277**: 143-145; 1979.

25. Rodríguez-Gil, J. E.; Gómez-Foix, A. M.; Fillat, C.; Bosch, F. and Guinovart, J.J. Activation by vanadate of glycolysis in hepatocytes from diabetic rats. Diabetes **40**: 1355-1359; 1991.

26. Meeks, M. J.; Landolt, R. R.; Kessler, W. V. and Born, G. S. Effect of vanadium on metabolism of glucose in the rats. J. Pharmaceutical Sci. **60**: 482-483; 1971.

27. Ramanadham, S.; Mongold, J. J.; Browsey, R. W.; Cros,G.H. and McNeill, J. H. Oral vanadyl sulfate in the treatment of diabetes mellitus in the rat. Am. J. Physiol. **257**: H904-H911; 1989.

28. Challiss, R.J.; Leighton, B.; Lozeman, F. J.; Budchoski, L. and Newsholme, E. A. Effects of chronic administration of vanadate to the rat on the sensitivity of glycolysis and glycogen synthesis in skeletal muscle to insulin. Biochemical Pharmacology **36** (3): 357-361; 1987.

29. Bernier, M.; Lard, D. and Lane, M. D. Effect of vanadate on the cellular accumulation of pp15, an apparent product of insulin receptor tyrosine kinase action. J. Biol. Chem. **263**: 13626-13634; 1988.

30. Bollen, M.; Miralpeix, M.; Ventura, F.; Toth, B.; Bartrons, R. and Stalmans, W. Oral adminstration of vanadate to streptozotocin-diabetic rats restores the glucose-induced activation of liver glycogen synthase. Biochem. J. **267**: 269-271; 1990.

31. Blondel, O.; Simon, J.; Chevalier, B. and Portha, B. Impaired insulin action but normal insulin receptor activity in diabetic rat liver: effect of vanadate. Am. J. Physiol. **258**: E459-E467; 1990.

32. Kadota, S.; Fantus, I. G.; Hersh, B. and Posner, B.I. Vanadate stimulation of IGF binding to rat adipocytes. Biochem. Biophys. Res. Commun. **138**: 174-178; 1986.

33. PoussUgur, J.; Chambard, J. C.; Franchi, A.; Paris, S. and Van Obberghen-schilling, E. Growth factor activation of an amiloride-sensitive Na^+/H^+ exchange system in quiescent fibroblasts: coupling to ribosomal protein S6 Phosphorylation. Proc. Natl. Acad. Sci. U.S.A. **79**: 3935-3939; 1982.

34. Gómez-Foix, A. M.; Rodriguez-Gil, J. E.; Fillat, C.; Guinovart, J. J. and Bosch, F. Vanadate raises fructose 2,6-bisphosphate concentrations and activates glycolysis in rat hepatocytes. Biochem. J. **255**: 507-512; 1988.

35. Villar-Palasi, C.; Guinovart, J. J.; Gómez-Foix, A. M.; Rodriguez-Gil, J. E. and Bosch, F. Effects of vanadate on protein kinases in rat hepatocytes. Biochem. J. **262**: 563-567; 1989.

36. Dubyak, G.R. and Kleinzeller, A. The insulin mimetic effects of vanadate in isolated rat adipocytes. Biochemistry **20**: 5795-5799; 1980.

37. Kasuga, M.; Zick, Y.; Blithe, D. L.; Crettaz, M. and Kahn, C. R. Insulin stimulates tyrosine phosphorylation of the insulin receptor in a cell-free system. Nature **298**: 667-669; 1982.

38. Rosen, O. M.; Herrera, R.; Olowe, Y.; Petruzzelli, L. M. and Cobb, M. H. Phosphorylation activates the insulin receptor tyrosine protein kinase. Proc. Natl. Acad. Sci. U.S.A. **80**: 3237-3240; 1983.

39. Rosen, O. M. After insulin binds. Science Wash. DC **237**: 1452-1458; 1987.

40. Van Obberghen, E. and Gammeltoft, S. Insulin receptor: structure and function. Experientia Basel **42**: 727-734; 1986.

41. Tamura, S.; Brown, T. A.; Dubler, R. E. and Larner, J. Insulin-like effectof vanadate on adipocyte glycogen synthase and on phosphorylation of 95,000 datton subunit of insulin receptor. Biochem. Biophys. Res. Commun. **113**: 80-86; 1983.

42. Tamura, S.; Brown, T.A.; Whipple, J. H.; Fujita-Yamagnchi, Y.; Dubler, D. E.; Chegn, K. and Larner, J. A novel mechanism for the insulin-like effect of vanadate on glycogen synthase in rat adipocytes. J. Biol. Chem. **259**: 6650-6658;1984.

43. Bosch, F.; Hatzoglou, M.; Park, E. A and Hanson, R. W. Vanadate inhibits expression of the gene for phosphoenolpyruvate carboxykinase (GTP) in rat hepatoma cells. J. Biol. Chem. **265**: 13677-13682; 1990.

44. Miralpeix, M.; Decaux, J-F.; Kahn, A. and Bartrons, R. Vanadate induction of L-type pyruvate kinase mRNA in adult rat hepatocytes in primary culture. Diabetes **40**: 462-464; 1991.

45. Wynshaw-Boris, A.; Short, J.; Loose, D. and Hanson, R. W. Characterization of the phosphoenolpyruvate carboxykinase (GTP) promoter-regulatory region. J. Biol. Chem. **261**: 9714- 0729; 1986.

46. Bosch, F.; Arivo, J.; Gómez-Foix, A. M. and Guinovart, J. J.: Glycogenolytic, noninsulin-like effects of vanadate on rat hepatocyte glycogen synthase and phosphorylase. J. Biol. Chem. **262**: 218-222; 1987.

47. Rodriguez-Gil, J. E.; J.; Gómez-Foix, A. M.; Arivo, J.; Guinovart, J. J and Bosch, F. Control of glycogen synthase and phosphorylase in hepatocytes from diabetic rats: effects of glucagon, vasopressin, and vanadate. Diabetes **38**: 793-798; 1989.

48. Green, A. The insulin-like effect of sodium vanadate on adipocyte glucose transport is mediated at a post-insulin-receptor level. Biochem. J. **238**: 663-669; 1986.

49. Mooney, R. A.; Bordwell, K. L.; Luhowskyi, S. and Casnellie, J. E. The insulin-like effect of vanadate on lipolysis in rat adipocytes is not accompanied by an

insulin- like effect on tyrosine phosphorylation. Endocrinology **124**: 422-429; 1989.

50. Bosch, F.; Ariño, J.; Gómez-Foix, A. M. and Guinovart, J. Glycogenolytic, noninsulin-like effects of vanadate on rat hepatocyte glycogen synthase and phosphorylase. J. Biol. Chem. **262**: 218-222; 1987.

51. Blair, J. B.; Cimbala, M. A.; Foster, J. L. and Morgan, R. A. Hepatic pyruvate kinase: regulation by glucagon, cyclic adenosine 3':5'-monophosphate, and insulin in the perfused rat liver. J. Biol. Chem. **251**: 3756-3762; 1976..

52. Haradn, K.; Saheki, S.; Wada, K. and Tanaka, T. Purification of four pyruvate kinase isozymes of rats by affinity elution chromatography. Biochim. Biophys. Acta. **524**: 327-339; 1978.

53. Noguchi, T.; Yamada, K.; Inoue, H. Matsuda, T. and Tanaka, T. The L- and R-type isozymes of rat pyruvate kinase are produced from a single gene by use of different promoters. J. Biol. Chem. **262**: 14366-14371; 1987.

54. Imamura, K. and Tanaka, T. Multimolecular forms of pyruvate kinase from rat and other mammalian tissues. J. Biochem. (Tokyo) **71**: 1043-1051; 1972.

55. Imamura, K.; Tanaka, T.; Nishina, T.; Nakashima, K. and Mima, S. Studies on pyruvate kinase deficiency. J. Biochem. (Tokyo) **74**: 1165-1175; 1973.

56. Noguchi, T.; Inoue, H. and Tanaka, T. The M_1- and M_2-type isozymes of rat pyruvate kinase are produced from the same gene by alternative RNA splicing. J. Biol. Chem. **261**: 13807-13812; 1986.

57. McDevitt, M. A.; Imperiale, M. J.; Ali, H. and Nevins, J.R. Requirement of a downstream sequence for generation of a poly(A) addition site. Cell **37**: 993-999; 1984.

58. Cognet, M.; Yu, C. L.; Vaulont, S.; Kahn, A. and Marie, J. Structure of the rat L-type pyruvate kinase gene. J. Mol. Biol. **196**: 11-25; 1987.

59. Scheidereit, C.; Geisse, S.; Westphal, H. M. and Beato, M. The glucocorticoid receptor binds to defined nucleotide sequences near the promoter of mouse mammary tumour virus. Nature (London) **304**: 749; 1983.

60. Short, J. M.; Wynshaw-Boris, A.; Short, H. P. and Hanson, R. W. Characterization of the phosphoenolpyruvate carboxykinase (GTP) promoter-regulatory region (II). J. Biol. Chem. **261**: 9721-9726; 1986.

61. Dynan, W. S. and Tijan, R. Control of eukaryotic messenger RNA synthesis by sequence-specific DNA-binding proteins. Nature **316**: 774-778; 1985.

62. Maniatis, T.; Goodburn, S. and Fischer, J. A. Regulation of inducible and tissue-specific gene expression. Science **236**: 1237-1244; 1987.

63. Edlund, T.; Walken, M.M.; Barr, P. J. and Rutter, W. J. Cell-specific expression of the rat insulin gene: evidence for role of two distinct 5'flanking elements. Science **230**: 912-916; 1985.

64. Veres, G.; Craigen, W. J. and Caskey, C. T. The 5'-flanking region of the ornithine transcarbamylasegene contains DNA sequences regulating tissue-specific expression. J. Biol. Chem. **261**: 7588-7591; 1986.

65. Widen, S. G. and Papaconstantinou, J. Liver-specific expression of the mouse α-fetoprotein gene is mediated by cis-acting DNA element. Proc. Natl.Sci. (USA) **83**: 8196-8200; 1986.

66. Casanova, J.; Copp, R. P.; Janocko, L. and Samuels, H.H. 5'-flanking DNA of the rat growth hotmone gene mediates regulated expression by thyroio hormone. J. Biol. Chem. **260**: 11744-11748; 1985.

67. Yamada, K.; Noguchi, T.; Matsuda, T.; Takenaka, M.; Monaci, P.; Nicosia, A. and Tanaka, T. Identification and characterization of hepatocyte-specific regulatory regions of the rat pyruvate kinase L gene. J. Biol. Chem. **265**: 19885-19891; 1990.

68. Vaulont, S.; Puzenat, N.; Levrat, F.; Cognet, M.; Kahn, A. and Raymondjean, M. Proteins binding to the liver-specific pyruvate kinase gene promoter. J. Mol. Biol. **209**: 205-219; 1989.

69. Decaux, J-F.; Antoine, B. and Kahn, A. Regulation of the expression of the L-type pyruvate kinase gene in adult rat hepatocytes in primary culture. J. Biol. Chem. **264**: 11584-11590; 1989.

70. Vaulont, S.; Munnich, A.; Decaux, J-F. and Kahn, A. Transcription and post-transcriptional regulation of L-type pyruvate kinase gene expression in rat liver. J. Biol. Chem. **261**: 7621-7625; 1986.

71. Munnich, A.; Marie, J.; Reach, G.; Vaulont, S.; Simon, M-P. and Kahn, A. *In vivo* hormonal control of L-type pyruvate kinase gene expression. J. Biol. Chem. **259**: 10228-10231; 1984.

72. Decaux, J-F.; Marcillat, O.; Pichard, A. L.; Henry, J. and Kahn, A. Clucose -dependent and -independent effect of insulin on gene expression. J. Biol. Chem. **266**: 3432-3438; 1991.

73. Thompson, K. S. and Towle, H. C. Localization of the carbohydrate response element of the rat L-type pyruvate kinase gene. J. Biol. Chem. **266**: 8679-8682; 1991.

74. GeneLight™ Plasmids Technical Manual. **Promega**.

75. Current Protocol in Molecular Biology (Ausubel, F. M.; Brent, R. et al eds). Vol. I: pp.1.7.5-1.7.6; Greene Publishing Associate, Inc. and John Wiley & Sons, Inc. (1994).

76. Felgner, P. L.; Gadek, T. R.; Holm, M.; Roman, R.; Chan, H. W.; Wenz, M.; Northrop, J. P.; Ringold, G. M. and Danielsen, M. Lipofection: A highly efficient, lipid-mediated DNA-transfection procedure. Proc. Natl. Acad. Sci. (USA) **84**: 7413-7417; 1987.

77. Innes, C. L.; Smith, P. B.; Langenbach, R.; Tindall, K. R. and Boone, L. R. Cationic liposomes (lipofectin) mediate retroviral infection in the absence of specific receptors. J. ?? of Virology Feb. 957-961; 1990.

78. Ponder, K. P.; Dunbar, R. P.; Wilson, D. R.; Darlington, G. J. and Woo, S. L. C. Evaluation of relative promoter strength in primary hepatocytes using optimized lipofection. Human Gene Therapy **2**: 41-52; 1991.

79. Jacoby, D. B.; Zilz, N. D. and Towle, H. C. Sequences within the 5'-flanking region of the S_{14} gene confer responsiveness to glucose in primary hepatocytes. J. Biol. Chem. **264**: 17623-17626; 1989.

80. Felgner, P. L. and Ringold, G. M. Cationic liposome-mediated transfection. Nature **337**: 387-388; 1989.

81. Magnuson, M. A.; Quinn, P. G. and Granner, D. K. Multihormonal regulation of phosphoenlpyruvate carboxykinase-chloramphenicol acetyltransferase fusion genes. J. Biol. Chem. **262**: 14917-14920; 1987.

VITA

Wenjun Huang

Candidate for the Degree of

Master of Science

Thesis: CONSTRUCTION AND EXAMINATION OF THE CHIMERIC GENES CONTAINING DELETIONS IN THE 5'-PROMOTER REGION OF THE L-PK GENE

Major Field: Biochemistry and Molecular Biology

Biographical:

Personal Data: Born in Wuhan, China, on September 26, 1963, the daughter of Guichao Huang and Qifang He.

Education: Graduated from Kongjian High School, Shanghai, China in July 1981; received Bachelor of Science degree in Pharmacy from Shanghai Medical University, Shanghai, China in July 1986. Completed the requirements for the Master of Science degree with a major in Biochemistry and molecular Biology at Oklahoma State University in December 1994.

Experience: Employed as a teacher by Shanghai Medical University, Department of Pharmacy, 1986 to 1991. As a graduate research assistant at Oklahoma State University, Department of Biochemistry and Molecular Biology, 1993 to June of 1994. As a technician at Oklahoma State University, Department of Biochemistry and Molecular Biology, July of 1994 to present.