STUDIES ON PYRUVATE KINASE IN YEAST

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SOYEON PARK

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Thesis Approved:

James B Blair

Thesis Adviser

Franklin R. Leach

Richard C Bissenburg

Wayne B. Powell

Dean of the Graduate College

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NOMENCLATURE

Ala	L-alanine
Ap	Ampicillin resistance gene
bp	base pair
β-ME	β-Mercaptoethanol
CAT	Chloramphenicol acetyltransferase
CIP	Calf Intestine Phosphatase
FBP	Fructose 1,6-Bisphophate
G6P	Glucose 6-Phophate
Kb	Kilo base pair
kDa	kilo Dalton
L-PK	L-type pyruvate kinase
PCR	Polymerase chain reaction
PEP	Phospho enol pyruvate
Tc	Tetracycline resistance gene
YEp24	Yeast Episomal Plasmid
YPA	Yeast plate containing Alcohol

CHAPTER I

Introduction

Pyruvate kinase deficiency mutants from different organisms have been isolated by several groups (Maitra *et al.* 1977, Lam *et al.* 1977, Graaff *et al.* 1992, Clifton *et al.* 1981). Maitra *et al.* 1977 have reported that mutants lacking pyruvate kinase activity in *Saccharomyces cerevisiae* which naturally occurred have lost more than 99 % of this activity of the wild type and were unable to grow on glucose but grew on ethyl alcohol. Yeast pyruvate kinase consists of a tetramer of an identical subunit like other pyruvate kinase isozymes. These yeast mutants are known to be recessive and segregated in tetramer forms some of which contain full pyruvate kinase activity and others which have less than 1 % of the pyruvate kinase activity. These mutants were first designated as pyk1 by Maitra *et al.* 1977. Pyk1-1 to pyk1-6 have been further designated by the recombinant fraction ratio constituted. They have suggested the possibility that the pyruvate kinase gene mutation is involved either in the

structural or a regulatory gene as the cis configuration. Pyk1-5 is one of the pyk1 recombinant groups constituted which does not express active pyruvate kinase. The sequencing data of the pyruvate kinase deficiency mutant, pyk1-5 are reported here to analyze the mutation site responsible for the enzyme inactivity.

The development of the overexpression systems using powerful promoters and various host/vector systems has made it possible to take advantages such as expression of cDNA for proteins of mammalian cells for biochemical, biophysical or crystallographic studies.

In this research, plasmid pLPK101 containing the rat L-type pyruvate kinase gene under the yeast pyruvate kinase promoter system in the YEp24 vector was constructed to express the active form of L-type pyruvate kinase in yeast. Pyk1-5 strain was chosen for yeast transformation because this mutant lacks pyruvate kinase activity and does not grow on glucose. Only transformants with plasmid pLPK101 recover their ability to grow on glucose in pyk1-5 and express the active pyruvate kinase form. After transformation, isolated proteins were enzyme assayed and Western Blotted. FBP cause allosteric activation for both yeast pyruvate kinase and L-type pyruvate kinase enzymes and ATP inhibits both pyruvate kinases. However, Ala inhibits L-type pyruvate kinase specifically. This distinct property between L-PK and yeast PK allows us to test the expression of L-PK in pyk1-5. The

expression of L-type pyruvate kinase could be also detected by antibodies specific for L-type pyruvate kinase.

Literature

To keep the level of the blood glucose constant in mammals is very important for life. This level is tightly and reciprocally regulated through the glycolytic and gluconeogenetic pathway. The glycolytic and gluconeogenetic pathway utilize common enzymes. Yet, some of the key enzymes - hexokinase, phosphofructokinse (PFK), fructose 1,6-bisphosphatase FBP), glucose 6-phosphatase (G6P), pyruvate kinase (PK) and pyruvate carboxylase- catalyze physiologically irreversible steps. Pyruvate kinase is one of the key enzymes in glycolytic pathway that catalyzes the transfer of the phosphoryl group of phosphoenol pyruvate (PEP) to ADP to generate ATP and pyruvate. It results in an overall exergonic reaction and therefore irreversibility of this reaction makes an important metabolic point for regulation. Pyruvate kinase requires the participation of both mono (K^+) and divalent (Mg^{++}) cations.

Four types of pyruvate kinase in mammals were confirmed and characterized by electrophoresis, immunologically and kinetically. They were named the M_1-, M_2-, L-, and R-type (Tanaka *et al.* 1967, Imamura *et al.* 1972, 1973, Nakashima *et al.*1974). All these isozymes consist of four identical

subunits of about 60 kDa, but differ in enzymatic properties as well as in regulation of their gene expression.

The M_2- type pyruvate kinase is the prototype of the PK isozyme since it is predominant in fetal tissues and in cancer cells and is also present in most adult tissues. This PK is kinetically similar to L-PK in that its PEP saturation profiles are sigmoidal and are activated allosterically by addition of FBP. The M_1- type PK is the major isozyme in skeletal muscle, heart and brain. The PEP saturation profile of the M_1-PK is Michaelis-Menten type, both in the presence and absence of FBP (Imamura *et al.* 1972). The M_1- and M_2-type isozymes of PK are produced from the same gene by alternative RNA splicing (Noguchi *et al.* 1982, 1986). These isozyme types were isolated, cDNA was cloned (Noguchi *et al.* 1984) and then exon sequences were determined and characterized (Takenaka *et al.* 1989).

The L and R type PK differ from the M_1- and M_2- types in amino acid compositions and immunological properties, suggesting that these two groups are products of distinct genes. The L-type is present in liver as the major component and in kidney and intestine as a minor component (Imamura *et al.* 1972, 1973). L-type is influenced by the diet and hormones and is allosterically activated in the presence of FBP (Hess *et al.* 1966). Erythrocyte pyruvate kinase was isolated through investigation of a hereditary deficiency of erythrocyte pyruvate kinase and differed in electrophoretic

mobility from the other three types of PK. It is called R-type pyruvate kinase. This isozyme is only expressed in erythroid cells (Imammura *et al.* 1973, Nakashima *et al.* 1974). R-type has very similar kinetic properties to those of L-PK except that the inhibition of L-PK by Ala is completely reversed to the sigmoidal curve by adding FBP, but that of R-PK is moderately reversed (Imamura *et al.* 1973). Actually, the L- and R-type isozymes are produced from a single gene by use of different promoters (Noguchi *et al.* 1987). The rat L-type PK gene possesses two alternative tissue-specific promoters, located 472 bp apart; the upstream L' promoter is erythroid-specific and the downstream L-promoter is hepatocyte-specific. The nucleotide sequence of the cloned L-type PK cDNA from rat has been determined, the complete amino acid sequences have been deduced (Lone *et al.* 1986, Cognet *et al.* 1987, Noguchi *et al.* 1987, Inoue *et al.* 1986) and the cDNA has been expressed from fertilized eggs in transgenic mice(Tremp *et al.* 1989) and from clonally derived cell lines produced by in vitro transformation of rat fetal hepatocytes (Yeoh 1990).

The crystal structure of cat muscle pyruvate kinase has been determined at a resolution of 2.6 Å by Stuart et al. 1979. More recently, the structure of pyruvate kinase has been crystallized using a rabbit muscle complexed with Mn^{++}, K^{+}, and pyruvate at a resolution of 2.9 Å by Larsen et al. The pyruvate kinase enzyme consists of four identical subunits and each subunit folds into three domains. Domain A

has an eight-stranded parallel β sheet and α helices motif. The α helices connect adjacent strands of the β sheet. The active site lies between Domain A and B. Domain B is made up of a closed anti-parallel sheet structure. Domain C is a five-stranded β sheet. These strands are also interconnected by α helices. Domain A can be dissected into eight β strand-α helix units starting from the N-terminus (Stuart *et al.* 1979, Larsen *et al.* 1994).

Structural and functional studies about these four isozymes in mammals have been extensively carried out since the four isozymes were found and clarified. The experiments were extended to reveal the regulation mechanism by effectors on these isozymes. Particularly, the interest of our lab is focused on L-type PK because this isozyme is the most regulated allosteric enzyme, activated by FBP and inhibited by ATP and Ala. It seems likely that L-type is associated with specialized liver function, and is involved in metabolism of the whole body. The rapid modulation of metabolic flux results from control of enzyme activity by allosteric effectors (Imamura *et al.* 1972, 1973). Nevertheless, little information is known about the binding sites of allosteric effectors and the regulation mechanisms of L-type pyruvate kinase.

L-type Pyruvate Kinase of Rat Liver

L-type pyruvate kinase is a liver-specific enzyme and plays an important role in glycolytic regulation. It is regulated by diet and hormones at the transcriptional level and also at the posttranslational level by phosphorylation and dephosphorylation.

cDNA cloning of rat L-PK was carried out by Noguchi *et al.* 1983. The complete nucleotide sequence of the L-PK mRNA and the deduced amino acid sequence were reported by Lone *et al.* 1986 who presented the 5'- noncoding region of L-PK mRNAs is very short. These results are in disagreement with those of Inoue et al. 1986 who reported more than 230 nucleotides of the 5'- noncoding region.

It was reported by Cognet *et al.* 1987 that L-type pyruvate kinase exists as a single gene in the rat haploid genome, composed of 11 exons and 10 introns with a fully conserved exon-intron structure, and with a total size of 13 Kb including 8360 base pairs of the L-PK, 3193 bp of the 5'-noncoding and 1468 base pairs of the 3'-noncoding region. This report differs from Noguchi *et al.* 1987 who reported that L-PK consists of 12 exons and 11 introns with a length of about 9.3 Kb.

The plasmids containing the 5'-uncoding region of rat L-type pyruvate kinase fused to the chloramphenicol acetyltransferase (CAT) gene were transfected into primary

hepatocytes by Decaux *et al.* 1989 to investigate the carbohydrate response element of the rat L-type pyruvate kinase. They have shown that those elements are necessary for glucose-stimulated expression of the L-PK gene and are sufficient to confer carbohydrate responsiveness to a heterologous promoter. They also suggested that nuclear factors bind to this region of DNA and regulate the rate of transcription.

Yamada *et al.* 1990 have identified and characterized three cis-acting elements (pKL-I, pKL-II, and pKL-III) required for hepatocyte-specific expression of the L-PK and trans-acting factors interacting with them by using transient DNA transfer experiments with L-type pyruvate kinase fused to the chloramphenicol acetyltransferase gene. These cis-acting elements behave like a unit to enhance expression from heterologous as well as homologous promoters in a manner that was independent of its orientation and position relative to the Cap site.

Cognet *et al.* 1987 demonstrated through computer analysis some elements in the L-type pyruvate kinase flanking regions that seem to play a regulatory role in other genes: an octanucleotide, 5'-ATGCAAAG-3' at -740, identical with that found in immunoglobin gene enhancers and promoters (Falkner *et al.* 1984). Several copies of the consensus 5'-(G)TGG$^{TTT}_{AAA}$(G)-3' and its inverse-complement (Weiher *et al.*

1983) are scattered throughout the 5'-and 3'-flanking regions. This element has been found in several cellular and viral enhancer elements (Irwin *et al.* 1986).

A comparison between the sequence and organization of the genes for the yeast pyruvate kinase enzyme using the 2885 nucleotides with the 11 L-PK exons has been made by Cognet *et al.* 1987. Exon 7 was reported to have 68% similarity to a region of the yeast gene, while exon 4 and 8 are 78% and 69% similar with respect to yeast sequences, and nucleotides 5178 to 5223 of the rat L-PK gene located at intron 1 showed 74% similarity to nucleotides 2178 to 2200 of the yeast gene. Similarity is also found in the 5'- and 3'- flanking regions between L- type and yeast pyruvate kinase genes.

As mentioned earlier, Noguchi *et al.* 1987 found that L- and R-type mRNAs are produced from the same gene by using alternative promoters. The expression of the L- and R-types is tissue-specific and is regulated during differentiation of hepatocytes and erythrocytes, respectively. The two isozyme mRNAs differ in the 5'-noncoding sequences including the coding region and the 3'-noncoding region length. The first and second exons are responsible for the 5'-terminal sequences specific for the L- and R-type, respectively. The rest of the exons are the same for both isozymes. L-type seems to use the canonical promoter TATAAA sequence upstream of the cap site. R type uses another promoter sequence, CCAATCT in addition to three other CAAT sequences around it

at the 5'-flanking region. The last exon contains several putative polyadenylation signals. Marie *et al.* 1986 has reported alternative use of these signals is responsible for producing the multiple mRNA species of the L-type.

Three mRNA species of 3.2, 2.2 and 2 Kb for L-type pyruvate kinase were found in liver and in other tissues. These mRNA species share identical 5'-flanking and coding sequences and only differ by the length of their 3'-noncoding region. The mechanism involved seems to be the alternative use of different polyadenylation sites. Their respective ratio is tissue-specific and development-specific varying from liver to kidney and reticulocytes (Marie *et al.* 1986).

The transcription of the L-type pyruvate kinase is controlled by glucose plus insulin positively and by glucagon plus cyclic AMP negatively. Adult rat liver cells in primary culture have been developed by Decaux *et al* 1989 to investigate the mechanisms of the nutritional and hormonal control of the L-type pyruvate kinase gene. Both glucose and insulin accumulated the metabolites as a positive effector to control at the levels of transcription for the expression of the L-type pyruvate kinase gene. The half-life of all three mRNA species varies from 24 hr in the presence of glucose and insulin in the culture medium to 1 hr with glucagon.

Transgenic mice and tissue culture systems have been utilized by Tremp *et al. 1989* and Decaux *et al.* 1989, respectively. The *in vivo* situation can be mimicked by

transgenic mice models. Two series of transgenes has been expressed in mice *by* Tremp *et al.* 1989 to determine whether the DNA fragment encompassing the gene contains all DNA elements sufficient for the *in vivo* control of this gene. Expression of rat L-type pyruvate kinase transgene from the dual erythroid- and liver- specific promoter in all transgenic mice showed similar patterns with those of the endogenous mouse gene. They have concluded that the integrated DNA carries all the elements responsible for hormonal and nutritional response as well as tissue specificity in expression of the transferred rat gene.

Transgenic mice carrying about 3 Kb of the 5'-flanking region of the rat L-type pyruvate kinase linked to the chloramphenicol acetyltransferase (CAT) structural gene have been also developed by Yamada *et al. 1990* to investigate tissue-specific expression and regulation by diet and insulin. This concluded that the 5'-noncoding region of the L-type pyruvate kinase gene contains all the cis-acting elements necessary for tissue-specific expression of the L-type pyruvate kinase and its accumulation by diet and insulin.

5' Non-coding Region of Yeast Pyruvate Kinase

Upsteam activating and repressing sequences of the yeast pyruvate kinase gene have been identified by Nishizawa *et al.* 1989 using deletion analysis of the 5'-noncoding sequences. Three cis-acting regulatory elements responsible for

transcriptional regulation of pyruvate kinase gene were demonstrated. $UAS_{pyk}1$ (upstream activating system) between positions at -653 and -634 is defined as an essential cis-acting positive regulatory element of the yeast pyruvate kinase promoter. $UAS_{pyk}2$ between -811 and -714 is required for full transcriptional activation of the yeast pyruvate kinase promoter. URS (upstream repressible sequence) is responsible for transcriptional repression of the PYK promoter when yeast cells utilize nonfermentable carbon source. The TATA sequence located at -199 is also an essential element for transcriptional activation of the PYK promoter. The two UAS and the TATA sequence are only required for transcriptional activation in the 5'-noncoding sequences.

3'Non-coding Region of Yeast Pyruvate Kinase

The mature mRNA in eukaryotic cells is formed by processing mRNA precursors including the capping of the 5' end, splicing and the proper formation of the 3'-end followed by the addition of a poly (A) tail. This formation of a proper 3' end is a very important step in mRNA maturation. The AAUAAA sequence known as a crucial polyadenylation signal in higher eukaryotes does not seem to influence the efficiency of 3'-end formation strongly in *Saccharomyces cerevisiae* (Dechiara *et al.* 1990). Therefore, 3'-end formation of yeast mRNA possibly uses different sequences and mechanisms.

The importance of the tripartite sequence TAG-- TA(T)GT--TTT for 3' end formation has been postulated *by* Zaret *et al.* 1982 with cyc1 mutant deficient these tripartite sequences in normal CYC1 gene. Two sequences similar to this consensus sequence are found at positions +147, GAT TATGT ---- ---TTT and position +410, CAG --- TATGT --- TTT from the 3' untranslated region of the yeast PK (Burke *et al.* 1983). TTTTTATA or TTTTTAT (Irniger *et al.*1991) has been also proposed to serve as a processing signal of pre-mRNA 3' ends. The potential TTTTTAT signal has been found at position +456 in yeast pyruvate kinase. There is more evidence for participation of the signal TATATA or TATCTA in the process of transcription termination and/or 3'-end processing (Russo *et al.* 1991). There is still a possibility for the participation of additional regulatory elements which may be located at or near the actual polyadenylation site from the observations that these sequences are necessary but not sufficient to direct 3'-end formation (Irniger *et al.* 1991). Some subset of genes in yeast are known to contain its sequence AATAAA in their 3' regions. The yeast pyruvate kinase gene also contain the sequences AAATAAA, AAATAA and AATAAA at position +89, +112 and +332, respectively. Considering the 3 species of yeast pyruvate kinase mRNA, these potential AAUAAA sequences may play a role in the 3'-end formation. Not much research on yeast pyruvate kinase 3'-noncoding sequences has been done.

YEp24 Vector Plasmid

Significance of the yeast plasmid has been increased by its use as an efficient vector for yeast transformation. Maximum transformation stability has been obtained from experiments with chimeric DNAs containing the yeast plasmid when one of the two inverted repeats is present. YEp24 (Yeast Episomal Plasmid) is one of the yeast plasmids that serves as a shuttle vector in both *E. coli* and in *Saccharomyces cerevisiae* to introduce constructs at high copy number. This vector plasmid is a pBR322 derivative. The presence of the pBR322 origin of replication makes it possible to work in *E. coli*. YEp24 vector plasmid also contains the yeast URA 3 gene for selection and the 2μ circle to ensure plasmid replication and segregation in yeast. The ampicillin resistance gene (Ap) of pBR322 is utilized as a selectable marker in *E.coli*. The tetracycline resistance gene (Tc) of pBR322 is separated from its promoter by the URA3 sequence (Burke *et al.* 1983, Hartly *et al.* 1980, Botstein *et al.* 1979). The yeast pyruvate kinase gene has been cloned into the BamHI site of the vector YEp24 by Burke *et al.* 1983. This cloned gene was provided to us by Dr. P. Tekamp-Olson. We have used this pyruvate kinase-clone in YEp24 as an expression system for a rat L-type pyruvate kinase coding region.

CHAPTER II

Materials and Methods

Materials

Rat liver poly A^+ RNA was obtained from the Clontech Laboratories, Inc. The RT-PCR kit was obtained from Perkin Elmer, Corp. The TA cloning kit with pCR2.1 vector was obtained from Invitrogen. The plasmid QIAprep Spin Plasmid Kit and QIAEXII Gel Extraction Kit were obtained from Qiagen, Inc. All other reagents were obtained from the Sigma Chemical Company Ltd. and Difco Laboratories and were of reagent grade. Oligonucleotides for RT-PCR and PCR were obtained from the Oklahoma State University Core facility. T4 DNA Ligase was obtained from Life Technologies, Inc. CIP(Calf Intestine phsophatase) was obtained from Promega, Corp. Pyk1-5 strain, HB101 strain and Goat antibodies specific for rat L-type and yeast pyruvate kinase were available in our laboratory. YEp24 containing the entire yeast pyruvate kinase gene was a generous gift of Dr. P.

Tekamp-Olson (Burke *et al.* 1983). Restriction enzymes were obtained from Promega, Corp., New England Biolabs, and Life Technologies, Inc. Media, designated as YPG, contains 1% yeast extract, 2% bactopeptone and glucose as a carbon source. Media, YPA, contains peptone and yeast extract supplemented with 150 mM ethyl alcohol.

Methods

Preparation Of Yeast pyk1-5 Cells

Pyk1-5 strain was streaked on the plate medium containing 150 mM ethyl alcohol (Maitra et. al. 1977) and incubated at 30°C. After 6 days, colonies appeared and a single yeast colony was then inoculated in 8 ml YPA medium and grown for 40 hours to saturation at 30°C. 75 ml YPA medium was inoculated with 8 ml of the saturated culture and incubated at 30°C. When the culture cell density was grown up to 2 x 10^6 cells/ml at 30°C, the culture was used for DNA isolation.

PK Genomic DNA Isolation from pyk1-5 Cells

DNA isolation was carried out as described by Philippsen *et al.* 1991. Cells were harvested by centrifuging suspensions at 4000 xg for 5 min at 4°C. The cell pellet was resuspended in 10 ml sterile water and pelleted again by centrifuging at 4000 xg for 5 min. The cells were

resuspended in 3 ml of 0.9 M sorbitol, 0.1 M EDTA, 50 mM dithiothreitol(pH 7.5) and 0.2 mg zymolase/ g cells and gently shaken for 1.25 hours at 37°C. Cells were then centrifuged at 4000 xg for 5 min and resuspended in 3.0 ml of 50 mM Tris-HCl, 50 mM EDTA(pH 8.0) by pipetting up and down which after the spheroplasts were lysed in 0.3 ml of 10 % SDS and incubated for 30 min at 65°C.

After 30 min, 1.0 ml of 5 M potassium acetate was added followed by incubation on ice for 1 hour and the white precipitates which consist of insoluble potassium dodecyl sulfate and the denatured protein was formed. The supernatant was removed by centrifuging at 15,000 xg for 30 min and at 10, 000 xg for 10 min and was resuspended in 4.0 ml of 50 % ethanol for at least 5 min followed by centrifuging for 10 min at 10,000 xg. The pellet was air-dried and then dissolved in 3.0 ml of 10 mM Tris-HCl, 1 mM EDTA(pH 7.5) and 150µl of 1 mg/ml DNase-free pancreatic RNase(Boehringer Mannheim, FRG) was added and incubated at 37°C for 30 min. After this, 3.0 ml of isopropanol was added to remove the precipitated DNA. DNA was washed with 50 % isopropanol and air-dried. DNA was dissolved in 0.5 ml of 10 mM Tris-HCl, 1 mM EDTA(pH 7.5) and the supernatant was removed by centrifuging and then same volume of ethyl alcohol was added and centrifuged again for 15 min. After centrifugation, the pellet was redissolved in 100 µl of TE

buffer(pH 8.0) and 0.9 % agarose gel electrophoresis was applied to confirm the DNA existence (Data not shown).

Amplification Of The Yeast Pyruvate Kinase Gene

150-250 ng of DNA was added to 100 µl of final volume containing 1X PCR buffer, 10 mM dNTPs mixture, 50 mM $MgCl_2$, 10 µM each of upstream and downstream primers ((+)/#1 and (-)/#1 in table 1) designed for this study , sterile water and 2.5 U of Tag DNA polymerase. DNA in the PCR component was denatured for 45 s at 94°C and annealed for 30 s at 55°C followed by extension for 2.5 min at 72°C. The amplification cycles were repeated 35 times. Final extension step for 10 min at 72°C was then performed. The PCR product was analyzed on 0.9 % agarose gel.

Oligonucleotide Primers For Yeast Pyruvate Kinase

Pairs of oligonucleotide primers were synthesized according to the published yeast pyruvate kinase nucleotide sequences (Burke et. al. 1983, McNally et. al. 1989) to carry out amplification and sequencing of yeast pyruvate kinase gene. Primers (+)/#1 and (-)/#1 in Table 1 are used for PCR amplification and also applied for the sequencing. The list

of primers synthesized for this study is summarized on Table 1.

Table 1. Oligonucleotide primers designed for amplification and sequencing of yeast pyruvate kinase gene in pyk1-5

Primer	5′ sequences 3′	Position(base)	Primer	5′sequence3′	Position
(+)/#1	GGATCCTGCTTG TGATGTCTTCCA AGTG	587 (28-mer)	(-)/#1	GGATCCGATTATCT TGCGATGGGAGG	2669 (26-mer)
(+)/#2	AAGACACCAAT CAAA	877 (15-mer)	(-)/#3	ACGGTAGAGACTT GC	2394 (15-mer)
(+)/#3	CAAGAACATCA CCAA	1301 (15-mer)	(-)/#4	CGAATGGGAAGAC ACCT	2220 (17-mer)
(+)/#4	TCCCAGCCCCA GAAGTC	1723 (17-mer)	(-)/#5	CGGTTTCAGCCATA GTG	1941 (17-mer)
(+)/#5	CGTGTCGCTGCT GTT	2064 (15-mer)	(-)/#6	GTGGACACCGTTCT TGA	1519 (17-mer)
(+)/#6	TCAAGAACGGTG TCCAC	1519 (17-mer)	(-)/#7	GGTGGGATTGGGTA GTC	1209 (17-mer)
(+)/#7	CTCTCACGGTTC TTACG	1067 (17-mer)	#7. 2nd	GGTGGGATTGGGTA GTC	1209 (17-mer)
(+)/#8	ACCAGATGCCC AAGAGC	2181 (17-mer)	#7. 3rd	GGTGGGATTGGGTA GTC	1209 (17-mer)
#8. 2nd	ACCAGATGCCC AAGAGC	2181 (17-mer)	(-)/#8	GGCAAGTAAGCGA TAGC	1983 (17-mer)
(+)/#9	CATGGTCCCCTT TCA	769 (15-mer)	(-)/#9	TGGGGCTGGGATTT CAA	1715 (17-mer)
(+)/#10	CCAAGGGTCCA GAAATC	1165 (17-mer)	(-)/#10	ACGACAATTGGAG AC	2589 (15-mer)
			(-)/#11	CTACCAGCGGAGA TGAC	1317 (17-mer)

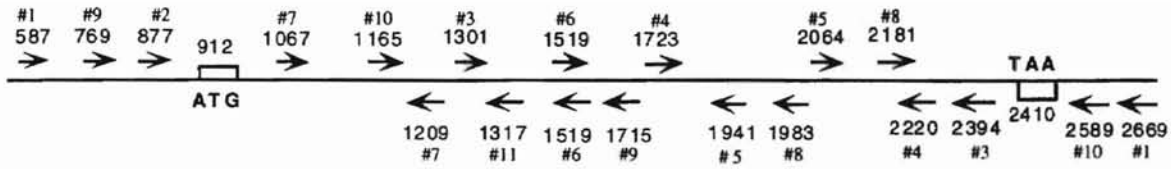

Fig 1. Sites of oligonucleotide primers designed for sequencing on pyk1-5 pyruvate kinase

DNA Sequencing of pyk1-5 Pyruvate Kinase

DNA was sequenced by the ABI Prism 373 DNA sequencer (PEApplied Biosystems) in the Core facility using the sequencing method of the Sanger dideoxynucleotide termination. The sequencing data was aligned by the Lasergene computer program (DNASTAR) for the sequencing analysis.

Oligonucleotide Primers For pLPK101

Each pair of oligonucleotide primers was designed to amplify DNA fragments of the rat L-type pyruvate kinase coding region and yeast 5'- and 3'- noncoding regions, respectively for the cloning. Each primer was designed to contain a restriction enzyme site at the terminal site to carry out the cloning as shown in Table 2 and Figure 2.

RT-PCR and PCR for pLPK101

By using the RT-PCR technique, the rat L-type pyruvate kinase cDNA was directly amplified from rat liver poly $(A)^+$ RNA. Reaction for reverse transcription was performed in 20 µl of final volume containing 200 ng mRNA, 1 mM of each dATP, dTTP, dGTP and dCTP, 1x PCR buffer, 1 U of RNase inhibitor and 50 µM of Oligo $d(T)_{16}$ or 15 µM downstream primer. Each tube was incubated at 70 °C for 10 min, chilled on ice for 2 min and then incubated at 42 °C for 50 min with 50 Units of

reverse transcriptase. After incubation, the reaction was carried out at 99 °C for 5 min and incubated at 5 °C for 5 min for the final reverse transcription reaction. For PCR, 0.4 µl of cDNA from the RT reaction was used in 100 µl of final volume which consisted of 1X PCR buffer, 0.2 mM each of dNTPs mix and upstream and downstream primers. To increase efficiency of PCR, "touch down" and "hot start" methods were used. After predenaturing at 95°C for 1 min, the temperature was lowered to 85 °C to add Tag DNA polymerase (Hot start method). Twenty cycles of 45 s each at 94 °C and 70 °C, and 1 min 30s at 72 °C were carried out, but the 70 °C annealing temperature was lowered -0.7 °C per each cycle (Touch down method). After finishing the first PCR stage, 15 cycles of 45 s each at 94 °C and 56 °C, and 1 min 30 s at 72 °C were performed and the final extension was carried out at 72 °C for 10 min.

Table 2. The pairs of oligonucleotide primers designed for pLPK101 construction

Gene source	oligonucleotide (5′ to 3′)	position	Direction	#
Rat PK	ACCGGTCAACGTAGCAGCATGGAAG PinAI	1 of rat PK gene	upstream	A
Rat PK	ACCGGTGATGGGGCTAGATGGCAGATGTA PinAI	1714 of rat PK gene	downstream	B
Rat PK	ACCGGTCAACGTAGCAGCATGGAAG PinAI	1 of rat PK gene	upstream	3
Rat PK	ACCGGTATTTCAGGATACGCTCAGCA PinAI	1626 of rat PK gene	downstream	4
YEp24+yeast PK	CGTCCTGTGGGATCCGATCCAAATGTAA BamHI	5′terminal site of yeast PK	upstream	1
yeast PK	ACCGGTGTTTTGATTGGTGTCTTGTAA PinAI	872 of yeast PK gene	downstream	2
Yeast Pk	ACCGGTCATGATTGAATGAAGATA PinAI	2424 of yeast PK gene	upstream	5
YEp24	GATGCGTCCGGCGTAGAGGATCC BamHI	3′terminal site of yeast PK	downstream	6

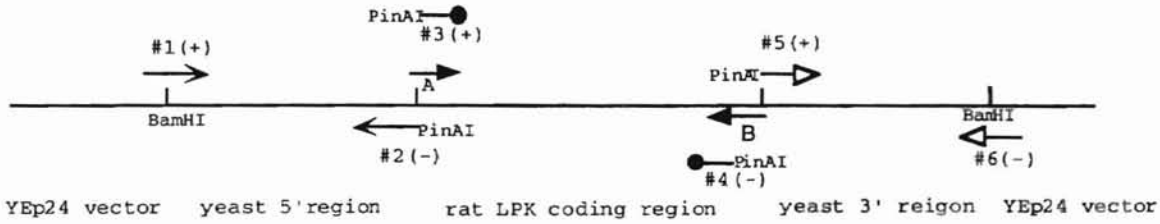

Fig 2. The sites of oligonucleotide primers designed for pLPK101 PCR

Cloning and Transformation

With InVαF' Cells

The TA cloning Kit with the pCR2.1 vector obtained from Invitrogen provides a straightforward strategy to insert the PCR product directly to a vector at the modified site of EcoRI. Most procedures were carried out as the instruction manual described. The ligation reaction was usually executed with 10 µl final volume set up with 1X ligation buffer, 4 Weiss units T4 DNA ligase, 50 ng pCR 2.1 vector, sterile water and fresh PCR product and incubated at 14°C for overnight. In general, inserted PCR products were set up as 1:1 and 1:3 molar ratio of insert-to-plasmid, respectively.

0.5 M β-ME and 2µl of each ligation reaction were added to each 50 µl vial of InVαF' competent cells and then incubated on ice for 30 min followed by heat shock for 30 s at 42 °C and 2 min on ice. 250 µl of SOC medium (2.0 % Tryptone, 0.5 % yeast extract, 10.0 mM NaCl, 2.5 mM KCl, 10.0 mM $MgCl_2$ and 20.0 mM glucose) was added, after which the vials was shaken at 37°C for 1 hour and spread with a proper amount (10 µl to 200 µl) on each LB agar plate containing 50 µg/ml of ampicillin, 1.6 mg of X-Gal and 4 µmol of IPTG and grown in a 37 °C incubator for 18 hours. White colonies were

selected and grown in LB (Luria-Bertani) culture media for 24 hours to determine the presence and orientation of insert by restriction analysis of the plasmid.

With HB 101 Competent Cells

To fuse the 5'-noncoding region of yeast to the pCR2.1 vector in which the 3'-noncoding region was cloned and to clone rat LPK between the 5'- and 3'- noncoding regions in the YEp24 vector, strain HB 101 of *E. coli* was employed as a bacterial transformer instead of strain InVαF' of *E. coli*.

The ligation reaction was set up with 20 µl final volume containing 1X ligation buffer, 0.5 µl ligase (Promega), sterile water, 2 µg vector and the insert fragments as 1:1, 1:2, and 1:3 ratio of insert-to-plasmid, respectively, followed by 15 °C incubation overnight. For transformation, strain HB 101 requires a 45 s heat shock step and β-mercaptoethanol is not necessary. The transformation procedure for HB 101 strain is otherwise the same as for strain InVαF'.

Plasmid DNA Preparation

E. coli containing plasmid was grown in 5 ml LB(Luria-Bertani) medium for 24 hours and plasmids was prepared by the plasmid QIAprep Spin Plasmid Kit (Qiagen Inc). The procedure

was derived from the modified alkaline lysis method of Birnboim and Doly 1979 and the silica-gel membrane technique. Pelleted overnight *E. coli* culture medium was resuspended by buffer P1 (50 mM Tris-HCl,pH 8.0: 10 mM EDTA; 100 µg/ ml RNase A) with RNase A, P2 (200 mM NaOH, 1 % SDS) and N3, which lysate and precipitate cells, and centrifuged in a micro centrifuge for 10 min. The supernatants were loaded on a silica-gel membrane tube, washed with buffer PB and PE and DNA was eluted by TE buffer (pH 8.0).

Phenol:Chloroform Extraction And Ethanol Precipitation

Phenol extraction and ethanol precipitation is the most widely accepted method to prepare highly concentrated nucleic acids. A volume of Phenol:Chloroform:Isoamyl alcohol equilibrated with pH 8.0 was added to the same volume of plasmid DNA dissolved in TE buffer (pH 8.0), vortexed vigorously, centrifuged for 5 min at 4 °C and the aqueous phase was transferred to a new tube. This plasmid DNA extraction step was repeated by using Chloroform :Isoamyl alcohol (pH 8.0) only. Salt, either 1/50 volume of 5 M sodium chloride, 1/9 volume of 3M sodium acetate (pH 7.5) or 1 volume of 4M ammonium acetate (pH 7.4), and 2.5 volume of 95 % ethanol was added into the DNA extracted aqueous phase and incubated on ice for 30 min to precipitate DNA followed

by centrifuging for 10 min, after which the pellet was dried and dissolved in either TE buffer (pH 8.0) or sterile water.

Recovery of DNA Fragments From Agarose Gel

QIAEX II Gel Extraction Kit (Qiagen Inc.) was used for this study to extract and purify DNA from standard agarose gel. The principle of this method is based on agarose gel solubilization and the silica-gel particle technology designed to adsorb nucleic acids selectively in the presence of high salt. 3 volumes of buffer QX I and 30 µl of QIAEX II silica-gel particles were added to 1 volume of the excised gel band with the particular DNA fragment and solubilized at 50 °C for 10 min. After centrifuging the sample for 30 sec, the pellet was washed with buffer QX1 and buffer PE and the pellet was dried for approximately 15 min. The pellet containing DNA was resuspended with TE buffer (pH 8.0), incubated at the proper temperature for 5 min as the manual instructed and the supernatant was collected after centrifugation for 30 sec.

Dephosphorylation Of Linearized Vector For Cloning

When the cloning vector contains the same restriction enzyme site at the both ends, the 5′ terminal site of the vector fragment was dephosphorylated by CIP (calf intestine

phosphatase) to prevent vector self-ligation. The vector was digested by a single restriction enzyme and phenol extraction-ethanol precipitation was carried out to concentrate DNA for the CIP reaction. 1X CIP buffer, 10 units CIP, sterile water and linearized vector were set up in a microcentrifuge tube and incubated for 1 hour at 37°C for a 5'- protruding or sticky end, or at 50°C in the case of a 5' blunt or recessed end, followed by the addition of 5 mM of EDTA which incubated another 10 min at 75°C. CIP treated vector was extracted with phenol and precipitated with ethanol again to get rid of CIP from DNA.

Yeast Transformation With Lithium Acetate

Yeast transformation was carried out by the lithium acetate method (Stearns *et al.* 1990) with the mutant of *Saccharomyces cerevisiae,* pyk1-5 which was characterized by an inability to grow on glucose. Transformed pyk1-5 can recover its ability to grow on glucose.

A single pyk1-5 colony was inoculated with 5 ml YPA medium(1 % yeast extract, 2 % bactopeptone and 150 mM ethyl alcohol as final concentration) containing 1% glycerol to increase the growth rate of the mutant. Two days later with the YPA medium was saturated, it was inoculated into 250 ml YPA medium and grown until the cell density reached to 1.7 X 10^6 to 2 X 10^6 cells/ml at 30°C. Cells were harvested and

resuspended in 10 ml sterile water. Cells were pelleted again and resuspended in 1.5 ml buffered lithium acetate solution (1:1:8 ratio of 10 X TE buffer: 10 X lithium acetate stock solution: sterile water). Up to 5 µg transforming DNA was introduced to each 200 µl pyk1-5 suspension tube with 200 µg carrier DNA followed by addition of 1.2 ml PEG solution(8 vol. 50% PEG, 1 vol. 10X TE buffer, 1 vol. 10X lithium acetate stock solution) and then shaken for 30 min at 30°C, which after the suspension was heat shocked exactly 15 min at 42°C, centrifuged 5 s at room temperature and resuspended in 200 µl to 1 ml of 1X TE buffer. Each YPG plate was incubated at 30°C until transformants appeared. Up to 200 µl of the suspension was spread on Difco YPG agar plates.

Enzyme Analysis

A yeast transformant was inoculated into 5ml YPG medium and growen at 30°C until saturated and this saturated culture was inoculated in 250 ml YPG medium and grown at 30°C to about OD_{600} ~0.4 to 0.6. Cells were harvested by centrifuging 5 min at 4000 x g at room temperature, weighed and 5 ml of 1.2 M sorbitol/ g cells was added, adjusted to pH 7.5. 25 mg of lyticase /g cells was added, adjusted to pH 6.8 and shaken for 1.25 hours, room temperature. The supernatant of the

sorbitol fraction was removed by centrifuging 10 min at 5000 x g and pellet was resuspended in 5 ml lysing buffer/g cells, adjusted to pH 7.5 and vortexed. Supernatant of lysing buffer fraction was removed by centrifuging 10 min at 5000 x g. The supernatant of the sorbitol fraction and lysing buffer fraction was mixed and dissolved in 2x saturated $(NH_4)_2SO_4$ and stored at 4°C until using. This mixed fraction was pelleted and dissolved in 1 ml of saturated $(NH_4)_2SO_4$ with 2 µl of β-mercaptoethanol to prepare the active enzyme.

PK assay stock solution consisted of 40 ml of 0.4 M Tris-Cl(pH 7.5), 4 ml of 2M KCl, 1.2 ml of 1 M $MgSO_4$ and 54.5 ml of deionized water. 20 ml of PK stock solution was mixed with 600 µl of 10 mg/ml NADH and 10 µl of 10 mg/ml LDH and adjusted to pH 7.5. 8 µl of the cell sample was mixed with 1 ml PK buffer (The PK stock containing NADH and LDH and adjusted to pH 7.5) and the reaction was started by the addition of 5mM PEP, 5 mM ADP and either FBP, ATP or alanine at the various concentration. PK activity was determined spectrophotometrically by the disappearance of NADH at 340 nm, room temperature.

Western Blotting Analysis

100 µl of each $(NH_4)_2SO_4$ saturated enzyme sample was pelleted, dissolved in 200 µl of sample buffer (0.125 M Tris

(pH 6.8) : 4% SDS : 20 % glycerol : 10 % β-mercaptoethanol (BME) : 2 crystals of bromophenol blue) and boiled for 1 min. 3 µl of each sample was loaded in each well on a polyacrylamide gel(9% resolving gel and 4% stacking gel). Rat liver control was prepared from 0.5 g of the homogenized adult rat liver followed by centrifuging with 2.5 ml of lysing buffer. Supernatant was dissolved by 1:2 dilution in the saturated $(NH_4)_2SO_4$ and 2 µl β-mercaptoethanol. The prepared 7.5 ml of the sample was pelleted and dissolved in 1 ml of the saturated $(NH_4)_2SO_4$. 100 µl of sample was pelleted again and dissolved in 200 µl of the sample buffer. 0.5 µl of the rat control was loaded to each well on a polyacrylamide gel. The gel was run for 3 hours at increasing voltage settings and then electroblotted on to PVDF membrane (Immobilon-P Millipore) for 1.5 hours at 25 V and 200 mA. Lanes with molecular weight standard were stained with coomassie blue. Lanes containing samples were blocked for 1.5 hours at room temperature in blocking buffer(25mM Tris (pH 8.0) : 125 mM NaCl : 4% BSA : 0.1% tween 20). Membranes were subjected to the 1 µl of 1° Ab, Goat anti-LPK serum or 1 µl of Goat anti-YPK serum for 1.5 hours at room temperature, followed by three-time washes with TBS(25mM Tris(pH 8.0) : NaCl) for 10 min, each. The 1 µl of 2° Ab, alkaline phosphatase-conjugated anti-goat IgG, was then

applied. Membranes were then visualized by treatment with BCIP(Bromochloroindoyl phosphate from Sigma) and NBT (Nitro Blue Tetrazolium).

CHAPTER III

Results and Discussion

Results

A. Sequencing of Yeast pyk1-5 Pyruvate Kinase

Genomic DNA of pyk1-5, a yeast mutant strain, was prepared and characterized by absorbancy of A_{260} nm/A_{280} nm and 0.9 % agarose gel electrophoresis (Data not shown). A total of 80 µl of 95 µg/ml of DNA was obtained. The pyruvate kinase gene was amplified by PCR with the entire genomic DNA of pyk1-5 as a template. The pair of oligonucleotide primers at the position 587 and 2669 as shown in table 1 was synthesized according to the published DNA sequences of yeast pyruvate kinase by McNally *et al.* 1989 and the 2.1 Kb DNA fragment was obtained. The amplified product was confirmed by 0.9 % agarose gel electrophoresis (Fig 3). This DNA fragment contains the structural gene of yeast pyruvate kinase as well as the first 325 bp of the 5′ noncoding sequences which contains the BamHI site at the 5′ terminal site and the first 257 bp of the 3′ noncoding sequences which

contains a BamHI site at the 3′ terminal site. The annealing temperature was optimized to 55 °C for this experiment. The first trial using an annealing temperature at 58.5 °C failed to yield the expected DNA size suggesting the setting of the annealing temperature is an important factor for PCR.

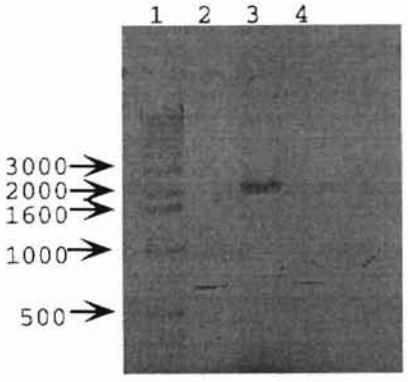

Fig.3

Figure 3

Amplification of yeast pyruvate kinase gene by PCR

Yeast pyruvate kinase gene, 2.1 Kb fragment, was amplified from pyk1-5 cells by PCR using the primer pair which was synthesized at position 587 and 2669, respectively (Table 1). Lane 1: 1 Kb DNA ladder. Lane 2 and 4: 0.7 Kb DNA control. Lane 3: 2.1 Kb yeast pyruvate kinase PCR product.

The entire coding region of the pyruvate kinase gene of pyk1-5 strain was sequenced at least twice in each direction. The sequencing result was aligned to the nucleotide sequences of the pyruvate kinase of *Saccharomyces cerevisiae* to determine the mutation site (Fig 4). The sequencing analysis indicated that the nucleotide at position 1714 is the potential mutation site. This site was sequenced four times with different primers and the result showing a change of the original sequence 'G' at this site to 'A' was found in all sequencing data examined. The amino acid codon at this site is changed from Gly 270 to Asp. This potential mutation site lies close to the active site on a subunit of yeast pyruvate kinase.

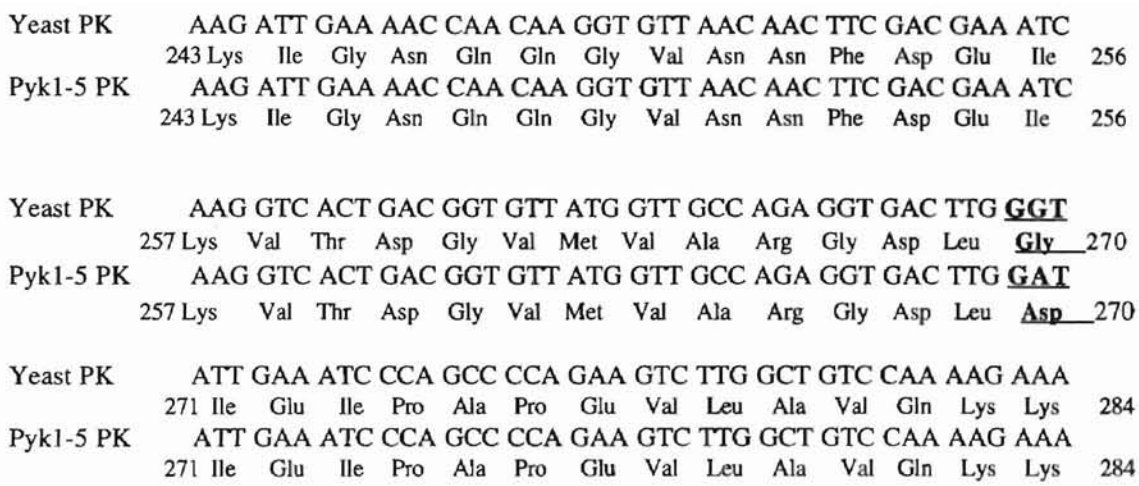

Fig 4. The result of sequencing of pyk1-5 pyruvate kinase - The mutation site Gly 270 to Asp displayed as bold characters-

B. The Expression of L-type Pyruvate Kinase

RT-PCR of Rat LPK

Reverse transcription-polymerase chain reaction(RT-PCR), the most up-date PCR technology makes it possible to amplify genes from low-abundance mRNA transcripts. The rat L-type pyruvate kinase coding region cDNA was amplified with a pair of oligonucleotide primers designated as #3 and #4 in Table 2 using rat liver poly(A)$^+$ RNA as the template by RT-PCR. The oligonucleotide primers were synthesized according to the published DNA sequences of rat liver pyruvate kinase by Cognet *et al.* 1987. A 1.7 Kb DNA fragment which contains the structural gene of rat L-type pyruvate kinase, the first 89 bp of the 3' non-coding region and the first 12 bp of the 5' noncoding region was obtained by this result and named as LPK1 (Fig 6).

The PCR product of LPK1 was sequenced to confirm the existence of the LPK1 gene using the oligonucleotide primers shown in Table 3 and Fig 5. Fig 7 presents the result of the sequencing analysis. There are three possible sites for mutation at amino acid 435, 492 and 829, respectively. Nucleotide changes at positions 435 and 492 do not affect an amino acid but the nucleotide change at position 829 is responsible for changing the amino acid 277 from Ile to Val. The PCR product encompassing rat LPK1 gene was sequenced once with each oligonucleotide primer so more sequencing analysis should be conducted to increase the reliability of the data.

The experiment was continued with this gene product because the existence of the complete LPK1 gene was observed.

Table 3. Oligonucleotide primers designed for sequencing the LPK1 gene

Position	Direction	Oligonucleotide sequences(5' to 3')	#
1	upstream	CAACGTAGCAGCATGGAAGGGC	1
176	upstream	GTAGCACCAGCATCATTG	2
235	downstream	CCTTGAGGCGGTCCACAGAG	3
889	downstream	CAAACTTCTTCACGCCTTCA	4
1301	downstream	TCCACGGCTCCAATCGCAGT	5
1731	downstream	GATGGGGCTAGATGGCAGATGTA	6

Fig 5. The sites of oligonucleotide primers designed for LPK1 gene sequencing

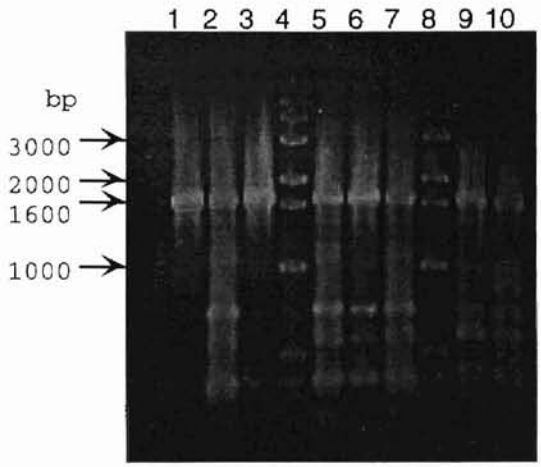

Fig. 6

Figure 6

Rat LPK1 gene product from RT-PCR.

5 µl of each reaction mixture was electrophoresed on a 0.9 % agarose gel and stained with ethidium bromide. Lane 4 and 8: 1 Kb DNA ladder. Lane 1 and 2: RT-PCR was performed with oligo $d(T)_{16}$ primers for reverse transcription and 200 µM dNTPs and either 37.5 nmol or 75 nmol of $MgCl_2$, respectively. Lane 3 and 5: A gene specific down-stream primer which is designated as "B" in Table 2, with the concentration of $MgCl_2$ and dNTPs the same as in lanes 1 and 2, were used to amplify rat L-type pyruvate kinase cDNA. Lane 6 and 7: As same as Lane 1 and 2 except 100 µM of dNTPs for the reaction. Lane 9 and 10: RT-PCR was carried out by downstream primer for reverse transcription with 100 µM of dNTPs for the reaction and either 1.5 µl or 3 µl of 25 mM $MgCl_2$, respectively. The 1.7 Kb PCR product of rat LPK appeared in the lanes as expected.

LPK CAG GGG GCT CCG GAG TCG GAG GTG **GAA** ATT GTG AAG GGC TCA
409 Gln Gly Gly Pro Glu Ser Glu Val **Glu** Ile Val Lys Gly Ser 450
LPK1 CAG GGG GCT CCG GAG TCG GAG GTG **GAG** ATT GTG AAG GGC TCA
409 Gln Gly Gly Pro Glu Ser Glu Val **Glu** Ile Val Lys Gly Ser 450

LPK CAG GTG CTG GTG ACG GTG GAC CCG AAG TTC CAG ACA AGG **GGT**
451 Gln Val Leu Val Thr Val Asp Pro Lys Phe Gln Thr Arg **Gly 492**
LPK1 CAG GTG CTG GTG ACG GTG GAC CCG AAG TTC CAG ACA AGG **GGC**
451Gln Val Leu Val Thr Val Asp Pro Lys Phe Gln Thr Arg **Gly 492**

LPK GAT GCA AAG ACA GTG TGG GTG GAC TAC CAC AAT ATC ACC CGG
493 Asp Ala Lys Thr Val Trp Val Asp Tyr His Asn Ile Thr Arg 534
LPK1 GAT GCA AAG ACA GTG TGG GTG GAC TAC CAC AAT ATC ACC CGG
493 Asp Ala Lys Thr Val Trp Val Asp Tyr His Asn Ile Thr Arg 534

LPK GTC GTT GCA GTG GGG GGC CGC ATC TAC ATT GAC GAC GGG CTC
535 Val Val Ala Val Gly Gly Arg Ile Tyr Ile Asp Asp Gly Leu 576
LPK1 GTC GTT GCA GTG GGG GGC CGC ATC TAC ATT GAC GAC GGG CTC
535 Val Val Ala Val Gly Gly Arg Ile Tyr Ile Asp Asp Gly Leu 576

.....

LPK CGG GAT CGG CTG GGG CCA GAA GGA CAG AAC **ATC** AAA ATT ATC
819 Arg Asp Ala Leu Gly Pro Glu Gly Gln Asn **Ile** Lys Ile Ile 832
LPK1 CGG GAT CGG CTG GGG CCA GAA GGA CAG AAC **GTC** AAA ATT ATC
819 Arg Asp Ala Leu Gly Pro Glu Gly Gln Asn **Val** Lys Ile Ile 832

Fig 7. The sequencing result of pLPK1 (LPK= the published sequences of rat L-type pyruvate kinase, LPK1= the sequenced LPK1 gene). Bold type indidcates a mutation site.

The amplification of LPK2 gene

The 1.6 Kb of rat liver PK cDNA was reamplified with 1.7 Kb of LPK1 gene RT-PCR product as a template (Fig 8). The pair of oligonucleotide primers were synthesized for this amplification according to the published nucleotide sequences of the rat L-type pyruvate kinase gene (Cognet *et al.* 1987) so that the resulting amplified L-type pyruvate kinase contained the first 12 bp(-1 to -12) of its original 5'-noncoding region and a PinAI site at its 5' terminal as well as the first 14 bp (+1 to +14) of its 3'-noncoding region and PinAI site at its 3' terminal and designated LPK2. The LPK2 gene was then cloned into plasmid pCR2.1 vector at its EcoRI site and this plasmid is designated pLPK2 (Fig 9). The published DNA sequence of the rat LPK gene by Cognet *et. al.* 1987 which was used to design primers for this study are in disagreement with those of Inoue *et al.* 1986 who reported 250 bp of nucleotides for 5'non-coding region.

RT-PCR with primers designed according to the published LPK gene sequences by Inoue et *al.* 1986 never produced the expected 1.7 Kb fragment of the rat L-type pyruvate kinase gene. This implies the 5'-noncoding sequence results by Inoue *et. al.* should be reconsidered (Data not shown).

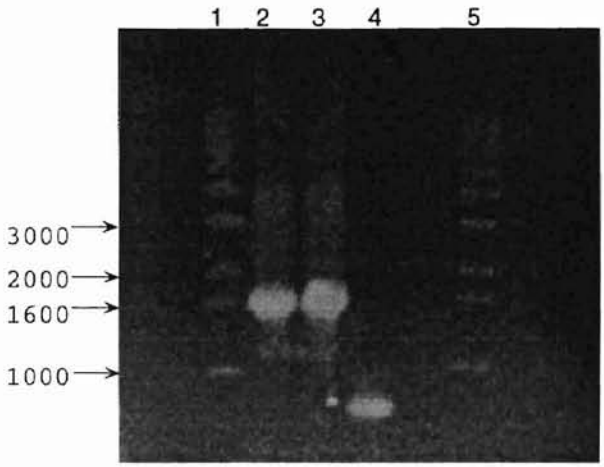

Fig. 8

Figure 8

Rat LPK2 amplified from LPK1 gene

Lane 2 and 3: 1.6 Kb fragment of rat LPK was amplified from the 1.7 Kb RT-PCR product of rat LPK which was inserted in the EcoRI site of the pCR2.1 vector. The pair of primers was designed from the sequences at position 1 and 1626 with a PinAI site at the 5' terminal. Lane 1 and 5: 1 Kb DNA ladder. Lane 4: DNA control, 769 bp fragment.

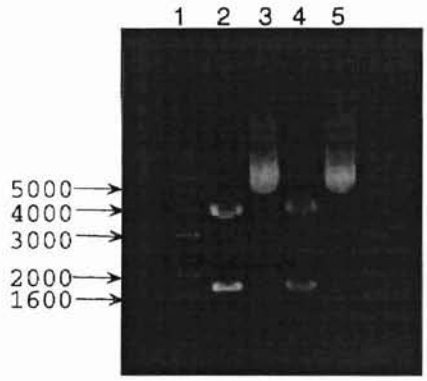

Fig. 9

Figure 9

Analysis of restriction enzyme digestion of 1.6 Kb rat L-PK PCR gene product cloned in pCR2.1 vector.

Lane 2 and 4: EcoRI digestion shows 2 bands of 3.9 Kb (the size of pCR2.1 vector) and 1.6 Kb (the size of rat LPK gene product) since the gene is inserted at the EcoRI site on pCR 2.1 vector. Lane 3 and 5: uncut cloned plasmid. Lane 1 and 6: 1 Kb DNA ladder.

PCR of 5'- and 3'- noncoding region of yeast pyruvate kinase

The yeast pyruvate kinase gene inserted in YEp24 has been reported to have 20- fold higher enzyme activity than the parental strain from yeast transformation (Burke *et al.* 1983). The same non-coding regions of the yeast pyruvate kinase gene with the promoter and termination sites was adapted to express the rat L-type pyruvate kinase gene in *Saccharomyces cerevisiae*. 5'- and 3'- noncoding regions of the yeast pyruvate kinase were amplified from the pPYK101 which carry out the entire yeast PK in YEp24 vector as a template, yielding 0.9 Kb and 2.0 Kb fragments, respectively with the pair of primers (Fig 10).

One pair of primers is located at BamHI site of pPYK101 plasmid and at the 3' terminal of Yeast PK promoter region containing PinAI restriction enzyme sequence to yield 0.9 Kb of the 5'-noncoding region of Yeast PK and designated as YPK5 (Fig. 2). The others are located at the 5' terminal containing PinAI site and at the 3' terminal site to produce the 2.0 Kb of 3'-noncoding region of yeast PK and designated as YPK3. PCR products of the 5'- and 3'- noncoding region were cloned into the pCR2.1 vector at the EcoRI site which is provided from Invitrogen, respectively and designated as pYPK5 and pYPK3. The presence of the inserts for YPK5 and YPK3 was confirmed by restriction enzyme digestion. The 1 Kb and 3.9 Kb fragments should be produced from pYPK5 by SpeI+EcoRV digestion since the SpeI and EcoRV sites are

located at position 259 and 296 in pCR2.1 vector(Fig 11). The 2.0 Kb and 3.9 Kb fragments should be produced from pYPK3 by EcoRI digestion since the YPK3 was inserted at the EcoRI site at position 263 in pCR2.1 vector.

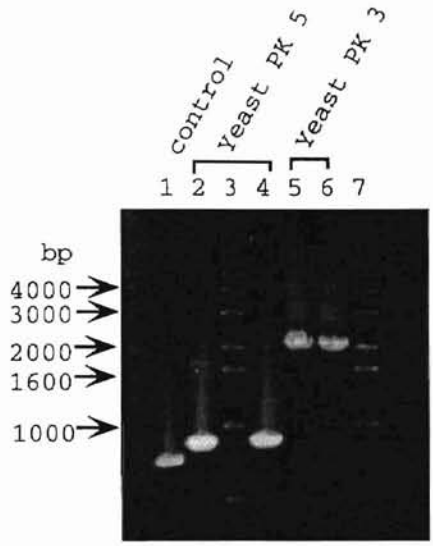
control
Yeast PK 5
Yeast PK 3
1 2 3 4 5 6 7
bp
4000
3000
2000
1600
1000

Fig. 10

Figure 10

Amplification of the 5'- and 3- noncoding region of yeast pyruvate kinase from pPYK101

Lane 1: Amplification of the control 769 bp DNA fragment. Lane 3 and 7: 1 Kb DNA ladder. Lane 2 and 4: Amplification of 925 bp of the 5' non-coding region of yeast PK from pPYK101 which was produced with an annealing temperature of 55°C and either 150 ng or 250 ng of DNA, respectively. Lane 5 and 6: Amplification of 1989 bp of the 3' non-coding region of yeast PK from pPYK101 which carried out with an annealing temperature of 55°C and either 150 ng or 250 ng of DNA concentration, respectively.

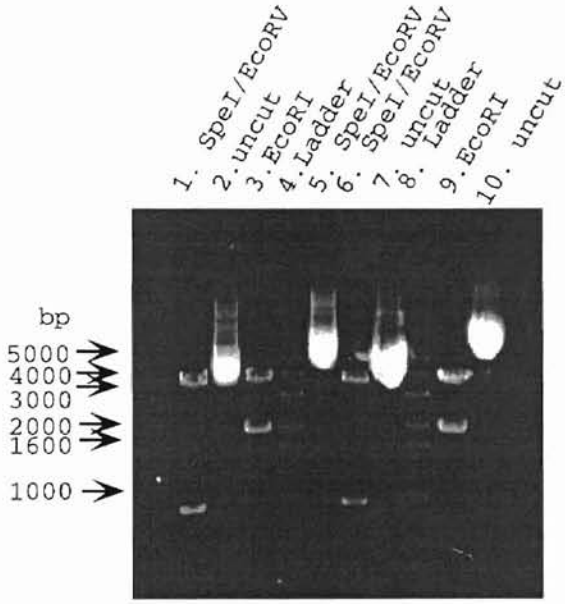
1. SpeI/EcoRV
2.uncut
3.EcoRI
4.Ladder
5. SpeI/EcoRV
6. SpeI/EcoRV
7. uncut
8. Ladder
9.EcoRI
10. uncut
bp
5000
4000
3000
2000
1600
1000

Fig. 11

Figure 11

Analysis of restriction enzyme digestion of the PCR product of the 5′- and 3′- noncoding region of the yeast PK which constructed into pCR2.1 vector at the EcoRI site, respectively.

Lane 4 and 8: 1Kb DNA ladder. Lane 1 and 6: SpeI/EcoRV digestion of the 5′ non-coding region of yeast PK cloned into the pCR2.1vector. Lane 2 and 7: uncut control for the 5′-noncoding region of the yeast PK in the pCR2.1 vector. Lane 3 and 9: EcoRI digestion of the 3′ noncoding region of yeast PK constructed in the pCR2.1 vector. Lane 5 and 10 : uncut control for the 3′-noncoding regionof the yeast PK in the pCR2.1 vector.

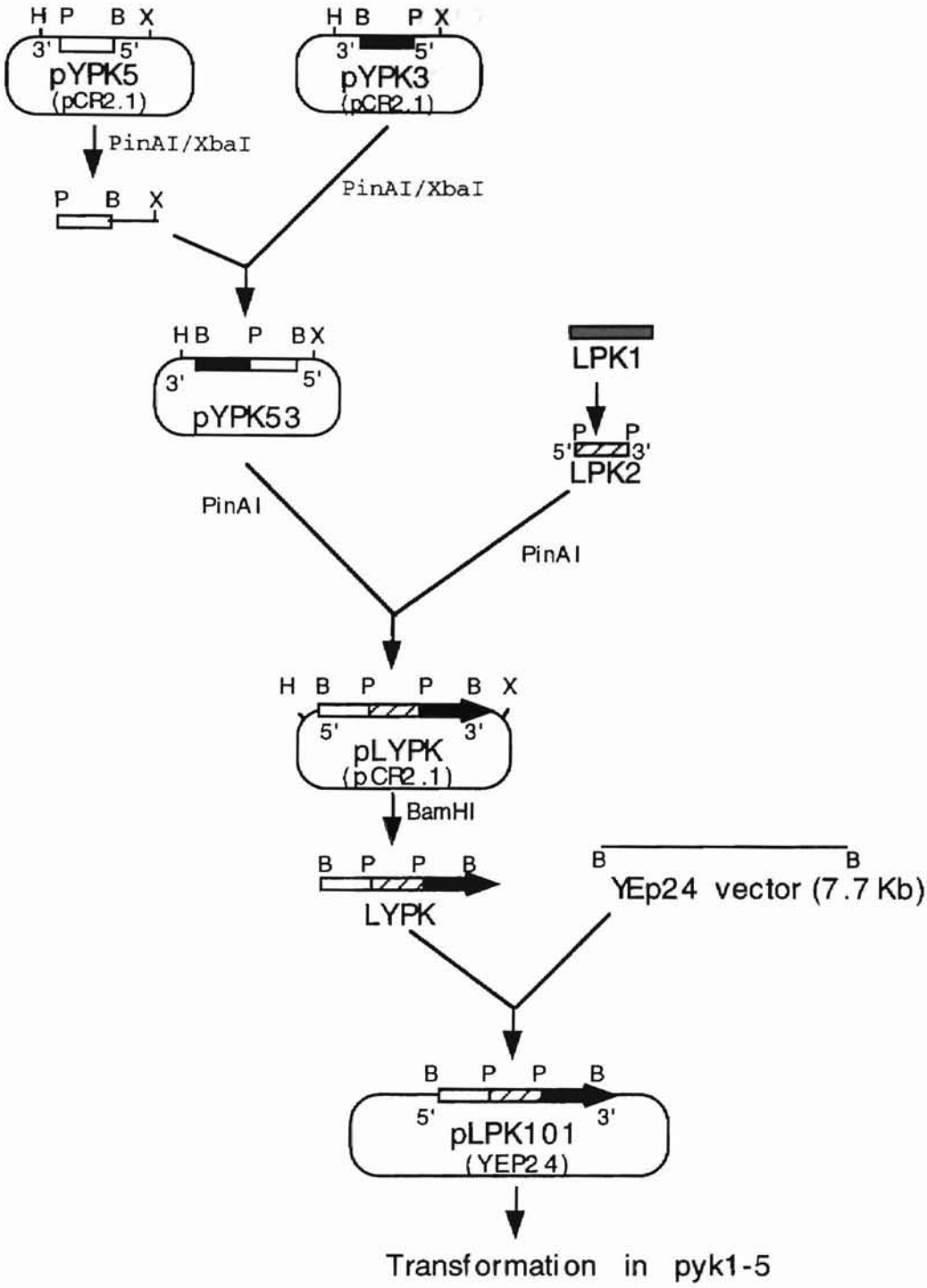

Fig 12. The strategy of pLPK101 plasmid construction (H; HindIII, P; PinAI, B; BamHI, X; XbaI)

The construction of pLPK101

The scheme of the construction of pLPK101 for this study is diagrammed in Fig 12.

To fuse the 5'- and 3'- noncoding regions in a single plasmid vector, pYPK5 was digested with PinAI+XbaI yielding the 0.9 Kb of 5' non-coding region and this fragment was then ligated to pYPK3 which had been digested with PinAI+XbaI. This fused plasmid was designated pYPK53 (Fig 13). When pLPK2 was digested with PinAI, the electrophoresis result indicated that pLPK2 was not digested by PinAI to yield the expected 1.6 Kb fragment. There is a possibility of modification of the PinAI sites on the primers during PCR and manipulation with the vector (Data not shown). To avoid this problem, new oligonucleotide primers were synthesized with same sequences as primers used for LPK2 amplification and the new PCR product was ligated in either the pCR2.1 vector or the pYPK 53 plasmid directly after digestion with PinAI. Analysis with PinAI digestion after cloning suggested that the PCR product of the rat LPK gene can be ligated in pYPK53 directly while can not be digested by PinAI due to unknown reasons.

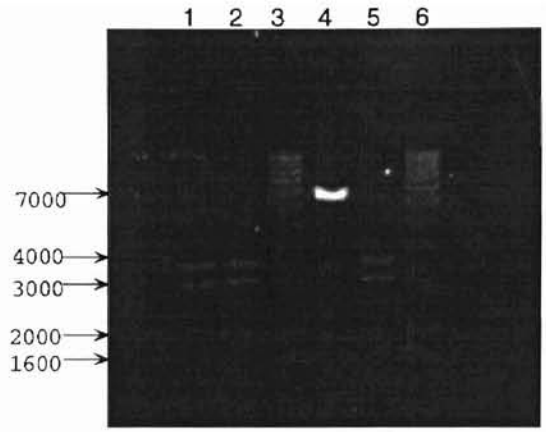

Fig. 13

Fig 13

Analysis of the restriction enzyme digestion of pYPK53 containing the fused 5'- and 3'- noncoding region of the yeast PK.

Lane 1,2 and 5: Each plasmid DNA was prepared from four colonies containing pYPK53 after *E. coli* transformation, digested with HindIII and XbaI to confirm the existence of the 5'- and 3'- noncoding region of yeast PK. 2.9 Kb fragment for the fused 5'- and 3'- noncoding region and 3.9 Kb fragment for the vector were generated. Lane 3 and 6: 1Kb DNA ladder. Lane 4: uncut control

The coding region of the rat L-type pyruvate kinase gene was inserted in pYPK53 at the PinAI site. Two orientation could be produced from this ligation as shown in Fig 15. The correct direction can be determined by BstXI digestion. In case of the correct direction, BstXI should produce a 3.9 Kb from the pCR2.1 vector and 3.2 Kb and 1.3 Kb from LYPK since BstXI is located at position 280 and 306 in pCR2.1 vector and at position 381 in rat liver PK. The digestion result in Lane 10 indicates the correct direction and used for the further cloning (Fig. 15). This cloned plasmid was designating pLYPK.

Attempts to clone the LPK gene in YEp24 failed several times for unknown reason. Hence, the YEp24 vector was prepared from pPYK101 by BamHI digestion followed by dephosphorylation. The coding region of the rat L-type pyruvate kinase gene embedded in the yeast 5'- and 3'-noncoding regions, a 4.5 Kb fragment, was obtained from pLYPK by BamHI digestion and cloned in the linearized YEp24 vector as shown in Fig 12. The construction was confirmed with BamHI digestion. This completed construction was designated pLPK101. The results of the BamHI digestions of transformants resulting from the ligation implied that LYPK was not completely separated from pCR2.1 vector by the gel electrophoresis, since the size of both fragments is only slightly different(3.9 Kb and 4.5 Kb, respectively) making the separation difficult, only one plasmid containing the entire pLYPK insert (Lane 3) was obtained among the 20

analyzed samples. The other lanes seem to be clones of pCR2.1. The transformant in Lane 3 was further analyzed with various restriction enzymes to confirm the existence and orientation of the inserted gene.

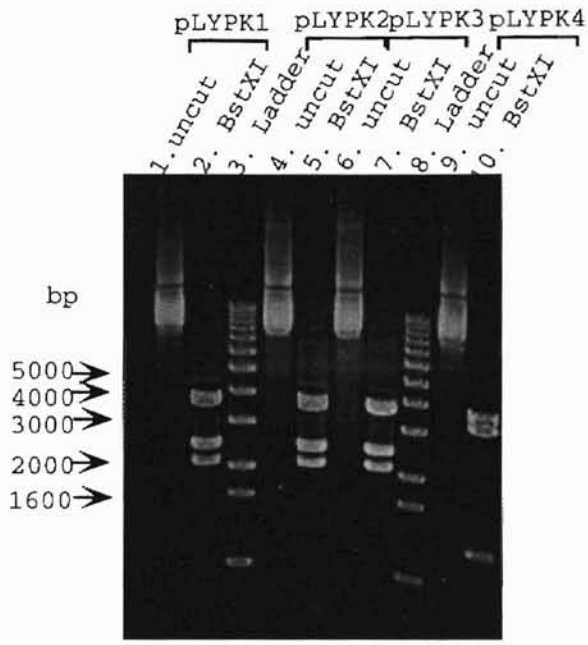
pLYPK1
pLYPK2
pLYPK3
pLYPK4
1. uncut
2. BstXI
3. Ladder
4. uncut
5. BstXI
6. uncut
7. BstXI
8. Ladder
9. uncut
10. BstXI
bp
5000
4000
3000
2000
1600

Fig. 14

Fig. 14

Analysis of the restriction enzyme digestion of pLYPK to determine orientation of LYPK gene.

Lane 3 and 8 : 1Kb DNA ladder. Lane 1: uncut control of pLYPK1. Lane 2: BstXI digestion with pLYPK1 generating 3.9 Kb, 2.2 Kb and 2.4 Kb fragments (inverted direction).
Lane 4 and 5: uncut control and BstXI digestion of pLYPK2.
Lane 6 and 7 : uncut control and BstxI digestion of pLYPK3.
Lane 9 and 10 : uncut control and BstXI digestion of pLYPK4 (correct direction).

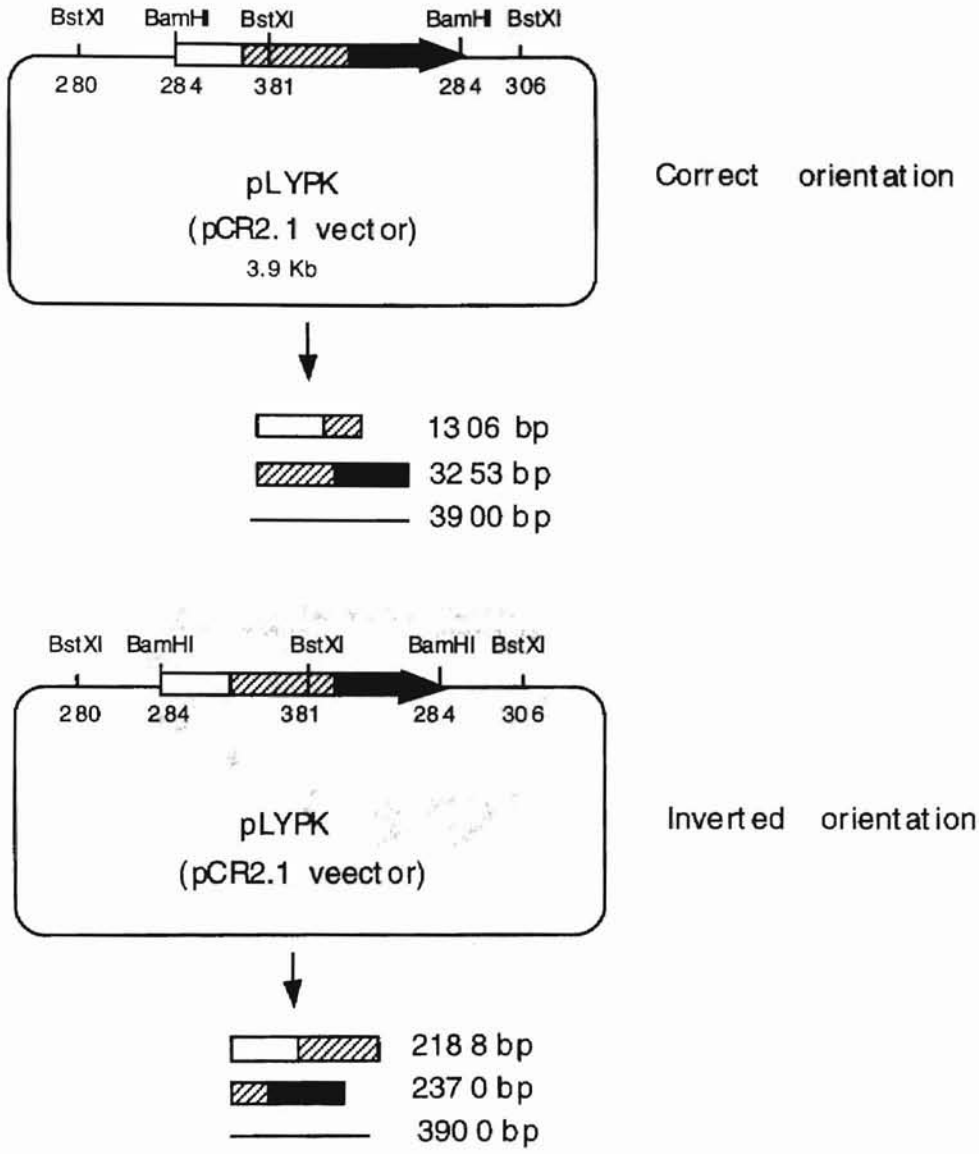

Fig 15. Orientation of pLYPK determined with BstXI digestion

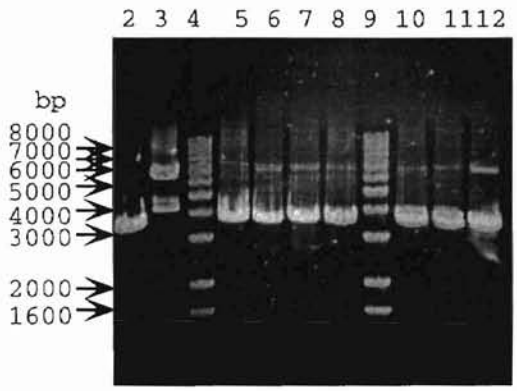

Fig. 16

Fig. 16

Analysis of the potential pLPK101 transformants with BamHI digestion

Plasmid from transformants of the YEp24-LYPK ligation obtained in HB101 cells were digested with BamHI and analyzed on a 0.9 % agarose gel. BamHI digestion should produce 4.5 Kb fragment from the LYPK gene and a 7.7 Kb fragment from the YEp24 vector gene. The expected fragment sizes were only observed in Lane 3 indicating this transformant has pLPK101 plasmid. The other lanes except Lane 3 seem to contain the pCR2.1 vector at 3.9 Kb. Lane 4 and 9: 1 Kb DNA ladder

Orientation of the pLPK101

The LYPK gene was cloned into the BamHI site of the YEp24 vector. To determine orientation of the insert, restriction enzymes were used to digest pLPK101. Fig 17 presents the results of the restriction enzyme digestion. The presence of the insert and its orientation in pLPK101 was determined by these results. As shown in Fig 18, the LYPK gene is oriented in the opposite direction as tetracycline resistance gene, in which the BamHI site is located on YEp24 vector (Fig 18).

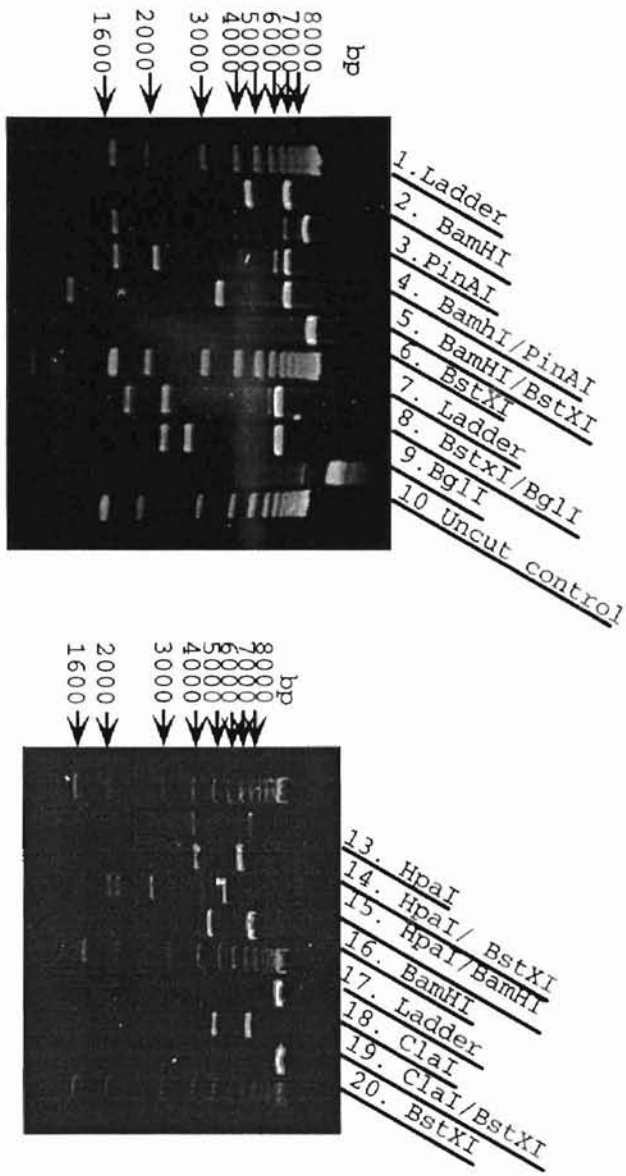
bp
8000
7000
6000
5000
4000
3000
2000
1600
1.Ladder
2. BamHI
3.PinAI
4. BamhI/PinAI
5. BamHI/BstXI
6. BstXI
7. Ladder
8. BstxI/BglI
9.BglI
10 Uncut control
bp
8000
7000
6000
5000
4000
3000
2000
1600
13. HpaI
14. HpaI/ BstXI
15. HpaI/BamHI
16. BamHI
17. Ladder
18. ClaI
19. ClaI/BstXI
20. BstXI

Fig. 17

Fig. 17

Analysis of the restriction enzyme digestion of pLPK101 to determine orientation.

Lane 1, 7 and 11: 1 Kb DNA ladder. Lane 2: BamHI. Lane 3: PinAI. Lane 4: BamHI/PinAI generating 7.7 Kb, 1.9 Kb, 1.6 Kb and 0.9 Kb fragments. Lane 5: BamHI/BstXI generating 7.7 Kb, 3.2 Kb, 1.3 Kb fragments. Lane 6: BstXI. Lane 8: BstXI/BglI generating 6.1 Kb, 2.3 Kb, 1.8 Kb and 0.9 Kb fragments. Lane 9: BglI generating 6.8 Kb, 2.7 Kb and 2.3 Kb fragments. Lane 10: uncut control of the pLPK101. Lane 12,17 and 21: 1Kb DNA ladder. Lane 13: HpaI. Lane 14: HpaI/BstXI. Lane 15: HpaI/BamHI. Lane 16: BamHI. Lane 18: ClaI. Lane 19: ClaI/BstXI producing 7.5 Kb and 4.7 Kb fragments. Lane 20: BstXI

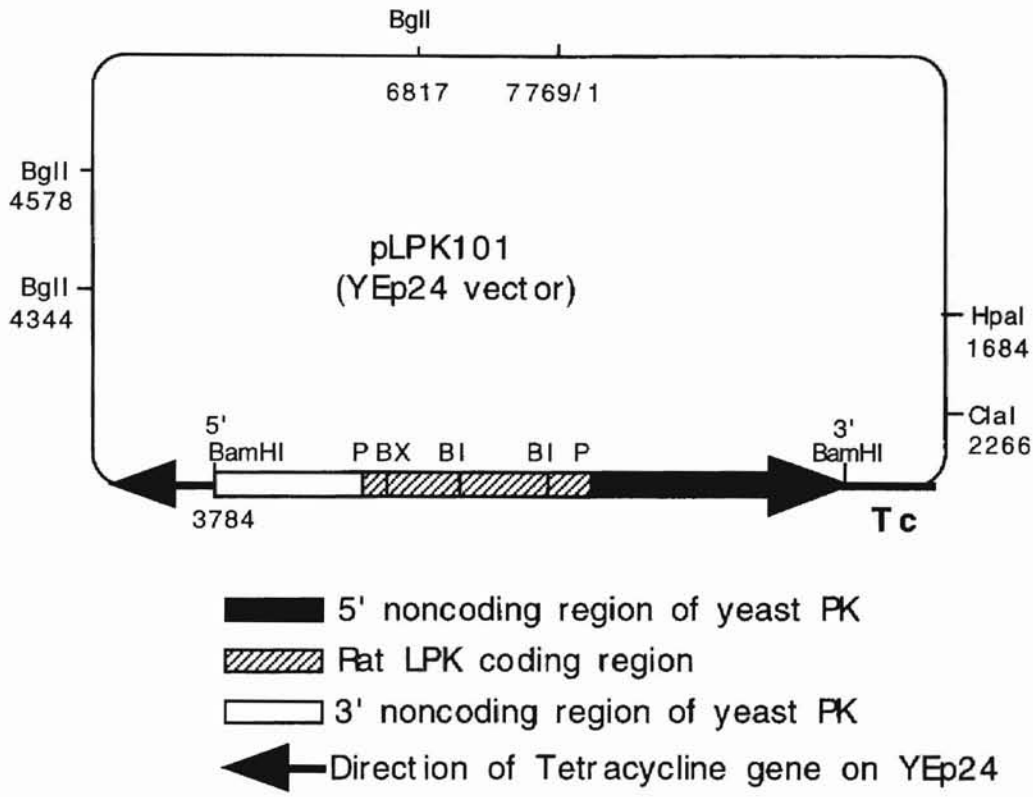

Fig 18. Orientation of the transformed pLPK101 plasmid (P: PinAI, BX: BstXI, Bl: BglI)

Yeast Transformation with pLPK101

The purpose of this study is to express the rat L-type pyruvate kinase protein under the control of the yeast pyruvate kinase promoter in yeast. Plasmid pLPK101 , bearing the rat L-type pyruvate kinase gene under the yeast pyruvate kinase promoter system provides a means of doing this.

Pyk1-5, a yeast mutant strain which is unable to grow on glucose and does not express active pyruvate kinase, was chosen to transform with pLPK101 and transformants were selected for their ability to grow on glucose.

pLPK101 was transformed into the yeast with different concentration of DNA, 460 ng, 2 μg and 4 μg, dissolved in 1 ml 1x TE buffer to optimize the DNA concentration for high transformation efficiency. Only a few colonies were obtained from each plate after 5 days suggesting efficiency of the expression system is low. The pPYK101 plasmid which carries the entire yeast pyruvate kinase sequences was also transformed into yeast as a control. All transformants tested gave similar responses to FBP, Ala and ATP, so the data on pyruvate kinase from one recombinant was chosen for this study. Pyruvate kinase enzymes were purified for further analysis by enzyme kinetics and Western Blot.

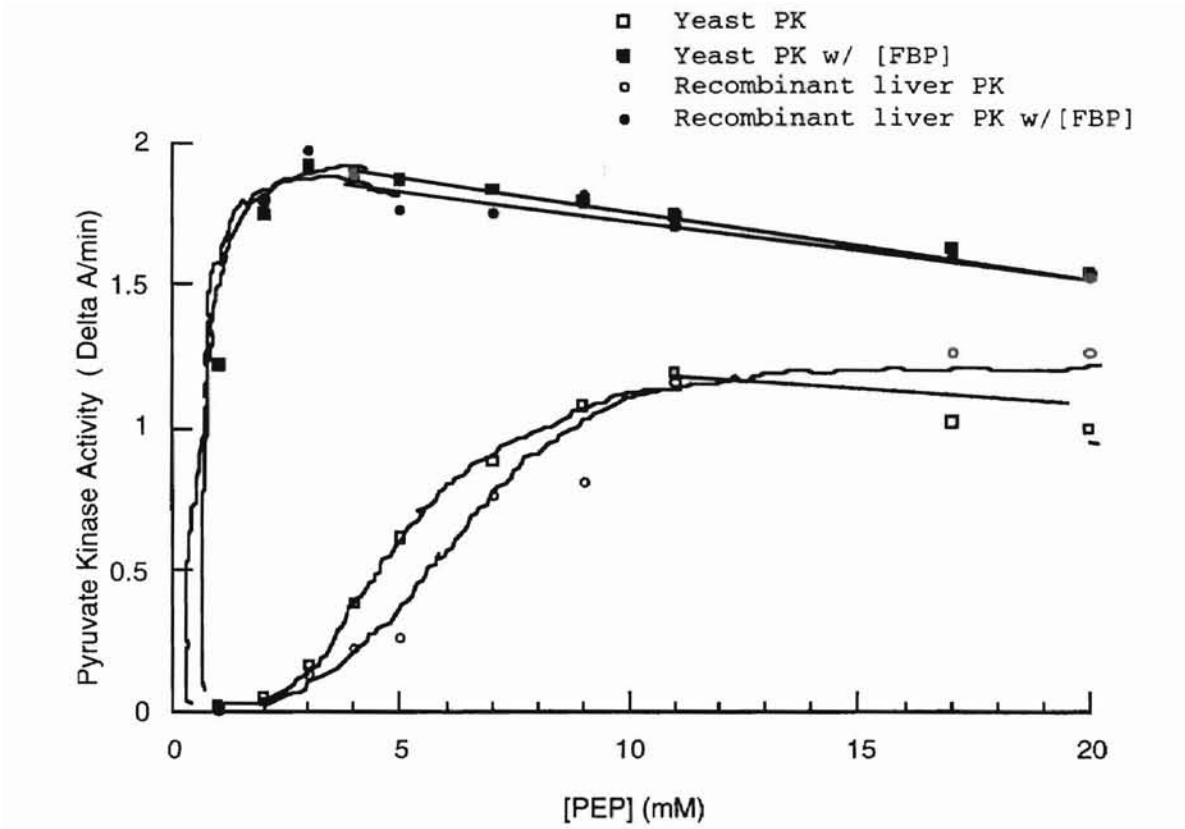

Fig 19. The plot of pyruvate kinase enzyme activities against [PEP] at the presence and absence of [FBP] measured with recombinant rat liver pyruvate kinase and yeast pyruvate kinase.

Table 4. Effect of Ala on the recombinant enzymes in the transformed pyk1-5

Yeast pyruvate kinase control					Recombinant rat liver PK enzyme				
Enzyme (µl)	[PEP] (mM)	[Ala] (mM)	Activity w/o [Ala] (ΔA/min)	Activity w/ [Ala] (ΔA/min)	Enzyme (µl)	[PEP] (mM)	[Ala] (mM)	Activity w/o [Ala] (ΔA/min)	Activity w/ [Ala] (ΔA/min)
10	5	5	0.250	0.283	10	5	5	0.669	0.619
10	5	10	0.305	0.287	10	5	10	0.684	0.623

Enzyme assays

Pyruvate kinase catalyses the conversion of PEP to pyruvate and this reaction can be coupled with the reaction of lactate dehydrogenase. Pyruvate kinase activities can be determined by absorbance at 340 nm by the consumption of NADH in the reaction of pyruvate by lactate dehydrogenase. Fig 19 shows enzyme activities as a function of the concentration of PEP in the absence and presence of FBP. Pyruvate kinase activities of recombinant liver enzyme and yeast control enzyme gave a sigmoidal response with increasing PEP concentration at 5 mM ADP. Both enzyme activities were stimulated by FBP as a result of a shift in [PEP] dependence.

$S_{1/2}$ refers to the substrate concentration at which half maximal activity is observed. $S_{1/2}$ of PEP is known to be 4.5 mM for yeast pyruvate kinase (Haeckel *et al.* 1968, Gancedo *et al.* 1967) and $S_{1/2}$ for rat liver pyruvate kinase is reported to be in the range from 0.5 to 0.8 mM (Taylor and Bailey 1967, Imamura *et al.* 1972 and Tanaka *et al.* 1967). In this study, the recombinant rat liver pyruvate kinase exhibited an $S_{1/2}$ value of approximately 6.5 mM PEP as shown in Fig 19. The yeast control gave an $S_{1/2}$ value of approximately 4.8 mM.

Table 5 shows the effect of Ala on rat liver pyruvate kinase activity at 5 mM PEP and 5 mM ADP. None of the recombinant pyruvate kinase transformed in pyk1-5 was inhibited by Ala even though we expected to see the inhibition on ala for the recombinant enzyme. A similar

experiment was also done with ATP. However, the data obtained was not interpretable so the report on ATP inhibition was excluded in this study.

Western Blot Analysis

The expression of rat L-PK in yeast was examined by Western Blot with polyclonal antibodies specific for rat liver pyruvate kinase proteins as well as polyclonal antibodies for yeast pyruvate kinase protein. Lane 2 to Lane 5 were detected using antibodies specific for rat liver pyruvate kinase proteins. As shown in Fig 20, proteins in Lanes 3 and 4 cross-reacted with antibodies against rat liver pyruvate kinase at the similar molecular weight to the protein detected from the rat liver extract control in Lane 5. This band is absent from the yeast pyruvate kinase control in Lane 2 suggesting that the liver pyruvate kinase protein is expressed in the recombinant liver pyruvate kinase yeast constructs. A lighter staining of a band migrating at the position of yeast pyruvate kinase also noted in Lane 2 to 4, indicating a weak cross reactivity of the antibody with yeast pyruvate kinase.

Lane 6 is the yeast control proteins and Lane 7 and 8 are proteins from recombinant rat liver pyruvate kinase. Lanes 6 to 9 were detected using antibodies specific for yeast pyruvate kinase. The cross-reacted patterns of proteins in Lane 7 and 8 are similar to those from yeast control in Lane 6 since we expected the observation of the

inactive proteins of yeast pyruvate kinase expressed in pyk1-5. We believe the multiple antibody reactive proteins seen with anti-yeast pyruvate kinase antibodies represent degradation products. The staining intensity observed in Lane 7 and 8 compared to those in Lane 6 indicates that high concentration of yeast pyruvate kinase proteins was expressed from yeast transformation. No proteins were detected from rat liver extract control by antibodies against yeast pyruvate kinase.

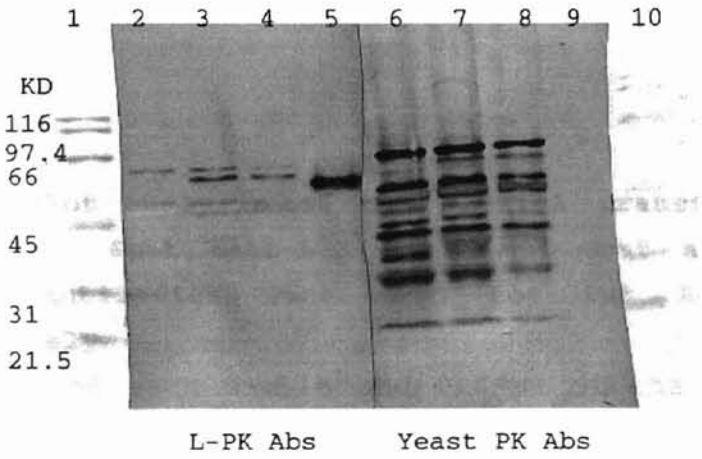

Fig.20

Fig. 20

Western blot analysis of the pLPK101 transformants expressed in pYK1-5. Goat anti-LPK serum and Goat anti-YPK serum as primary antibodies were used for rat Ab and yeast Ab, respectively

3 µl of each sample and 0.4 µl of the rat liver control were loaded in each lane. Lane 1 and 10: SDS-PAGE weight standard(BioRad). Lane 3, 4, 7 and 8: recombinant rat liver pyruvate kinase. Lane 2 and 6: yeast control. Lane 5 and 9: rat liver extract control. Lane 2 to 5 cross-reacted with antibodies against rat liver pyruvate kinase. Lane 6 to 9 cross-reacted with antibodies against yeast pyruvate kinase.

Discussion

Sequence Analysis of Pyruvate Kinase from pyk1-5

Three dimensional structures of the M1 pyruvate kinase from cat and rabbit muscle have been reported (Muirhead *et at.* 1986, Larsen *et al.* 1994). Protein sequences were ~94% similar between the M1 pyruvate kinases from cat and rabbit. There are the strong sequence similarities among the pyruvate kinase sequences of different species in the active site and the sites close to the active site. The active site of each subunit is located in the cleft between Domain A and B. The binding site for PEP is close to the active site and lies in the loops which connect strands Aβ4-6 to Aα4-6. The MVARGDL**G** motif belongs to the strand Aβ6 and Aα6 at position 263 from yeast PK and is one of the most highly conserved motifs. The mutation from 270 Gly to Asp in yeast pyruvate kinase observed in this study is located in this motif.

Pyruvate kinase requires two metal ions (Mg^{2+} and K^{+}) for its activity. The MVARGDL**G** motif is suggested to be related to the divalent cation binding site. In the cat enzyme, main chain carbonyl oxygens of Ala and Arg in this motif were reported as probable ligands by Muirhead *et al.* 1986 to produce an octahedral complex with Mg^{2+}. The movement of the main chain carboxyl group would allow Arg to closer to the phospho-binding sites. Larsen *et al.* 1994 crystallized the

enzyme complexed with Mn^{2+} and K^{+} and suggested the carboxyl group of Asp in this motif as one of the probable ligands.

The mutation from 270 Gly to Asp of yeast pyruvate kinase might suggest that the new negatively charged side chain of Asp could effect the ligands suggested for the enzyme-bound Mg^{2+} by Muirhead et. al. and Larsen *et al.* on the MVARGDL**G** motif. Hence, the interference with normal conformational changes would be responsible for the loss of enzyme activity.

pLPK101 Transformation

The pLPK101 is the yeast vector construct which carries rat liver pyruvate kinase coding region with yeast promoter and termination regions. The construction of pLPK101 was confirmed by restriction enzyme digestion. pLPK101 was transformed into pyk1-5, a yeast mutant strain which has no detectable pyruvate kinase activity. After transformation, pyk1-5 was complemented to a phenotype which can survive on glucose. Transformation with recombinant L-type pyruvate kinase also led to production of an active pyruvate kinase in cell extracts.

It is expected that this pyruvate kinase activity is the L-type pyruvate kinase, expressed from the recombinant liver pyruvate kinase cDNA. However, the kinetic properties observed with transformants raise questions because this recombinant protein was not inhibited by Ala as is characteristic of L-type pyruvate kinase. However, the

results from Western Blot analysis indicates that the L-type pyruvate kinase is expressed in the recombinant yeast since the protein is detected at a molecular weight similar to rat L-type pyruvate kinase.

More work is essential to clarify these issues. One possibility is that the expressed L-type pyruvate kinase enzyme forms a mixed tetramer with inactive yeast pyruvate kinase produced by the pyk1-5 mutant and this mixed tetramer influences the activity of the L-type pyruvate kinase.

Four subunits are associated in a tetramer along *q* and *r* axis. The intersubunit contact regions involve residues of Domain A related by r axis and Domain C related by *q* axis. Helices Aα6, Aα7 and Aα8 mostly contribute to the intersubunit contact region along the r interface and the residues taking part in this contact region are highly conserved in pyruvate kinase from different species. Each helical region has 55%, 83% and 60% similarity, respectively, between yeast pyruvate kinase and rat liver pyruvate kinase. The Aα7 helix is especially known to be strictly conserved among pyruvate kinase sequences (Fothergill and Michels 1992, Matteri *et al.* 1995, Muirhead *et al.* 1986). The intersubunit contact region also is involved in the strand Cβ5 along the q interface and 45% similarity exists between yeast pyruvate kinase and rat liver pyruvate kinase in the region. Because of the high sequence similarity in helices Aα6 to Aα8 in the

contact region in the rat and yeast pyruvate kinases, it seems plausible to suggest that the subunit of rat liver PK could contact with the subunit of yeast PK to form a mixed tetramer. Hence, the inactive yeast PK enzyme might effect the kinetic properties for the expressed rat liver PK giving no response to ala.

Another possible explanation of the current findings is that an inactivated yeast pyruvate kinase in pyk1-5 has been mutated giving an active enzyme form of yeast pyruvate kinase. However, the Western Blot analysis is not consistent with this interpretation since the expression of rat liver pyruvate kinase was indicated from the Western Blot analysis. More work will be needed to eliminate this possibility.

A mutation may also have occurred during the manipulation steps like RT-PCR and the cloning. The LPK1 PCR product was sequenced along only one direction and the sequence difference between LPK1 and rat liver PK published by Cognet et al. 1987 was found from sequence alignment. This result indicates the possibility of mutation. There are three potential mutation site on LPK1 gene and only a mutation from 277 Ile to Val is responsible for amino acid alternation (Fig. 6). This alternation site belongs to Domain A on yeast pyruvate kinase and this does not participate in the formation of secondary structural elements and this possible amino acid alternation does not seem to be important since the property of amino acid side chain did not changed. It is not clear this result is occurred because of

sequencing error or the different strains. This sequencing analysis requires more work for the reliable data and this sequencing difference will be considered for the future research.

A better approach is transformation with a "Knock out" yeast strain which does not produce yeast pyruvate kinase proteins. Our Lab is currently working on this approach and better interpretation may be obtained from this approach.

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VITA

Soyeon Park

Candidate for the Degree of
Master of Science

Thesis: STUDIES ON PYRUVATE KINASE IN YEAST

Major field: Biochemistry and Molecular Biology

Biographical: September 1991 to July 1995, YoungNam University, Korea; received Bachelor of Science degree in Biochemistry; received Academic Scholarship in 1991 and 1994. August 1995 to May 1998, Oklahoma State University, U. S. A.; received Master of Science degree in Biochemistry and Molecular Biology.