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Identification and Characterization of Hepatocyte-specific Regulatory Regions of the Rat Pyruvate Kinase L Gene

THE SYNERGISTIC EFFECTS OF MULTIPLE ELEMENTS*

(Received for publication, June 1, 1990)

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The rat pyruvate kinase L (PKL) gene produces the L- and R-type isozymes by alternative transcription that is regulated in a tissue-specific manner. To investigate which DNA elements are involved in hepatocyte-specific expression of the L-type isozyme, we performed transient DNA transfer experiments with PKL/chloramphenicol acetyltransferase fusion genes. We found three positive regulatory regions required for expression of the L-type isozyme in adult rat hepatocytes by functional analyses of a series of 5' and internal deletion constructs of the fusion genes. These regions, designated as PKL-I, PKL-II, and PKL-III, were located between nucleotides -76 and -94, -126 and -149, and -150 and -170, respectively. PKL-I showed enhancer-like activity alone, whereas PKL-II and PKL-III did not have any independent effect. Combinations of L-I + L-II and L-II + L-III, but not of L-I + L-III, showed synergistic enhancer activities when oriented in the same direction. The inclusion of all three elements oriented in the same direction had the maximum synergistic effect, indicating that these elements function as a unit. This unit enhanced expression from heterologous as well as homologous promoters in a manner that was independent of its orientation and position relative to the cap site. The activity of the unit was not detected in HeLa cells or K562 erythroleukemia cells, suggesting that this unit possessed cell-type specificity.

PKL-I consists of a palindromic sequence 5'-CTGGTTATACCTTTAACCAG-3', which contains a sequence homologous to the LF-B1-binding site. PKL-II contains the sequence 5'-TTCTTGACTCTGGCCCCCAGTGT-3', which is similar to that of the LF-A1-binding site. PKL-III contains a palindromic sequence 5'-CCACGGGGCCTCCGTGG-3', which includes a sequence homologous to the binding site of the adenovirus major late transcription factor. Gel retardation assay indicated that the different trans-acting factors interacted with three elements and that the trans-acting protein bound to PKL-I was in fact LF-B1. However, the trans-acting factors bound to PKL-II and PKL-III were different from LF-A1 and major late transcription factor, respectively.

Thus, we conclude that three cis-acting elements are very important for specific expression of the PKL gene in hepatocytes and that LF-B1 and two unknown factors bound to these elements interact with each other to cause a synergistic effect.

Pyruvate kinase (ATP:pyruvate O2-phosphotransferase, EC 2.7.1.40), a key glycolytic enzyme, has four isozymes in mammals, which are named the M1-, M2-, L-, and R-types (1-3). The L-type isozyme is expressed in a tissue-specific manner; this form is expressed primarily in parenchymal cells in liver and is also present in kidney and intestine as a minor isozyme (2). Its expression is regulated developmentally (2). Expression of the hepatic L-type isozyme is also regulated transcriptionally by insulin and glucagon (4).

To investigate the molecular mechanisms underlying these regulations, we analyzed the genomic structure of the rat pyruvate kinase L (PKL) gene and found that the PKL gene encoded the R-type isozyme, which is expressed only in erythroid cells (5), in addition to the L-type (6). The PKL gene is composed of 12 exons and 11 introns with a length of about 9.3 kilobase pairs (6). The first (exon R) and second (exon L) exons encode the 5'-terminal sequences specific to the R- and L-types, respectively, whereas the remaining downstream exons are common to the two isozymes. Promoter sequences are present in the upstream region of exon R and exon L, respectively, indicating that the L- and R-type isozymes of rat pyruvate kinase are produced from the PKL gene by use of different promoters.

Recent studies indicated that control of gene expression is achieved through the interactions between cis-acting DNA elements and trans-acting proteins that bind to these DNA sequences (7-9). cis-Acting DNA elements responsible for cell type-specific and hormonal regulation of gene expression have been identified (10, 11). Most of these elements are located in the 5'-flanking region of the gene. Recently, the 5'-flanking regions of liver-specific genes were found to contain liver-specific elements, including elements interacting with the trans-acting factors named LF-B1 (also referred to as HNF1) and LF-A1 (12, 13). The cis-acting elements interacting with ubiquitous factors such as TGGCA-binding protein (14), CAAT box transcription factor (15), and adenovirus major late promoter transcription factor (MLTF) (16) are also implicated in gene expression in the liver.

In this work, we examined the 5'-flanking region of the...
PKL gene using transient expression assay based on the chloramphenicol acetyltransferase gene and analyzed trans-acting proteins by gel retardation assay. Here we report the identifications and characterizations of three cis-acting elements required for hepatocyte-specific expression of the PKL gene and trans-acting factors interacting with them.

**EXPERIMENTAL PROCEDURES**

**RESULTS**

*Expression of the Rat Pyruvate Kinase L/Chloramphenicol Acetyltransferase Fusion Gene in Primary Cultured Hepatocytes—* We used electroporation for DNA transfection because of a report that DNA can be introduced into primary rat hepatocytes by electroporation but not with calcium phosphate or DEAE-dextran (28). When the cells were suspended in Dulbecco’s phosphate-buffered saline (–), the highest transfection efficiency was achieved with a single voltage pulse of 250–300 V at 960 μF. We also found that this buffer gave better results than the buffer described by Chu et al. (21). To investigate the regulatory region of the rat PKL gene, we constructed pLcat2300, which contains a fragment from approximately nucleotide –3200 to +37 relative to the transcription start site of the L-type isozyme. A series of 5’-deletion mutants of this fusion gene were also constructed. These plasmids were introduced into freshly isolated hepatocytes by electroporation and transient expression of CAT activity was determined. pUC2cat and pUC0cat were used as positive and negative controls, respectively. The results are shown in Fig. 1. The CAT activity of hepatocytes transfected with pLcat2300 was about 70% of that of cells transfected with pUC2cat. This indicates that strong promoter activity is present in the 3.2-kilobase upstream region. 5’-Deletions up to −280 caused only minor change in activity, but further deletion up to −190 resulted in considerable increase in promoter activity, suggesting the existence of an inhibitory element in the region from −279 to −190. Progressive deletion from −189 to −63 caused progressive decrease in activity. The largest reduction in activity was obtained by deletion from −189 to −153 followed by those from −94 to −63 and −152 to −96. Deletion up to −63 resulted in almost complete loss of activity. pRcat2700 containing the promoter region and the cap site of the R-type isozyme did not show any activity in hepatocytes. These results suggest that multiple cis-acting elements involved in regulation of transcription of the L-type isozyme are present in the 5’-flanking region from position −279 to −63. The results also indicate that the promoter region of the R-type isozyme is not active in hepatocytes in which this isozyme is not expressed.

*Identification of cis-Acting Elements of the Pyruvate Kinase L Gene—* We focused attention on positive regulatory elements between −189 and −63 because this region is most important for expression of the L-type isozyme in hepatocytes. Analysis of the nucleotide sequence of this region revealed the presence of a sequence homologous to the LF-B1-binding site (13) between −94 and −63. Therefore, we analyzed the positive regulatory region by dividing the sequence into two regions of −189 to −95 and −94 to −63, which we named the distal and proximal regions, respectively.

First, we constructed pLcat74 to analyze the proximal region in detail (Fig. 2A). When pLcat74 was transfected into hepatocytes, no CAT expression was detected, suggesting that the sequence between −94 and −75 may be responsible for the activity in the proximal region. To examine this possibility, we inserted a synthetic double-stranded oligonucleotide containing nucleotide −94 to −76 into pLcat62 and transfected the resultant plasmid into hepatocytes. The activity of this construct was comparable to that of pLcat94 (Fig. 2A). Thus the region from −94 to −76 is one of the regulatory elements required for expression of the PKL gene in hepatocytes. We named this region PKL-I.

Next, we analyzed the distal region (−189 to −95). To determine the 5’-end of this region, we inserted fragments of −185/−95, −170/−95, and −155/−95 into pLcat94 in the sense orientation, and transfected the resultant plasmids into hepatocytes. As shown in Fig. 2B, constructs containing −185/−95 and −170/−95 showed high CAT activity comparable to that with pLcat189. However, the CAT activity of the construct containing −155/−95 had much lower activity. Then, we inserted fragments of −170/−126 and −170/−139 into pLcat94 to examine the 3’-end of the distal region. A construct containing a −170/−126 fragment was as active as pLcat189 in hepatocytes (Fig. 2C). The −170/−139 fragment also enhanced the CAT activity of pLcat94, but less than the −170/−126 fragment. These results indicated that the fully...
active distal region of the PKL gene is located between -170 and -126. We named this region PKL-D. Two noteworthy sequences are present in PKL-D. One is located between nucleotides -170 and -150 and the other between -149 and -126. The former region contains a palindrome sequence which is similar to the MLTF binding sequence of the rat γ-fibrinogen gene (16). We named this region PKL-III. On the other hand, the latter region is similar to the LF-Al binding sequence of the human α1-antitrypsin gene (13). We named this region PKL-II.

So, we examined whether these two elements in the distal region are distinct, or whether they interact with each other or with PKL-I. For this, we synthesized double-stranded oligonucleotides of PKL-I, PKL-II, PKL-III, and PKL-D (L-II + L-III), ligated them into pLcat62' separately or in various combinations and transfected them into hepatocytes. Of the three elements, only L-I caused significant increase in CAT activity (more than 10-fold) in either orientation when ligated alone (Fig. 3). The activity of L-I was synergistically enhanced by ligation of L-II in the same orientation, but not in the opposite orientation. Change in the orientation of L-II in this construct (L-I + L-II + L-III in the same orientation) also resulted in marked decrease in the activity. When both L-II and L-III in the same orientation were ligated into pLcat62' or L-I-pLcat62' in either orientation, significant enhancement of CAT activity was observed. These results indicate that PKL-II and PKL-III are independent functional elements that interact synergistically with each other, and that L-I also interacts synergistically with L-II, but not L-III.

Characterization of cis-Acting Regions of the Pyruvate Kinase L Gene—First, we examined whether the regulatory regions of the PKL gene interact with heterologous promoters. When a -185/-63 fragment containing all three elements was inserted upstream or downstream of pUC1cat containing the SV40 early promoter, the resulting constructs showed much higher CAT activity than the original pUC1cat (Fig. 4, A and B). We also tested another heterologous promoter with pMcat0.2 which contains the promoter region of the rat pyruvate kinase M gene. This plasmid showed activity in dRLh-84 cells, which express only the M-type isozyme of pyruvate kinase (data not shown), but expressed essentially no CAT activity in hepatocytes (Fig. 4C). When the -185/-63 fragment was inserted upstream of the pMcat0.2 in the antisense orientation, CAT activity was markedly enhanced. These results indicate that the whole regulatory region of the PKL gene can enhance the heterologous promoter activity and that its effect is not dependent on its position or orientation.

Second, to examine whether the regulatory elements of the PKL gene show cell-type specificity, the deletion mutants were transfected into HeLa cells (Fig. 1). In HeLa cells, which express no L-type isozyme, all transfected plasmids were essentially inactive. We obtained similar results in K562 cells, which express the R- and M-type isozymes but not the L-type isozyme (data not shown). Thus, we conclude that the regulatory elements of the PKL gene show cell-type specificity.
Interaction of the regulatory regions with heterologous promoter. The −63/−185 fragment was inserted upstream (A) or downstream (B) of pUC1cat, or upstream of pMcat0.2 (C) in the orientation shown by the arrow, which is the 5′ to 3′ direction. The plasmids were transfected into hepatocytes and CAT activities were determined after 48 h. The activity is shown as a percentage of that of pUC1cat in A and B, and as a percentage of that of the most active plasmid in C because pMcat0.2 showed essentially no activity. Black and hatched boxes indicate the promoter regions from the SV40 and pyruvate kinase M gene, respectively. The directions of transcription are shown by arrows. CM, chloramphenicol; Ac-CM, its acetylated products.

Interaction of Regulatory Elements with trans-Acting Factors—To identify the trans-acting factors that bind to the three cis-acting elements of the PKL gene, we carried out gel retardation assays. First, we analyzed the L-I region, which contains a homologous sequence to the LF-B1-binding site. Proteins extracted from Escherichia coli cells transformed by the procaryotic expression vector with or without LF-B1 cDNA were used for the assays. The band of L-I bound to protein was observed by incubation with the extract containing LF-B1 (Fig. 5) but not with the extract without it (data not shown). The retarded band disappeared on addition of 200-fold excess of unlabeled L-I oligonucleotide. The retarded band also disappeared on addition of 50-fold excess of the ALB oligonucleotide, which is the LF-B1-binding site of the human albumin gene. However, the L-II and L-III oligonucleotides did not compete with L-I. These results indicate that LF-B1 is a trans-acting factor binding to L-I and that its binding affinity to L-I is lower than that to the ALB oligonucleotide.

Second, we examined the L-II, which contains a homologous sequence to the LF-Al-binding site of the human α1-antitrypsin gene. Incubation of the labeled L-II oligonucleotide with liver nuclear extracts resulted in formation of a retarded band (Fig. 6A). The intensity of the band was reduced dose-dependently by unlabeled L-II oligonucleotide but not by the L-I and L-II oligonucleotides and the binding site for MLTF (Fig. 6A). Conversely, the retarded band of the labeled MLTF oligonucleotide disappeared on addition of 50-fold excess of unlabeled MLTF oligonucleotide but was not affected by the L-III oligonucleotide (Fig. 7B). These results indicate that the L-III-binding factor is not MLTF and also differs from LF-B1 and L-II-binding factor.

DISCUSSION

Primary cultured hepatocytes are the best system to use in study of the regulation of hepatocyte-specific gene expression.
Regulatory Elements of Rat Pyruvate Kinase L Gene

because they retain all the functions of hepatocytes in vivo, unlike established cell lines derived from liver cells. However, so far hepatocytes have rarely been used for DNA transfer experiments because no efficient and reproducible procedure for transfection has been available. Recently, Ginot et al. (29) reported that plasmid DNA could be introduced into hepatocytes in culture efficiently by the calcium phosphate precipitation technique. Here, we demonstrated that adult rat hepatocytes could be transfected efficiently with DNA by electroporation. Using this method, we identified three positive elements: PKL-I, PKL-II, and PKL-III in the upstream region of the cap site for the L-type isozyme of the rat PKL gene. These elements, which have synergistic effects on homologous promoters, and probably heterologous ones too, are necessary for hepatocyte-specific expression of the PKL gene. Interestingly, these regions are located in the first intron of the PKL gene, that is between exon R and exon L. Very recently, Tremp et al. (30) produced two series of transgenic mice carrying either the entire rat pyruvate kinase were localized in the sequence from about nucleotide +37 in the PKL gene in transgenic mice (45).

Thus our present results by transient expression assay reflect gene expression of the L-type isozyme in vivo.

The positions and sequences of these regulatory regions are shown schematically in Fig. 8. PKL-I alone has an enhancer-like property, whereas PKL-II and -III do not have any independent effects. In combination, L-I + L-II and L-II + L-III, but not of L-I + L-III, showed synergistic enhancer activities, and the combination of all three elements had the greatest synergistic effect. However, the orientations of these elements were very important for their effects: the synergistic effects of these elements were observed only when they were oriented in the same direction. Thus, we concluded that the three elements function as a unit. This unit exerted its effect on not only a homologous promoter but also a heterologous promoter in a manner that was independent of its orientation or position relative to the cap site. Thus, a protein bound to L-I must interact with one bound to L-II, which in turn must interact with a protein bound to L-III. But proteins bound to L-I and L-III apparently did not interact with each other. PKL-I consists of a palindromic sequence 5'-CTGGTTATAGTTCATTAAACCAG-3', which contains a sequence homologous to the LF-B1 binding consensus sequence, 5'-GGTAAAATTATACA-3' (12). LF-B1 is a liver-specific nuclear protein that appears to be involved in the transcription of liver-specific genes such as α1-antitrypsin, fibrinogen, α-fetoprotein, albumin, and transthyretin (31-38). Moreover, LF-B1 has been reported to play a predominant role in hepatocyte-specific gene expression of albumin (38). Very recently, its cDNA was cloned (27). From gel retardation assay, we conclude that this trans-acting protein interacts with PKL-I.

PKL-II contains the sequence 5'-TCCTGGGACTCTG-GGGCCACTCCGTGG-3' located between nucleotides -149 and -126. This sequence is similar to the binding site of LF-A1, which is a trans-acting protein required for the expressions of several genes in hepatocytes, including the α1-antitrypsin, apolipoprotein A-I, and haptoglobin-related genes (13, 39). The 5'-TGCCC-3' of this motif is also arrayed tandemly in the 5'-flanking region of the rat glucokinase gene (40). We found that deletion of this motif caused decrease in the promoter activity for the glucokinase gene (41). Despite of this similarity in sequences, however, the trans-acting factor bound to PKL-II is not LF-A1. The factor is also different from LF-B1- and L-III-binding protein. Ochoa et al. (42) reported that the trans-acting factors bound to the two putative LF-A1-binding sites of the human transferrin gene were different from LF-A1. It remains to be determined whether the PKL-II-binding protein is identical with their factors.

PKL-III consists of a palindromic sequence, 5'-CGGCAC-CCCGACTCCCGCTTG-3', located between -170 and -150. PKL-III has homology with the 5'-flanking region of the rat γ-fibrinogen gene. Chodosh et al. (16) reported that the adenovirus major late transcription factor, MLTF, binds to this sequence of the rat γ-fibrinogen promoter. However, the trans-acting protein bound to PKL-III was found not to be MLTF and also differed from LF-B1 and L-II binding factor.

![Diagram](image-url)  
**Fig. 8. Schematic representation of the regulatory regions of the rat PKL gene.** Nucleotide residues are numbered negatively from the start site of transcription of the L-type isozyme. Arrows within PKL-I and PKL-III indicate palindromic sequences.
Vaulont et al. (43) recently reported that LF-B1 binds to the L-1 box (which we named PKL-I), NF1 to the L-2 box, LF-A1 to the L-3 box (PKL-II), and MLTF to the L-4 box (PKL-III) of the rat PKL gene. They also reported that in vitro transcription of the PKL gene depended primarily on the binding of LF-B1, and that the binding of LF-A1 also slightly stimulated its transcription, but that MLTF and NF1 did not contribute to its transcription (44). Our transfection experiments, however, showed that deletion of the NF1 binding site, located between −116 and −99, did not affect the enhancer activity appreciably, whereas deletion of PKL-III resulted in marked reduction in CAT activity. In addition, PKL-II and PKL-III showed synergistic enhancer activities. Moreover, our conclusions from binding experiments were not consistent with the report of Vaulont et al. (43) except in the case of LF-B1. The reason for this discrepancy remains to be determined. It is noteworthy that we used functionally defined oligonucleotides that were shorter than those used in their work.

Besides the positive regulatory regions, our results suggest the presence of a negative element between nucleotide −279 and −190. This region seems to play a minor role, but nevertheless its characteristics require study.

Acknowledgments—We thank Dr. Shuichi Katagiri for a gift of the human erythroleukemia K562 cell line and Drs. Hiroshi Nakajima and Tomoyuki Yamashita for helpful discussions.

References

Regulatory Elements of Rat Pyruvate Kinase L Gene

Supplemental Material: IDENTIFICATION AND CHARACTERIZATION OF REGIONAL-SPECIFIC REGULATORY REGIONS OF THE RAT PYRUVATE KINASE L GENE: THE TRAIT THAT REGULATES EXPRESSION IN VARIOUS SPECIES, ORGANs, GENDERS, AND DEVELOPMENTAL STAGES. The study investigated the regulatory elements of the rat pyruvate kinase L (PKL) gene. The researchers isolated RNA from various tissues and analyzed transcriptional activity under different conditions. The results showed significant differences in expression levels across different tissues and stages, indicating the presence of tissue-specific regulatory elements.

EXPERIMENTAL PROCEDURES

Materials - 6o of tissue homogenate, 94% polyacrylamide gel, Transblot Gradient, 200 U/ml of protease, and restriction endonucleases were obtained from Takara Shuzo Co. Acetic acid (POA) was purchased from Sigma-Aldrich. Acetic acid (A) was purchased from Sigma. Egg's minimum essential medium, RPMI1640 medium, and Dulbecco's PBS were obtained from Kanto Chemicals. Williams' medium E was from Flow Laboratories. Fetal bovine serum was obtained from HyClone Laboratories. [¹⁴C]Chloramphenicol (40 Ci/mmol) was obtained from DuPont-New England Nuclear.

Plasmids - The constructions of gplc357, a promoter-less CFP vector, and gplc357, a CFP vector with SV40 early enhancer and promoter, were described previously (15). A 1.8-kb BamHI fragment containing the 5'-flanking region of the PKL gene (from nucleotide -1400 to +37 relative to the cap site of the L-type) was isolated by a series of ligations of the subfragments and creation of a KpnI site at position +37 with KlboI linker. This fragment was isolated after agarose gel, visualized on an ultraviolet transilluminator, and sequenced with a dideoxy chain terminator reaction. The sequence of the BamHI/KpnI fragment was determined by a standard dideoxy chain terminator reaction.

Regulatory Elements of Rat Pyruvate Kinase L Gene

Theoretical predictions of the regulatory elements were performed by comparing the sequences of CCFP and SV40 early enhancer and promoter. The results showed significant differences in expression levels across different tissues and stages, indicating the presence of tissue-specific regulatory elements. The study also investigated the regulatory elements of the rat pyruvate kinase L (PKL) gene in different developmental stages. The results showed significant differences in expression levels across different stages, indicating the presence of developmental-specific regulatory elements.