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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*

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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 palindrome sequence 5′-CTGGTTATACTTTAACCAG-3′, which contain a sequence homologous to the LF-B1-binding site. PKL-II contains the sequence 5′-TTCCTGGACTCTGGCCCCCAGTGT-3′, which is similar to that of the LF-A1-binding site. PKL-III contains a palindrome sequence 5′-CCACGGGGCACTCCCGTGG-3′, which include 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 O^2-phosphotransferase, EC 2.7.1.40), a key glycolytic enzyme, has four isozymes in mammals, which are named the M_1-, M_2-, 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)[1] 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

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[1] The abbreviations used are: PKL, pyruvate kinase L; CAT, chloramphenicol acetyltransferase; MLTF, major late transcription factor.

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[2]

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 pLcat3200, 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 pLcat3200 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 −95. 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. 2*A*). When pLcat74 was transfected into

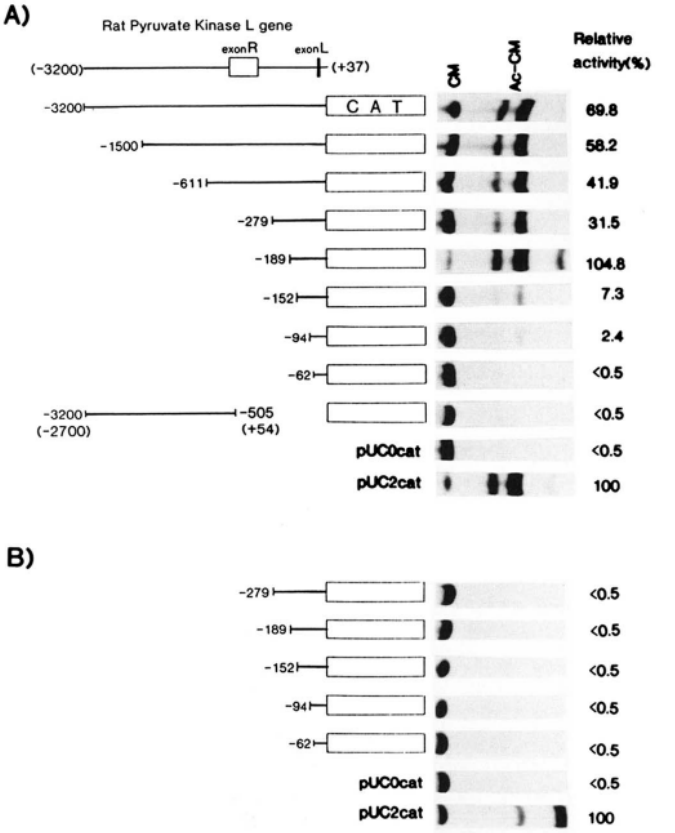

FIG. 1. **Expression of 5′-deletion mutants of the PKL/CAT fusion gene in hepatocytes and HeLa cells.** The series of 5′-deletion constructs shown on the left were transfected into hepatocytes (*A*) and HeLa cells (*B*), and CAT activities were determined after 48 h. The 5′-end points of the PKL gene in the constructs are indicated in base pairs relative to the transcription initiation site for the L-type pyruvate kinase. The *numbers in parentheses* for pRcat2700 show positions relative to the cap site of the R-type isozyme. The relative CAT activities of the deletion constructs are expressed as percentages of that of pUC2cat. Experiments on each plasmid were repeated at least three times with essentially the same results. Representative results are shown. Exon R and exon L indicate the exons specific for R-type and L-type pyruvate kinase, respectively. *CM*, chloramphenicol; *Ac-CM*, its acetylated products.

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. 2*A*). 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. 2*B*, 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. 2*C*). The −170/−139 fragment also enhanced the CAT activity of pLcat94, but less than the −170/−126 fragment. These results indicated that the fully

[2] Portions of this paper (including "Experimental Procedures" and Table I) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

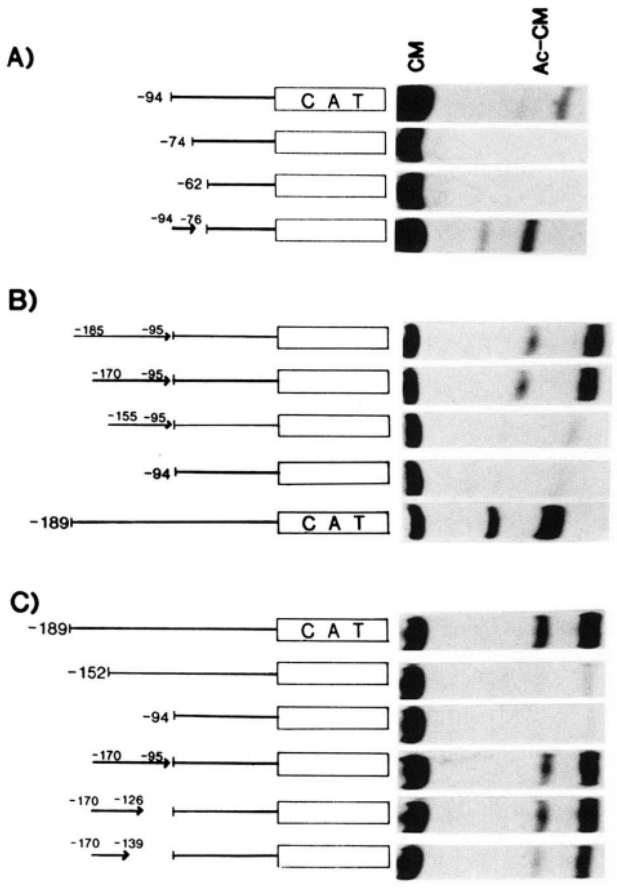

FIG. 2. **Identification of the proximal (*A*) and distal (*B* and *C*) regulatory regions of the PKL gene.** The PKL/CAT fusion genes shown on the left were transfected into hepatocytes and CAT activities were determined after 48 h. A single copy of synthetic double-stranded oligonucleotide between −94 and −76 was inserted into pLcat62′ in the sense orientation (*A*). Various fragments of the PKL gene with the 5′- (*B*) or 3′- (*C*) deletions shown on the left were inserted into pLcat94 in the sense orientation, and the resultant plasmids were transfected into hepatocytes. Experiments on each plasmid were repeated at least three times with essentially the same results. Representative results are shown. *CM*, chloramphenicol; *Ac-CM*, its acetylated products.

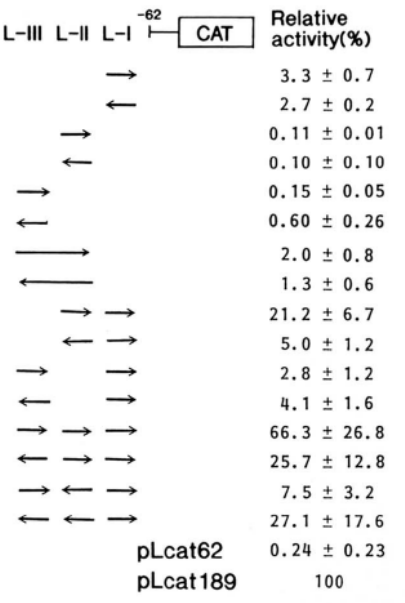

FIG. 3. **Identification and interaction of three *cis*-acting elements of the PKL gene.** A single copy of each double-stranded oligonucleotide was ligated to pLcat62′ in the orientation shown by *arrows* on the left, which is the 5′ to 3′ direction. The plasmids were transfected into hepatocytes and CAT activities were determined after 48 h. The relative activities of the constructs are expressed as percentages of that of pLcat189. Data are the mean ± S.E. for three independent experiments.

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-A1 binding sequence of the human α_1-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 or by ligation of L-III. This enhanced activity of L-I + L-II was further increased synergistically by addition of L-III 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_2-type isozyme of pyruvate kinase (data not shown), but expressed essentially no CAT activity in hepatocytes (Fig. 4*C*). 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_2-types 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.

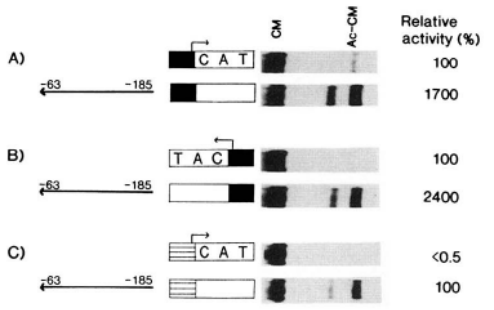

FIG. 4. **Interaction of the regulatory regions with heterologous promoter.** The −63/−185 fragment was inserted upstream (*A*) or downstream (*B*) of pUC1cat, or upstream of pMcat0.2 (*C*) in the orientation shown by the *arrow*, which is the 5′ to 3′ direction. The plasmids were transfected into hepatocytes 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.

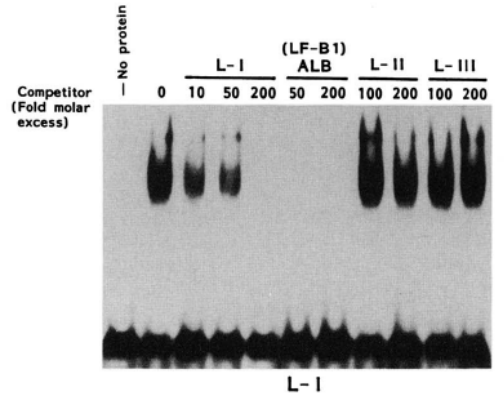

FIG. 5. **Gel retardation assay with labeled L-I oligonucleotide.** Samples of 0.1 ng of labeled L-I oligonucleotide were incubated with 0.4 μg of *E. coli* extract expressing LF-B1. The species and molar ratios of competitor DNAs are indicated at the top. *ALB*, human albumin gene promoter region.

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[3] 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-A1-binding site of the human α_1-antitrypsin gene. When labeled L-II oligonucleotide was incubated with rat liver nuclear extracts, a retarded band was detected (Fig. 6*A*). The formation of this band was competitively inhibited by addition of excess unlabeled L-II oligonucleotide but not by addition of α_1-antitrypsin, which is known to bind to LF-A1 (Fig. 6*A*), and L-I oligonucleotide. Addition of 200-fold excess of L-III oligonucleotide resulted in slight reduction in the intensity of the band. However, the formation of a retarded band of labeled L-III was not inhibited by 200-fold excess of L-II oligonucleotide (see below). On the other hand, the retarded band of the labeled α_1-antitrypsin oligonucleotide bound to the liver nuclear protein was markedly decreased by addition of 200-fold excess of α_1-antitrypsin, but not by addition of L-II oligonucleotide (Fig. 6*B*). Thus, the *trans*-acting factor bound to the L-II oligonucleotide is not LF-A1 and also differs from LF-B1- and L-III-binding protein.

Third, we investigated the L-III, which contains a sequence similar to the MLTF-binding site of the rat γ-fibrinogen gene. Incubation of the labeled L-III oligonucleotide with liver nuclear extracts resulted in formation of a retarded band (Fig. 7*A*). The intensity of the band was reduced dose-dependently by unlabeled L-III oligonucleotide but not by the L-I and L-II oligonucleotides and the binding site for MLTF (Fig. 7*A*). 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. 7*B*). 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

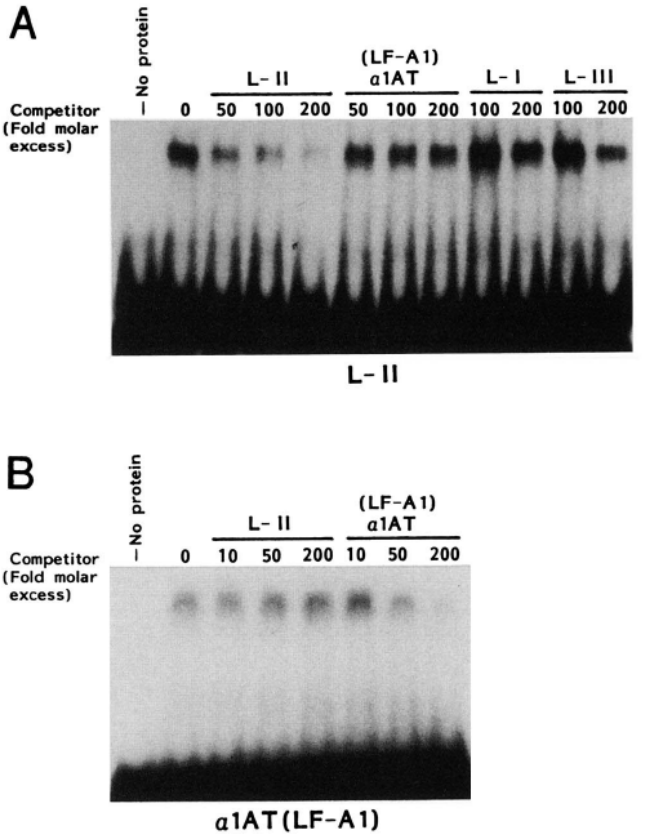

FIG. 6. **Gel retardation assay with labeled L-II (*A*) or α_1-antitrypsin oligonucleotides (*B*).** Samples of 0.1 ng of probe DNA were incubated with 5 μg of rat liver nuclear extract. The species and molar ratios of competitor DNAs are indicated at the top. $\alpha_1 AT$, human α_1-antitrypsin gene promoter region.

[3] P. Monaci and A. Nicosia, unpublished results.

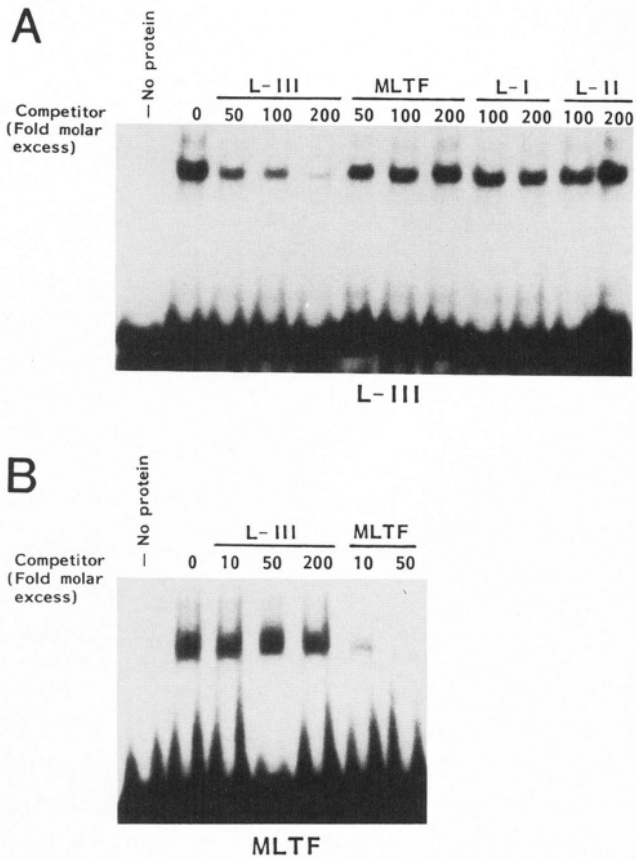

FIG. 7. **Gel retardation assay with labeled L-III (*A*) or MLTF oligonucleotides (*B*).** Samples of 0.1 ng of probe DNA were incubated with 5 μg (*A*) or 30 μg (*B*) of rat aliver nuclear extract. The species and molar ratios of competitor DNAs are indicated at the top. *MLTF*, adenovirus major late promoter region.

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 regulatory elements designated as PKL-I, PKL-II, and PKL-III in the upstream region of the cap site for the L-type isozyme of the 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 gene or a minigene devoid of exons 3–10 (two to nine by their numbering system), with 3.2 kilobases of the 5′-flanking region and 1.4 kilobases of the 3′-flanking sequence. They showed that the transgenes were expressed in a tissue-specific manner. We also observed that all the *cis*-acting elements responsible for tissue-specific expression of the L-type pyruvate kinase were localized in the sequence from about nucleotide −3000 to +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 palindrome sequence 5′-CTGGTTA-TACTTTAACCAG-3′, which contains a sequence homologous to the LF-B1 binding consensus sequence, 5′-GTTAAT-NATTAAC-3′ (12). LF-B1 is a liver-specific nuclear protein that appears to be involved in the transcription of liver-specific genes such as α_1-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′-TTCCTGGACTCTGG-CCCCCAGTGT-3′ located between nucleotide −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 α_1-antitrypsin, apolipoprotein A-I, and haptoglobin-related genes (13, 39). the 5′-TGGCCC-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 palindrome sequence, 5′-CGCCAC-GGGGCACTCCCGTGG-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.

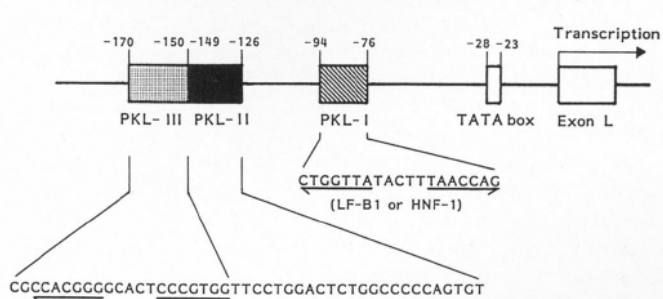

FIG. 8. **Schematic representation of the regulatory regions of the rat PKL gene.** Nucleotide residues are numbered negatively from the start site of transcription of the L-type isozyme. *Arrows* within PKL-I and PKL-III indicate palindrome sequences.

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.

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Supplemental Material to "IDENTIFICATION AND CHARACTERIZATION OF HEPATOCYTE-SPECIFIC REGULATORY REGIONS OF THE RAT PYRUVATE KINASE L GENE: THE SYNERGISTIC EFFECTS OF MULTIPLE ELEMENTS".

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EXPERIMENTAL PROCEDURES

Materials - T4 DNA ligase, T4 polynucleotide kinase, Klenow fragment, T4 DNA polymerase, and restriction endonucleases were obtained from Takara Shuzo and Toyobo. Sequenase, a sequence kit, was from United States Biochemical. Acetyl coenzyme A was from Sigma. Eagle's minimum essential medium, RPMI1640 medium, and Dulbecco's PBS(-) were obtained from Nissui Seiyaku, and Williams' medium E was from Flow Laboratories. Fetal bovine serum was obtained from HyClone Laboratory. [α-^{32}P]dATP (3000 Ci/mmol), [γ-32P]ATP (3000 Ci/mmol), and [^{14}C]chloramphenicol (60 mCi/mmol) were obtained from DuPont New England Nuclear.

Plasmids- The constructions of pUC0cat, a promoter-less CAT vector, and pUC2cat, a CAT vector with SV40 early enhancer and promoter, were described before (17); these plasmids were used as negative and positive controls, respectively. A plasmid containing an EcoRI/MspI fragment (about nucleotide -3200 to +37 relative to the cap site of the L-type) of the PKL gene inserted into the EcoRI/HindIII site of pUC18 was constructed by a series of ligations of the subfragments and creation of a HindIII site at position +37 with HindIII linker. This fragment was isolated after blunt-ending the EcoRI site and was inserted into the SmaI/HindIII site of pUC0cat (pLcat3200). This fragment was also digested with appropriate restriction endonucleases to produce 5'-deletion fragments. These fragments were -1468/+37, -611/+37, -279/+37, -94/+37, -74/+37, and -62/+37 and their 5'-ends were blunted by Klenow fragment or T4 DNA polymerase. These fragments were inserted into the SmaI/HindIII site of pUC0cat to produce pLcat1500, pLcat611, pLcat279, pLcat94, pLcat74, and pLcat62, respectively. A -185/+37 (BamHI/HindIII) fragment was ligated into the BamHI/HindIII site of the pUC0cat (pLcat189). A fragment of -152/+37 was generated by 5'-deletion of a -185/+37 fragment by T4 DNA polymerase and inserted into the SmaI/HindIII site of pUC0cat (pLcat152).

pLcat94 and pLcat62 were digested with KpnI and were blunt-ended by treatment with T4 DNA polymerase. A -185/-95 fragment was blunt-ended by Klenow fragment and introduced into these plasmids. Fragments of -170/-95, -155/-95, -170/-126, and -170/-139 were blunt-ended by T4 DNA polymerase or Klenow fragment and introduced into the blunt-ended KpnI site of pLcat94.

A blunt-ended fragment of -185/-63 was also inserted into the SmaI site of pUC1cat or the blunt-ended KpnI site of pMcat0.2. Plasmid pUC1cat was constructed by inserting a SphI/BamHI fragment of pSV2cat containing the SV40 promoter and CAT gene into the BamHI site of pUC19 in either orientation after creating a BglII site at the SphI site with BglII linker. Plasmid pMcat0.2 contains the promoter region of the rat pyruvate kinase M gene (-197 to +51 relative to the cap site) inserted into the SmaI/SalI site of pUC0cat.

Double-stranded oligonucleotides of L-I, L-II, L-III, L-D, ALB, α1AT, and MLTF (Table 1) were synthesized in a DNA synthesizer (Applied Biosystems, 380A). These oligonucleotides were inserted into the appropriate cloning sites of pLcat62', which was constructed by ligation of a -62/+37 fragment of the PKL gene to the HincII/HindIII site of pUC0cat.

A EcoRI/Nsp7524I fragment (about -3200 to -505 relative to the cap site of the L-type or about -2700 to +54 relative to the cap site of the R-type) was subcloned in pUC18 after creating a HindIII site at position +54 with HindIII linker. This fragment was then isolated after blunt-ending the EcoRI site and inserted into the SmaI/HindIII site of pUC0cat to produce pRcat2700.

The 5'- and 3'-end points of each construction were confirmed by DNA sequencing of denatured plasmid DNAs by the chain termination method (18,19).

Cell Culture and DNA Transfections - A human cervical carcinoma cell line, HeLa cells, was obtained from the Japanese Cancer Research Resources Bank. HeLa cells were grown in Eagle's minimum essential medium supplemented with 10 % fetal bovine serum. Human K562 erythroleukemia cells were grown in RPMI1640 medium supplemented with 10 % fetal bovine serum. Hepatocytes were prepared from male Sprague-Dawley rats (170-190 g body weight) that had received high-carbohydrate diet as described by Seglen (20). Freshly isolated hepatocytes were washed with Eagle's minimum essential medium and then with Dulbecco's PBS(-), and were suspended in the latter at a concentration of 5 x 10^6 cells/ml. DNA transfection was carried out by electroporation essentially as described (21) with some modifications. A mixture of 0.8 ml of cell suspension, 20 μg of plasmid DNA, and 100 μg of sonicated salmon sperm DNA was put into an electroporation cuvette and stood on ice for 15 min. The cells were then exposed to a single voltage pulse of 200-350 V at 960 μF using a Gene Pulsar system from Bio-Rad. The cuvette was stood on ice for 15 min, and then the cells were plated in 6cm culture dishes. Hepatocytes were cultured in Williams' Medium E supplemented with 10 % fetal bovine serum, 10^{-6} M insulin, 10^{-6} M dexamethasone, 100 μg/ml streptomycin, and 100 units/ml penicillin and K562 cells in the medium described above. The medium was changed 3 and 24 h after plating the cells. Plasmid DNA was transfected into HeLa cells by the strontium phosphate method (22). Each transfection experiment was repeated at least three times. Plasmid DNAs were prepared by lysozyme/Triton X-100 procedure and banded by ultracentrifugation on a cesium chloride/ethidium bromide equilibrium gradient (23), and analyzed on agarose gels to check the purity and quality of supercoiled DNA.

CAT Assays - Cells were incubated for 48 h after transfection, harvested and disrupted by freeze-thawing. CAT assays were performed as described by Gorman et al. (24) using equal amounts of cell extract in terms of protein, but protein extracts were preheated at 60°C for 10 min (25). Quantitative results were obtained by cutting out the areas of the chromatogram corresponding to the acetylated forms of [^{14}C]chloramphenicol and counting them in a liquid scintillation counter.

Preparation of Nuclear Extracts and Gel Retardation Assay - Liver nuclear extracts from Sprague-Dawley rats were prepared as described by Gorski et al. (26). LF-B1 cDNA containing the entire coding sequence (27) was cloned in the procaryotic expression vector of pT7-7 to produce pT7-B1[3]. E. coli strain BL21(DE3) transformed with pT7-B1 or pT7-7 were cultured in NZCYM medium for 2 h and then treated with isopropylthio-β-D-galactoside for 3 h. The cultured E. coli cells were collected by centrifugation and resuspended in 50 mM Tris-HCl, 2 mM EDTA, pH 8.0, and proteins were extracted from the cells by sonication. Gel retardation assay was carried out by incubating a labeled double-stranded oligonucleotide (0.1 ng) with the indicated amounts of nuclear or E. coli proteins in 25 mM Hepes buffer, pH 7.6, containing 60 mM KCl, 7.5 % (v/v) glycerol, 0.1 mM EDTA, 1 mM dithiothreitol, 5mM $MgCl_2$, and 3 μg poly(dI-dC). After incubation for 30 min at room temperature, 5 μl of 0.05 % bromphenol blue in 20 % Ficoll was added and the samples were loaded onto 4 % acrylamide gel in 0.5 x Tris-borate buffer, pH 8.3 and separated by electrophoresis at 10 V/cm for 2.5-3 h at 4°C. In competition experiments, the indicated amounts of a double-stranded oligonucleotide were added to the reaction mixture. The sequences of L-I, L-II, L-III, L-D, MLTF, ALB, and α1AT are shown in Table 1.

Table I. Nucleotide sequences of double-stranded oligonucleotides used in this study

A. From the rat PKL promoter				
L-I	-94	CTAGCTGGTTATACTTTAACCAG		-76
		GACCAATATGAAATTGGTCGATC		
L-II	-149	GATCTTCCTGGACTCTGGCCCCCAGTGT		-126
		AAGGACCTGAGACCGGGGGTCACACTAG		
L-III	-170	CCGGCGCCACGGGGCACTCCCGTGG		-150
		GCGGTGCCCCGTGAGGGCACCGGCC		
L-D	-170	GATCCGCCACGGGGCACTCCCGTGGTTCCTGGACTCTGGCCCCCAGTGT		-126
		GCGGTGCCCCGTGAGGGCACCAAGGACCTGAGACCGGGGGTCACACTAG		
B. From the human albumin promoter				
ALB	-72	GGGATGAGTCTAGTTAATAATCTACAATT		-47
		TACTCAGATCAATTATTAGATGTTAACCC		
C. From the human α1-antitrypsin promoter				
α1AT	-131	GATCCCAGCCAGTGGACTTAGCCC		-108
		GGTCGGTCACCTGAATCGGGCTAG		
D. From the adenovirus major late promoter				
MLTF	-65	CCGGTAGGCCACGTGACCGGGT		-48
		ATCCGGTGCACTGGCCCAGGCC		