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EXPRESSION OF LIVER TYPE PYRUVATE KINASE IN INSULINOMA CELLS: INVOLVEMENT OF LF-B1 (HNF1)

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The messages for LF-B1, which interacts with the cis-acting element of PKL-I to play an essential role in expression of L-type pyruvate kinase (PK) in the liver, and L-type PK were found to be present in RIN-m5F insulinoma cells as well as the liver, kidney and small intestine, although the levels of the two mRNAs in these tissues were not correlated. Gel retardation assay suggested that similar nuclear proteins bound to two other cis-acting elements, PKL-II and PKL-III, were expressed in both liver and insulinoma cells, and that additional PKL-III-binding proteins were present only in RIN-m5F cells. Thus, we suggest that the mechanism of L-type PK expression in pancreatic B cells is similar to that in the liver.

In mammals, four isozymes of pyruvate kinase (PK, EC 2.7.1.40) have been identified (1), named L-, R-, M_1-, and M_2-types, and shown to be encoded by two genes per haploid genome (2-4): the PKL gene produces the L- and R-type isozymes by use of alternative promoters (4), whereas the PKM gene produces the M_1- and M_2-types by alternative RNA splicing (2,3). As the expression of these isozymes is tissue-specific and changes during development, it is of interest to examine how the expression of these two genes is regulated at the transcriptional and splicing levels.

The L-type isozyme is mainly expressed in parenchymal liver cells and is also present in the kidney and small intestine as a minor species (1). We have identified three cis-acting elements in the upstream region of the cap site for the L-type isozyme of the PKL gene that are necessary for expression of the L-type isozyme in hepatocytes (5). The upstream region containing the three elements also directs tissue-specific expression of a CAT reporter gene in transgenic mice (6). These elements, which are named PKL-I, PKL-II, and PKL-III, have little if any activity singly, but in combination show synergistic enhancer activities when oriented in the same direction. Thus, these elements act as an enhancer unit. We also found that different trans-acting proteins interacted with these elements and that the trans-acting protein bound to PKL-I was LF-B1 (HNF1).

Some characteristic liver proteins, such as glucokinase (7,8) and liver type glucose transporter (9) are also expressed in pancreatic B-cells and their derived cell lines. Here we report that L-type PK is expressed in RIN-m5F cells (10), a rat insulinoma cell line, by a similar mechanism to that in the liver.

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Abbreviation: PK, pyruvate kinase.

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MATERIALS AND METHODS

Materials - Restriction endonucleases and other enzymes were obtained from Takara Shuzo and Bethesda Research Laboratories. RPMI1640 medium was purchased from Nissui Seiyaku, and fetal bovine serum from Hyclone Laboratories. Nitrocellulose filters (Nitroplus) were obtained from Micron Separations Inc. [α-^{32}P]dATP (3000 Ci/mmol) and [γ-^{32}P]ATP (3000 Ci/mmol) were from Du Pont-New England Nuclear.

Cell Culture - RIN-m5F insulinoma cells were cultured in RPMI 1640 medium supplemented with 10 % fetal bovine serum.

Isolation of Total RNA and Northern Blot Analysis - Total RNA was isolated from rat tissues and cells by the acid guanidine-phenol-chloroform method (11). Northern blot analysis of total RNA was carried out as described (12,13). The probes used for L-type PK, glucokinase and LF-B1 were as described (12,13), and were labeled with [α-^{32}P]dATP using random oligonucleotide primers (14).

Primer Extension - A 116 bp Taq I fragment of pLPK21 (4,15) was labeled at the 5' end with [γ-^{32}P]ATP by T4 polynucleotide kinase. Primer extension was performed as described (4). The products were analyzed by electrophoresis on 6 % polyacrylamide/urea gel using sequence ladders of M13 mp18 DNA as markers.

Gel Retardation Assay - Nuclear extracts were prepared from rat liver and RIN-m5F cells (16) and used for gel retardation assay. The assay conditions and nucleotide sequences of double-stranded oligonucleotides used were as described previously (5) except for use of the adenovirus major late transcription factor (MLTF)-binding site of the rat γ-fibrinogen gene (17) which is

-87 CCGGGAGACCCCGTGACCAGTT -70
CTCTGGGGCACTGGTCAAGGCC

RESULTS AND DISCUSSION

LF-B1 mRNA is reported to be present in various tissues including liver, kidney, small intestine and spleen (18). We confirmed this by Northern blot analysis (Fig. 1A). In contrast with an earlier report of results obtained by RNase protection assay (18), however, we found that the mRNA was most abundant in the kidney followed in order by the small intestine and liver. The LF-B1 mRNA of these tissues gave two bands of about 3.2 and 3.6 kb, but that of spleen gave only one band of 3.6 kb. As multiple mRNA species are produced by alternative use of multiple polyadenylation signals (19), only the most 3' signal must be used in the spleen. LF-B1 mRNA was also detected in RIN-m5F insulinoma cells at higher level than in hepatocytes (Fig. 1B). We also detected two mRNA species hybridizable to the coding sequence of the LF-B1 cDNA at low levels in the testis. However, their sizes, 3.0 and 2.4 kb, were smaller than those of LF-B1 mRNAs, and we do not yet know whether they encode LF-B1 or a related protein. We previously showed that LF-B1 played an essential role in expression of L-type PK in hepatocytes (5). As the message for LF-B1

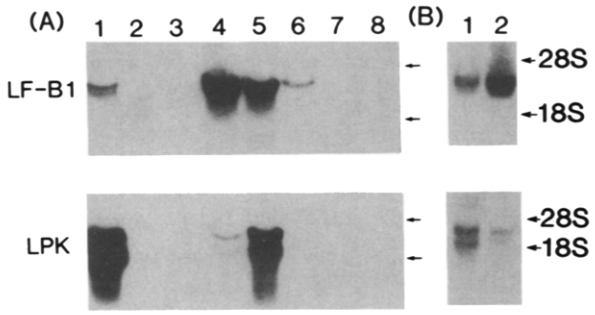

Fig. 1. Northern blot analysis of total RNA isolated from various tissues and cells. (A) Samples of 30 μg of RNA were analyzed. Lane 1, liver; lane 2, testis; lane 3, adipose tissue; lane 4, kidney; lane 5, small intestine; lane 6, spleen; lane 7, brain; lane 8, skeletal muscle. (B) Samples of 20 μg of RNA were analyzed. Lane 1, hepatocytes isolated from a normal rat; lane 2, RIN-m5F cells. LPK, L-type pyruvate kinase.

was present in the kidney and small intestine, this protein is probably also essential for expression of L-type PK in these tissues. However, the levels of the L-type PK and LF-B1 mRNAs were not correlated. Thus, the expression of L-type PK is unlikely to be predominantly regulated by the level of LF-B1. This conclusion is consistent with our previous finding that the expression of LF-B1 does not simply regulate that of L-type PK in the liver (13). The absence of the L-type PK mRNA in spleen may be explained by the absence of PKL-II- and/or PKL-III-binding protein.

The presence of LF-B1 mRNA in RIN-m5F cells prompted us to examine whether L-type PK is expressed in these cells. On Northern blot analysis, two bands that hybridized with the L-type PK cDNA were detected in insulinoma RNA and their sizes were similar to those in hepatocyte RNA. But, their levels were lower in insulinoma cells than in hepatocytes isolated from normal rats. The B-cell type glucokinase mRNA was also detected in this insulinoma (data not shown), as reported by others (20). However, it was not certain whether these two bands are actually those of the message for L-type PK because the probe used hybridizes equally well to the R-type PK mRNA (4). This problem was examined by a primer extension analysis using primer common to the two isozyme mRNAs. The L-type PK mRNA should produce an extended band of 158 bases (4), whereas the R-type isozyme mRNA should generate a longer band of 261 bases (13). As shown in Fig. 2, a major band of 158 bases was detected in RIN-m5F mRNA as well as liver mRNA, but not in muscle mRNA. Thus, we conclude that the L-type PK mRNA is expressed in RIN-m5F cells.

From the viewpoint of tissue (cell)-specific gene expression we were interested to see whether nuclear proteins similar to those in hepatocytes bind to the two cis-acting elements, PKL-II and PKL-III, in insulinoma cells. We examined this problem by gel retardation assay. As reported previously (5), incubation of labeled L-II oligonucleotide with rat liver nuclear extracts gave a retarded band and the formation of this band was competetively inhibited by addition of 200-fold excess of unlabeled L-II oligonucleotide (Fig. 3A). However, addition of the LF-A1 binding site of the human α1-antitrypsin gene (α1AT) (22) did not reduce the intensity of the band, although PKL-II contains a sequence homologous to the LF-A1-binding site (5). When the same amount of insulinoma nuclear extract was incubated with the labeled L-II, a similar retarded band was obtained and its intensity was higher than that of the liver extract, suggesting a higher content of L-II-binding factor in RIN-m5F cells than in the liver. The formation of this band was also competitively inhibited by excess L-II oligonucleotide, but not by α1AT oligonucleotide. These results suggest that a similar nuclear protein bound to the PKL-II region is expressed in both insulinoma cells and liver, and that this protein, which is different from LF-A1, is responsible for expression of the L-type isozyme in RIN-m5F cells.

Labeled L-III oligonucleotide gave a retarded band on incubation with rat liver nuclear extracts and the intensity of this band was markedly reduced by addition of 200-fold excess of unlabeled L-III oligonucleotide, but not by addition of the binding site of the adenovirus major late transcription factor (MLTF) (23) and the MLTF-binding site of the rat γ-fibrinogen gene (γFBG) (17). Consistent with our previous observations (5), these results suggest that the L-III-binding protein is not MLTF, although PKL-III contains a sequence homologous to the MLTF-binding site. On the other hand, three retarded bands of L-III were obtained on incubation with nuclear extracts from insulinoma cells. The intensities of these bands were much lower than that of the bands from liver extracts, when the same amounts of nuclear extracts were used. The mobility of band B corresponded to that of the band of the liver extract. Addition of excess unlabeled L-III oligonucleotide resulted in less reduction in the intensities of the three retarded bands, especially that of

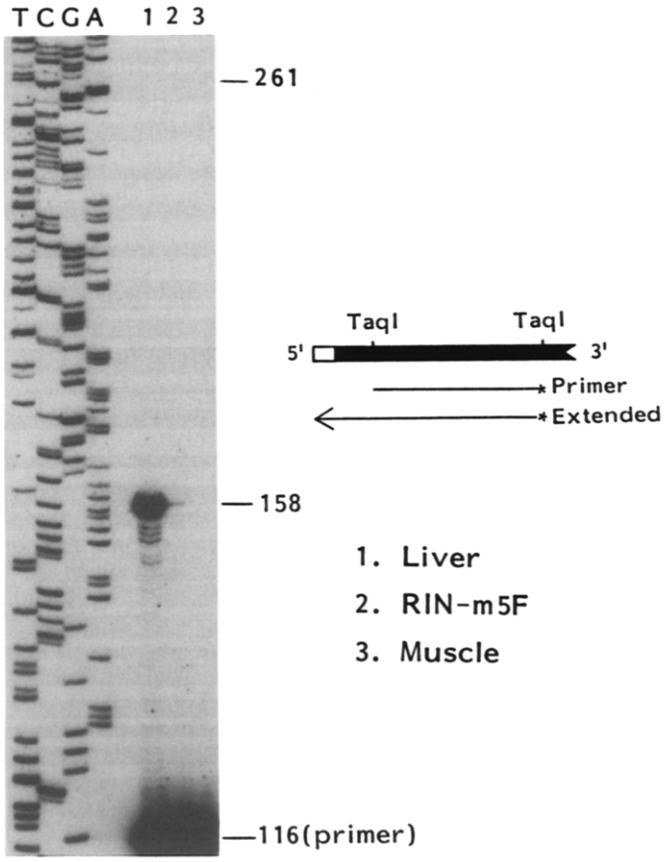

Fig. 2. Primer extension analysis of RIN-m5F insulinoma RNA. The ^{32}P probe was hybridized with RNA from the tissues shown. The cDNA was extended by reverse transcriptase and the products were analyzed on sequence gel using the sequence ladders of mp 18 DNA as molecular size markers. The cDNA fragment used is indicated by the bar on the right; the asterisks show the position of the ^{32}P label.

band B, than of the bands of liver extracts, although the MLTF-binding sites of the adenovirus and γ-fibrinogen genes did not compete with the formations of these bands. These results suggested that multiple nuclear proteins bound to PKL-III were present in RIN-m5F cells and that these proteins were different from MLTF. The protein that formed the retarded band in liver extracts is probably similar to that of the band B in insulinoma cells. But, its level appears to be much lower in insulinoma cells than in the liver. Other proteins forming bands A and C appear to be present only in insulinoma cells and their levels also appear to be lower in insulinoma cells. These lower levels of L-III-binding proteins may be responsible for the lower expression of the L-type PK mRNA in RIN-m5F cells than in hepatocytes. Further identifications and characterizations of the L-II- and L-III-binding proteins are necessary.

We examined the PK activity in insulinoma cells using specific antibodies, and found that the L-type isozyme accounted for less than 10 % of the total PK activity in RIN-m5F cells, and the rest being that of M_2-type (data not shown). We have not examined the PK isozyme in pancreatic B cells. But we believe that L-type PK is expressed in B cells since the L-type isozyme in hepatocytes decreases by transformation and

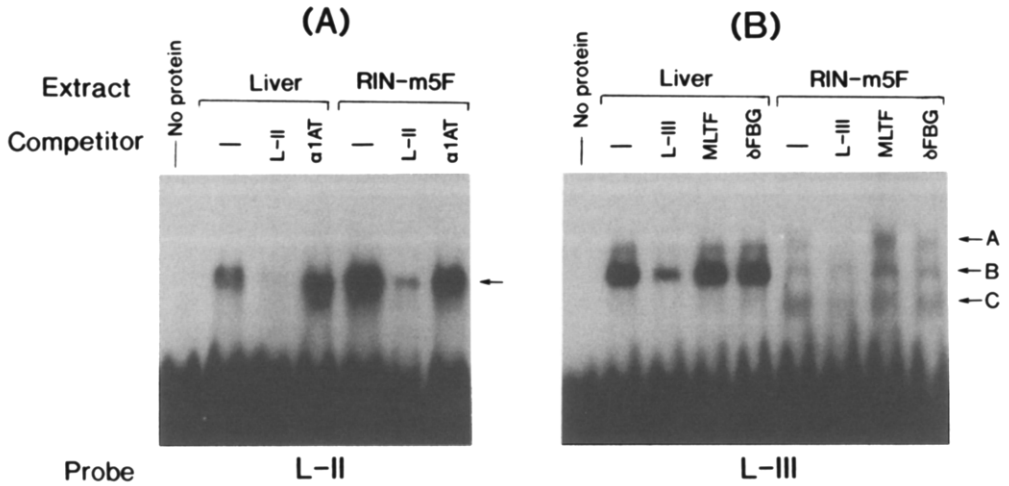

Fig. 3. Gel retardation assay with labeled L-II (A) or L-III oligonucleotides (B). Samples of 0.1 ng of probe DNA were incubated with nuclear extracts (2.5 μg in A and 10 μg in B) prepared from rat liver or RIN-m5F cells. The competitor DNAs shown at the top were used in 200-fold molar excess. α1AT, human α1-antitrypsin gene promoter region; MLTF, adenovirus major late promoter region; γFBG, rat γ-fibrinogen gene promoter region.

its extent is correlated with the differentiation state of hepatoma cells (13,26). In addition, very recent report indicated that LF-B1 mRNA was present in pancreas (27). However, the L-type isozyme level may not be higher in B cells than in RIN-m5F cells since the PK in rat pancreas islets is reported to be mainly the M_2-type (21). Thus, physiologically the L-type isozyme may have little significance in pancreatic B cells although these data suggest that L-type PK is expressed in B cells by a similar mechanism to that in the liver. Further studies are required to confirm these problems.

We showed previously that expression of L-type PK in the liver is stimulated by insulin mainly at the transcriptional level and by fructose at both the transcriptional and post-transcriptional levels (24,25). The effect of insulin on L-type PK required ongoing protein synthesis and metabolism of glucose (12), whereas the effect of fructose was attributable to an intermediate common to the metabolism of both fructose and glycerol (25). Therefore, it will be very interesting to examine whether gene expression of L-type PK in B cells is also regulated by insulin and carbohydrates.

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