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Characterization and Purification of Carbohydrate Response Element-Binding Protein of the Rat L-Type Pyruvate Kinase Gene Promoter

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The L-III transcriptional regulatory element of the rat pyruvate kinase L gene is located between −170 and −150 base pairs upstream of the hepatocyte-specific transcription initiation site. As the L-III element is not only necessary for cell type-specific expression but also for transcriptional stimulation by carbohydrates, it is also referred to as a carbohydrate-response element. Electrophoretic mobility shift assays using rat liver nuclear extract showed that L-III element-binding protein (L-IIIBP) was observed as multiple bands. These bands disappeared when the nuclear extract was preincubated at 60°C for 5 min and were competed with unlabeled L-III oligonucleotide but not with unlabeled adenovirus major late promoter E box oligonucleotide. In addition, these bands were not affected in the presence of antiserum against upstream stimulating factor (USF). Thus, we conclude that L-IIIBP is different from USF. Then, heat-labile L-IIIBP was purified from rat liver nuclear extracts. Purified L-IIIBP exhibited two bands on sodium dodecyl sulfate/polyacrylamide gel electrophoresis by silver staining. Ultraviolet crosslinking experiment showed that both bands had binding activity to the L-III oligonucleotide.

Key Words: **L-type pyruvate kinase; gene expression; *trans*-acting protein.**

The rat pyruvate kinase (PK) L gene encodes both R- and L-PK isozymes and is expressed in a tissue-specific manner using two promoter systems (1). The PKL gene consists of 12 exons and 11 introns. The first exon (exon R) and the second exon (exon L) contain nucleotide sequences specific to R-PK and L-PK, respectively. Other exons are common to both isoenzymes. The regulatory elements necessary for cell type-specific expression of R-PK in erythroid cells are located upstream of exon R (2–4). On the other hand, 170 bp upstream region from exon L contains a regulatory unit necessary for L-PK expression in the liver, kidney, small intestine, and pancreatic-β cells (5,6). This unit is also responsible for transcriptional stimulation of the PKL gene by carbohydrates (7). The unit consists of three positive *cis*-acting elements, which are L-I (94 to −76), L-II (−149 to −125), and L-III (−170 to −150), respectively (5). In the context of a natural promoter, both L-II and L-III elements are required for carbohydrate responsiveness (8–10). While the L-III element itself possesses responsiveness to carbohydrate (10), the L-II element functions as an accessory element (8). The L-III element contains two copies of imperfect palindromic E box sequence (5′-CACGGG-3′) separated by 5 bp. Sequences similar to these two elements were also present in the 5′-flanking region of the rat S_{14} gene, another carbohydrate responsive gene, and these sequences were also responsible for carbohydrate stimulation of the S_{14} gene promoter (11, 12). Whereas hepatocyte nuclear factor (HNF) 1 binds to the L-I element, HNF4 and nuclear factor (NF)-1 family member proteins bind to the L-II element (5, 13). Indeed, HNF1, HNF4, and NF-1 family members regulate the transcriptional activity of the PKL/chloramphenicol acetyltransferase (CAT) fusion gene (13, 14). While Kahn's group has reported that the upstream stimulating factor (USF) binds to the L-III element (15), we found that an unknown protein different from USF interacted with the L-III element by electrophoretic mobility shift assays (EMSA) (5). In addition, Kaytor et al. reported that the artificial sequences, which is not recognized by the USF, displayed responsiveness to glucose stimuli (16). This observation suggests that *trans*-acting factor different from USF may mediate

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TABLE 1

Oligonucleotide Sequences Used in This Study

Oligo name	Nucleotide sequences	Carbohydrate responsiveness
L-III	CCGGCGC**CACGGG**GCACT**CCCGTG**G GCG**GTGCCC**CGTGA**GGGCAC**CGGCC	Yes
lBamL3	GATCGGCGC**CACGGG**GCACT**CCCGTG**GTT CCGCG**GTGCCC**CGTGA**GGGCAC**CAACTAG	Yes
mlBamL3-A	GATCGGCGC**CACGGG**TAACT**CCCGTG**GTT CCGCG**GTGCCC**ATTGA**GGGCAC**CAACTAG	Yes
mlBamL3-B	GATCGGCGC**CACGGG**GCACTCCCTTTGTT CCGCG**GTGCCC**CGTGAGGGAAACAACTAG	No
MLP	CCGGTAGGC**CACGTG**ACCGGGT ATCCG**GTGCAC**TGGCCCAGGCC	No

Note. Bases mutated and E box sequences are indicated by underlining and bolding, respectively.

the carbohydrate responsiveness. Accordingly, the identification and characterization of the L-III element-binding proteins (L-IIIBP) is important to understand the molecular mechanisms underlying the stimulation of the gene transcription by carbohydrates.

In this study, we carried out the further characterization of the L-IIIBP. Here, we report the purification of L-IIIBP which is different from the USF.

MATERIALS AND METHODS

Materials. Streptoavidin magnetic particles were purchased from Dynal. T4 polynucleotide kinase and Klenow fragment were obtained from New England Biolabs. γ-^{32}P-ATP (111 TBq/mmol) was from DuPont/New England Nuclear. Poly (dA-dT) was from Boehringer Mannheim. HiLoad Sephacryl S-100HR, DEAE-Sepharose Fast Flow, Heparin-Sepharose CL-6B, Nick column, and Rainbow marker were products of Amersham Pharmacia Biotechnology Inc. Biotin-14-dATP was from GIBCO/BRL. Molecular weight marker, pre-made polyacrylamide gels (Multi-gel), and 2D-silver stain kit "Daiichi" were purchased from Daiichi Pure Chemicals.

Oligonucleotides and preparation of sequence-specific affinity column. Oligonucleotides used in this study are listed in Table 1. They were synthesized by a 380A DNA synthesizer (Applied Biosystems Inc.). These oligonucleotides were annealed with their complementary oligonucleotides to produce double-stranded DNA as described previously (5). For the preparation of L-III sequence-specific affinity column, double-stranded lBamL3 oligonucleotide was phosphorylated by T4 polynucleotide kinase and ligated with T4 DNA ligase reaction. Average 200-300 bp of concatemer lBamL3 oligonucleotide (100 μg) was resuspended in 50 mM Tris-HCl, pH 7.2, 10 mM $MgSO_4$, 1 mM dithiothreitol, 0.1 mM Biotin-14-dATP, 0.5 mM dGTP, 0.5 mM dCTP, 0.5 mM dTTP, and 50 μg/ml bovine serum albumin (BSA). After adding 20 U Klenow fragment, the mixture (100 μl) was incubated at 37°C for 15 min and subjected to a Nick column equilibrated with 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.5 M NaCl (TEN 0.5) to remove unincorporated nucleotides. Biotinylated DNA was mixed with streptoavidin paramagnetic particles in TEN 0.5 and coupling reaction was carried out by rotating at room temperature for 60 min. After washing with buffer C (25 mM Tris-HCl, pH 8.0, 1 mM EDTA, 5 mM $MgCl_2$, 0.1% Nonidet P-40, 1 mM dithiothreitol and 10% glycerol) containing 2.5 M KCl, it was stored in buffer C containing 1 M KCl at 4°C (13).

Preparation of rat liver nuclear extract and purification of the L-IIIBPs. Male Sprague-Dawley rats (6 weeks-old) were used. Liver nuclei and nuclear extract were prepared as described previously (13). All operations were carried out at 4°C. L-IIIBP activity was monitored by EMSA with a ^{32}P-labeled L-III oligonucleotides. Crude nuclear extracts were prepared from 254 rat livers and subjected to HiLoad Sephacryl S-100HR 26/60 column equilibrated with buffer C containing 0.2 M KCl. Active fractions were pooled. After 0.33 g/ml of $(NH_4)_2SO_4$ was added to pooled fractions, they were stirred for 30 min on ice. After centrifugation, the precipitates were resuspended in buffer C containing 0.1 M KCl and dialyzed against the same buffer. The dialysate was then applied to DEAE-Sepharose Fast Flow column equilibrated with buffer C containing 0.1 M KCl. It was washed with buffer C containing 0.3 M KCl to remove contaminated DNA. These flow through fractions were diluted to 0.1 M KCl in buffer C and applied to a Heparin-Sepharose CL-6B column equilibrated with buffer C containing 0.1 M KCl. It was washed with 3 bed volume of the same buffer and step-wisely eluted with 0.3 M KCl and 0.6 M KCl in buffer C. Active fraction (0.3 M KCl fraction) was pooled, precipitated with ammonium sulfate, and dialyzed against 0.1 M KCl in buffer C. Finally, L-IIIBP was purified by L-III sequence-specific affinity column chromatography. Poly (dA-dT) and PMSF were added to the dialyzed purified fraction to final concentrations of 26.7 μg/ml and 1 mM, respectively. After the mixture stood on ice for 15 min, it was centrifuged to remove the insoluble protein at 4°C for 5 min. Then, the supernatant was mixed with a L-III sequence-specific affinity particles and incubated at room temperature for 15 min. After magnetic separation, the particles were washed thoroughly with buffer C containing 0.3 M KCl. L-IIIBP activities were eluted with buffer C containing 1 M KCl. This fraction was diluted with buffer C to 0.1 M KCl and subjected to the second-round affinity column chromatography. The procedures were same as the first-round except for use of a final concentration of 6.4 μg/ml poly (dA-dT). The active fraction was subjected to a 10/20% sodium dodecyl sulfate (SDS)/polyacrylamide gradient gel electrophoresis (PAGE). Separated proteins were visualized by silver stain.

EMSA. EMSA were carried out as described previously (13). For the heat stability experiment, nuclear extract was heated at 60°C or 90°C for 5 min before being added to the binding reaction. Competition experiments included a 200-fold molar excess of unlabelled competitor DNAs. For supershift experiments, normal rabbit IgG (1 μl) or anti human USF antibody (1 or 0.1 μl) was added to the binding mixture. The anti human USF antiserum was a gift from Dr. Michele Sawadogo (MD Anderson Cancer Center, Texas, U.S.A.). In the case of the affinity-purified fraction, the reaction was carried out

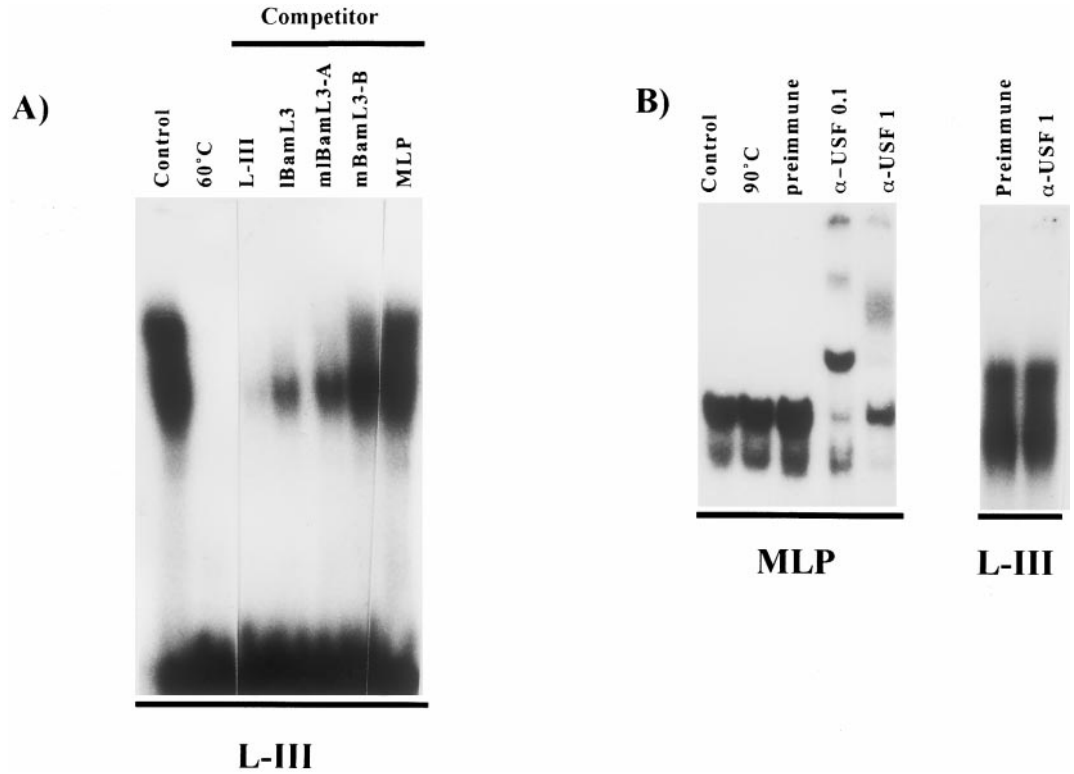

FIG. 1. Characterization of the L-IIIBP. End-labeled L-III or MLP oligonucleotide were incubated with 5 μg of rat liver nuclear extract, respectively. For heat treatment, nuclear extract was incubated at 60°C (A) or 90°C (B) for 5 min, respectively. The mixture contained a 200-fold molar excess of the competitor DNAs shown at the top (A). Preimmune serum or an antiserum directed against USF (0.1 or 1 μl) was added to the binding reaction as indicated (B). The acrylamide concentration of the gels were 6% (A) or 4% (B), respectively.

under the same conditions except for the presence of 10 μg of BSA and 20 ng of poly (dA-dT).

Ultraviolet crosslinking. Purified L-IIIBPs from 2 nd round of the sequence-specific affinity and ^{32}P-labeled L-III oligonucleotide were mixed in the same condition of EMSA. Binding reaction was carried out at room temperature for 30 min. Reactions were irradiated for 15 min to ultraviolet ray using a Spectrolinker (Spectronics Corporation, XL-1000). After crosslinking, samples were recovered by trichloroacetic acid precipitation and subjected to a 12.5% SDS/PAGE. The gel was dried and exposed to Kodak XAR film at −80°C.

RESULTS AND DISCUSSION

Characterization of L-IIIBP. First, we attempted to further characterize L-IIIBP using EMSA. When the ^{32}P-labeled L-III oligonucleotide was mixed with rat liver nuclear extract, L-IIIBP activities were observed as multiple bands on a 6% polyacrylamide gel (Fig. 1A). We examined whether these bands were identical to the USF protein. The USF protein has two unique properties. One is that the USF protein binds to the E box sequences (5′-CACGTG-3′) of the adenovirus major late promoter (MLP) in a high affinity manner. The other is that the USF protein is a heat-stable protein. When the labeled MLP oligonucleotide was mixed with the liver nuclear extract, two major bands were observed (Fig. 1B). These bands disappeared by the addition of 200-fold molar excess of unlabeled MLP oligonucleotide but not unlabeled L-III oligonucleotide (5). Binding was still observed when nuclear extract was incubated with 90°C for 5 min. In contrast, the L-IIIBP activities decreased by the addition of 200-fold molar excess of unlabeled L-III oligonucleotide but not unlabeled MLP oligonucleotide (Fig. 1A). All bands disappeared by preheating the nuclear extract at 60°C for 5 min. These results indicate that the L-IIIBP preferentially binds to the L-III oligonucleotide than to the MLP oligonucleotide and is a heat-labile protein. Then, to confirm that heat-labile L-IIIBP does not contain USF protein, we carried out the supershift assays using anti human USF antiserum. As shown in Fig. 1B, the USF activities binding to the MLP oligonucleotide were specifically supershifted with the addition of anti human USF antiserum. In contrast, USF antiserum did not affect mobility of complexes between heat-labile L-IIIBP and the L-III oligonucleotide (Fig. 1B). From these results, we conclude that L-IIIBP is different from the USF protein. However, it remains to be determined whether the fast-migrating bands are degradated forms of the slow-migrating proteins or distinct proteins.

Next, to examine whether L-IIIBP is a candidate

protein involved in the carbohydrate responsiveness, we carried out competition experiments using EMSA. Oligonucleotides and their responsiveness to carbohydrate are listed in Table 1. The lBamL3 oligonucleotide contains 2 bp longer nucleotide sequences at each 5′- and 3′-end than the L-III oligonucleotide and its 5′-end is replaced by 5′-GATC for the biotinylation by the Biotin-14-dATP to create a sequence-specific affinity column. The mlBamL3-A oligonucleotide contains mutated nucleotide sequence between two imperfect palindromic E boxes (5′-CACGGG-3′). It has been reported that nucleotide sequences of these oligonucleotides can confer the glucose responsiveness to the reporter gene (10). The mlBamL3-B is mutated at 3′-side of two imperfect palindromic E boxes (5′-CCCGTG-3′) and this mutant does not have the glucose responsiveness by reporter assays (10). The MLP oligonucleotide contains a perfect E box, 5′-CACGTG-3′, and does not confer the glucose responsiveness. As shown in Fig. 1A, L-IIIBP activities were competed out by the addition of 200-fold molar excess of unlabeled L-III, lBamL3, and mlBamL3-A oligonucleotides. In contrast, L-IIIBP activities were partially competed with the addition of 200-fold molar excess of unlabeled mlBamL3-B oligonucleotide and were not competed with the MLP oligonucleotide. Our results showed that L-IIIBP preferentially bound to two copies of imperfect palindromic E box motif separated by 5 bp (5′-CACGGGNNNNNCCCGTG-3′) in a high affinity manner and a single copy of 5′-CACGGG-3′ in a weak affinity manner, and that L-IIIBP did not bind to a copy of perfect E box 5′-CACGTG-3′. These results suggest that L-IIIBP is a candidate for carbohydrate response factor.

Purification of L-IIIBP. Finally, we purified the L-IIIBP from 4,640 mg of crude nuclear extract which was prepared from 254 male Sprague-Dawley rat livers. The purification procedures included gel filtration, anion-exchanger chromatography, heparin affinity chromatography, and two rounds of DNA sequence-specific affinity chromatography (see Materials and Methods). However, the purification of L-IIIBP was very difficult since it is an extremely unstable protein. The purified L-IIIBP was subjected to a SDS/PAGE and was visualized by silver staining. As shown in Fig. 2A, two major bands were observed in the purified fraction from two-round sequence-specific affinity chromatography. Their molecular masses were 26 and 24 kDa, respectively. EMSA using this fraction showed that their binding to the L-III oligonucleotide was competed with the L-III oligonucleotide but not the MLP oligonucleotide and inactivated by heating at 60°C for 5 min, although the observed bands were apparently faster-migrating than that of the starting materials (Fig. 2B). These binding mixtures were subjected to the UV crosslinking. The crosslinked L-III/L-IIIBP complexes were analyzed on a SDS-PAGE. The bands, 34 and 32 kDa, were competed with the L-III oligonucleotide but not the MLP oligonucleotide (Fig. 2C). As the probe oligonucleotide showed Mr 8,000, estimated molecular mass of L-IIIBP was 26 and 24 kDa. These sizes coincided with molecular mass of purified L-IIIBP. Although L-IIIBP activities in the EMSA using purified L-IIIBP were detected as three major bands, the formation of homo- and hetero-dimer between 26 and 24 kDa proteins resulted in the bands.

In this study, we characterized and purified the L-IIIBP. L-IIIBP is a heat-labile protein and preferentially binds to single or two copies of 5′-CACGGG-3′ rather than a single copy of 5′-CACGTG-3′ which is the recognition sequence of the basic helix-loop-helix (bHLH) proteins. To explain discrepancy between the present results and previous observations made by Kahn's group for detection of L-IIIBP, the following possibilities can be considered. First, the length and nucleotide sequences of oligonucleotide used were different. Whereas we used 5′CG<u>C</u>CACGGGGCAC-TCCCGTGG-3′ which correspond to −170 to −150 bp, they used the longer oligonucleotides which correspond to −173 to −140 bp. Oligonucleotides we used were 13 bp shorter than theirs and had an additional C residue (underlined). However, when we used their oligonucleotide as a competitor in the EMSA, the L-IIIBP activities were competed out in a high affinity manner, similar to our probe (data not shown). Thus, this possibility can be eliminated. Second, it is possible that the quality of nuclear extracts from both groups is different. Bendall and Molloy reported the base preferences for DNA binding by human USF (17). They showed that a 43 kDa form of human USF binds to the E box sequence (5′-CACGTG-3′) in a high affinity manner. Although USF binds to an imperfect E box sequence (5′-CACGGG-3′) weakly, its binding to the cognate sequences is dependent upon the absence of $MgCl_2$ in the binding mixture. We observed that bacterially synthesized USF protein bound to the L-III oligonucleotide even in the presence of 5 mM $MgCl_2$ (data not shown). However, this binding affinity was very weak in comparison with USF binding to MLP oligonucleotide. Accordingly, it is supposed that only when nuclear extract contains the enriched USF activity, USF could bind to the L-III element. Thus, the quality of nuclear extracts may be very critical to detect L-IIIBP activity.

Although L-PK, S_{14}, or other carbohydrate-regulatable genes are expressed and regulated in a tissue-specific manner, many of other genes including a USF-binding site (E box) in their functional promoter are not regulated by carbohydrates. Moreover, the substitution of the L-III element with a consensus USF-binding site in the L-PK promoter resulted in loss of the carbohydrate responsiveness (9). It appears that USF itself is not a carbohydrate

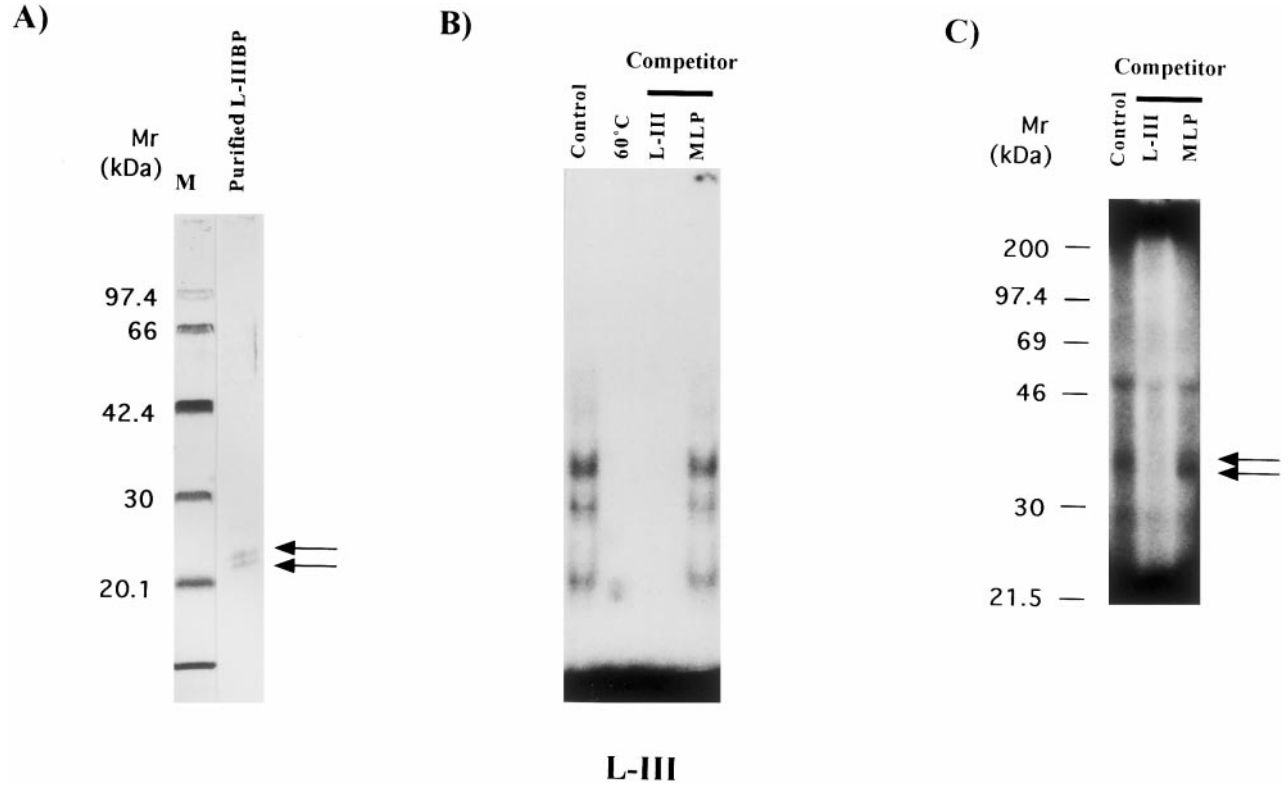

FIG. 2. Analysis of the purified L-IIIBP. (A) Purified L-IIIBP was separated by SDS/PAGE on a 10/20% gradient gel and the gel was developed with silver stain. M indicates molecular-mass markers. The arrows indicate the positions of purified L-IIIBP. (B) The purified protein was analyzed by EMSA using the L-III oligonucleotide as a probe in the presence or absence of a 200-fold molar excess of the indicated competitors. The purified L-IIIBP was also preincubated at 60°C for 5 min before the binding reaction. (C) The purified L-IIIBP was subjected to the binding reaction in the presence or absence of the competitor DNAs, then UV-irradiated. After TCA precipitation, the sample was subjected to a 12.5% SDS/PAGE. The gel was dried and exposed to a X-ray film with an intensifying screen at −80°C. Molecular-mass were indicated on the left. Arrows indicate the products.

response factor. Kaytor et al. reported that USF was not involved in the glucose-mediated regulation of the L-PK gene, based upon results with dominant negative forms of USF and with ChoRE mutants (16). The present study suggests that the purified L-IIIBP, which is different from USF, is involved in carbohydrate regulation of the PKL gene transcription. It has been reported that okadaic acid, an inhibitor of protein phosphatase 1 and 2A, and 5-amino-4-imidazolecarboxamide riboside, an agonist of 5′-AMP-activated protein kinase, inhibit stimulation of the PKL and S14 gene transcription by carbohydrate (18, 19). In addition, carbohydrates such as glucose and fructose are suggested to act on these genes through some their metabolites (20). Several metabolites including glucose-6-phosphate and xylulose-5-phosphate have been proposed as such candidates (21, 22). Thus, it is very likely that the L-IIIBP activity is regulated by phosphorylation/dephosphorylation mechanism, which is stimulated by some metabolite of carbohydrates. Very recently, Hasegawa et al. has reported that a novel heat-labile protein (designated as glucose response element binding protein, GRBP) binds to the L-III element and that its binding is increased by a high carbohydrate-diet (23). It remains to be determined whether GRBP is identical to L-IIIBP.

To analyze the physiological function of L-IIIBP *in vivo*, we are performing the determination of their peptide sequences and molecular cloning of the corresponding cDNAs.

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