

REVIEW ARTICLE

Nutrient and hormonal regulation of pyruvate kinase gene expression

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Mammalian pyruvate kinase (PK), a key glycolytic enzyme, has two genes named *PKL* and *PKM*, which produce the L- and R-type isoenzymes by means of alternative promoters, and the M₁- and M₂-types by mutually exclusive alternative splicing respectively. The expression of these genes is tissue-specific and under developmental, dietary and hormonal control. The L-type isoenzyme (L-PK) gene contains multiple regulatory elements necessary for regulation in the 5' flanking region, up to position –170. Both L-II and L-III elements are required for stimulation of L-PK gene transcription by carbohydrates such as glucose and fructose, although the L-III element is itself responsive to carbohydrates. The L-II element is also responsible for the gene regulation by polyunsaturated fatty acids. Nuclear factor-1 proteins and hepatocyte nuclear factor 4, which bind to the L-II element, may also be involved in carbohydrate and polyunsaturated fatty acid regulation of the L-PK gene respectively. However, the L-III-element-binding protein that is involved in carbohydrate regulation remains to be clarified, although in-

volvement by an upstream stimulating factor has been proposed. Available evidence suggests that the carbohydrate signalling pathway to the L-PK gene includes a glucose metabolite, possibly glucose 6-phosphate or xylulose 5-phosphate, as well as phosphorylation and dephosphorylation mechanisms. In addition, at least five regulatory elements have been identified in the 5' flanking region of the *PKM* gene up to position –279. Sp1-family proteins bind to two proximal elements, but the binding of proteins to other elements have not yet been clarified. Glucose may stimulate the transcription of the *PKM* gene via hexosamine derivatives. Sp1 may be involved in this regulation via its dephosphorylation, although the carbohydrate response element has not been determined precisely in the *PKM* gene. Thus glucose stimulates transcription of the *PKM* gene by the mechanism which is probably different from the L-PK gene.

Key words: Carbohydrate, glycolysis, isoenzyme, transcriptional regulation.

INTRODUCTION

Since the glycolytic pathway is a fundamental system for energy metabolism in organisms, glycolytic enzymes are present in all mammalian cells or tissues. However, individual tissues utilize these enzymes in unique ways. For example, the liver predominantly converts pyruvate, a glycolytic product, into fatty acid via acetyl-CoA, whereas glucose is metabolized to lactate or CO₂ and water for energy production in muscle and brain. In addition, liver and kidney are the only tissues which generate glucose from C₃ compounds such as pyruvate, lactate and glycerol. Thus, when gluconeogenesis is stimulated by starvation, glycolysis must be suppressed in these two tissues. In order to fulfil these demands, most glycolytic enzymes, including pyruvate kinase (PK; ATP:pyruvate 2-O-phosphotransferase, EC 2.7.1.40) exists in multiple forms (isoenzymes). Generally these are expressed in a tissue-specific manner and have distinct properties.

PK is a rate-controlling glycolytic enzyme and catalyses the formation of pyruvate and ATP from phosphoenolpyruvate and ADP. In mammals, PK exists as four isoenzymes, which are referred to as the L-, R-, M₁-, and M₂-types respectively [1]. The L-PK is predominantly expressed in the liver, but is also present in the kidney, small intestine and pancreatic β -cells [1,2]. The

expression of the R-PK is restricted to erythrocytes. While M₁-PK is expressed in the skeletal muscle, heart and brain, M₂-PK is expressed in other tissues. The expression of the PK isoenzymes is developmentally regulated. The M₂-PK is the only detectable isoenzyme in early fetal tissues and, during development, it is gradually replaced by the L-, R-, or M₁-types. In contrast, in transformed cells and regenerating liver, the tissue-specific isoenzymes are present at decreased levels or absent altogether and are replaced by the M₂-type isoenzyme.

All PKs are cytosolic enzymes and function as a tetramer. The biochemical properties and molecular characteristics of the PK isoenzymes are listed in Table 1. The number of amino acid residues deduced from the nucleotide sequences of each cDNA is 574 for R-PK [3], 543 for L-PK [4,5], 531 for M₁-PK, and 531 for M₂-PK [6] respectively. The nucleotide sequences of R- and L-PK cDNA are nearly identical, except for the 5'-untranslated region, a portion of the coding sequences, and the length of the 3'-non-coding sequences (Figure 1a). In addition, the nucleotide sequences of M₁- and M₂-PK cDNA are also identical, except for 160 nucleotide sequences within the coding regions (Figure 1b). These nucleotide sequences encode the amino acid sequences which are responsible for intersubunit contact [7–9]. The sequence similarity of this region of M₂-PK cDNA with L-PK cDNA is higher than that of M₁-PK cDNA. With exception of M₁-PK, the

Abbreviations used: PK, pyruvate kinase; FBP, fructose-1,6-bisphosphate; PKA, cyclic AMP-dependent protein kinase; PUFA, polyunsaturated fatty acids; CAT, chloramphenicol acetyltransferase; NF-1, nuclear factor-1; HNF, hepatocyte nuclear factor; EMSA, electrophoretic-mobility-shift assay; L-IIBP, L-II-element-binding protein; COUP-TF, chicken ovalbumin upstream promoter–transcription factor; bHLH, basic helix–loop–helix; L-IIIIBP, L-III-element-binding protein; USF, upstream stimulating factor; S₁₄, Spot 14; ChoRE, carbohydrate-response element; GIRE, glucose-response element; LZ, leucine zipper; PPAR, peroxisome-proliferator-activated receptor; RXR, retinoid X receptor; GLUT, glucose transporter; FAS, fatty acid synthase; T₃, 3,3',5-tri-iodo-L-thyronine; CREB, cAMP-response-element-binding protein.

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Table 1 Biochemical properties and molecular characteristics of the rat pyruvate kinase isoenzymes

Abbreviations: PEP, phosphoenolpyruvate; PKA, protein kinase A.

Biochemical property	Isoenzyme ... R	L	M ₁	M ₂
Molecular mass of subunit (kDa)	62	59	58	58
Number of amino acids	574	543	531	531
Kinetics with regard to PEP	Sigmoidal	Sigmoidal	Hyperbolic	Sigmoidal
K _m (mM) for PEP	1.0–1.4	0.3–0.9	0.08	0.4
K _m (mM) for ADP	0.4–0.6	0.1–0.4	0.3	
Activation by FBP (mM)	Yes (0.4)	Yes (0.06–0.1)	No	Yes (0.1–0.4)
K _i (mM) for ATP	0.04–0.06	0.1–0.15	3–3.5	2.5
Phosphorylation by PKA	Yes	Yes	No	No
Inhibition by amino acids				
K _i (mM) for L-alanine		1.0		0.6
K _i (mM) for L-phenylalanine		5.0		0.5
Tissue distribution	Erythrocytes	Liver Kidney Small intestine Pancreatic β -cells	Muscle Heart Brain	Widely distributed Fetal tissues Tumour
mRNA size (kb)	2.1	3.2, 2.8, 2.2, 2.1	2.2	2.2
Gene	<i>PKL</i>	<i>PKL</i>	<i>PKM</i>	<i>PKM</i>

mammalian PKs exhibit allosteric properties, being activated homotropically by phosphoenolpyruvate and heterotropically by fructose 1,6-bisphosphate (FBP). The intersubunit contact domain is responsible for the allosteric properties of the three PK isoenzymes. In fact, the replacement of a single amino acid in this region results in the acquisition of allosteric properties and activation by FBP in the case of M₁-PK, and substantial loss of both homotropic and heterotropic allosteric effects in the case of M₂-PK [10,11]. Kato et al. cloned a human cytosolic thyroid hormone-binding protein (p58) cDNA using monoclonal antibodies against p58 [12]. Interestingly, nucleotide sequence analysis revealed that the p58 protein is identical with the M₂-PK subunit. Although the monomer has 3,3',5-tri-iodo-L-thyronine (T₃)-binding activity and only 5% pyruvate kinase activity, the tetramer does not bind T₃. Conversion of the p58 protein into the tetramer is reversible and is controlled by an allosteric effector of pyruvate kinase, FBP. We speculate that the switching mechanism for the dual function of the M₂-PK protein, as based on the participation of FBP, has been acquired as an evolutionary trait.

The expression of PK activity is regulated by both quantitative (protein synthesis and degradation) and qualitative (phosphorylation–dephosphorylation) alterations by various hormones and nutrients. In the present review we focus on the regulation of PK gene expression by hormones and nutrients.

ORGANIZATION OF RAT PK GENES

Mammalian PK isoenzymes are encoded by two genes, the *PKL* or *PKM* genes respectively. Whereas the R- and L-PK isoenzymes are encoded by the *PKL* gene [3,13], the M₁- and M₂-PK isoenzymes are encoded by the *PKM* gene (Figure 1) [14,15].

The rat *PKL* gene is approx. 9.3 kb in length and consists of 12 exons and 11 introns [3]. The first exon (exon R) and the second exon (exon L) encode the 5'-non-coding and N-terminal sequences which are specific to the R-type and L-type PK isoenzymes respectively. The other exons are common to both isoenzymes. The third exon encodes the amino acid sequence, Arg-Arg-Ala-Ser, which is the target sequence for cAMP-dependent protein kinase (PKA). These isoenzymes are

produced from the *PKL* gene via tissue-specific promoters. Whereas transcription is initiated from the second exon in the liver, kidney, small intestine and pancreatic β -cells, it is initiated from the first exon in erythroid cells. In the latter case, the second exon is spliced out as an intron. Hereafter the term 'L-PK gene' specifically refers to the gene for the L-type isoenzyme in the *PKL* gene.

In contrast, the rat *PKM* gene is approx. 20 kb in length and also consists of 12 exons and 11 introns [14]. Exons 9 and 10 encode sequences which are specific to M₁- and M₂-isoenzymes respectively [6,14]. The other exons are common to both isoenzymes. Unlike the *PKL* gene, the target amino acid sequence that is phosphorylated by PKA is not encoded by any exons. These isoenzymes are produced by mutually exclusive alternative splicing from a common primary transcript. Exon 9 is selected in the skeletal muscle, heart and brain, while exon 10 is selected in other adult tissues as well as in tumour cells. The regulatory mechanisms for the tissue-specific alternative splicing remain to be determined, although, in an earlier study, we showed that the 5' splice site of exon 9 (M₁) was hardly recognized by the splicing machinery in M₂-expressing cells, but not by M₁-expressing cells [16].

The human *PKM* and *PKL* genes and the chicken *PKM* gene have also been cloned and their nucleotide sequences have been determined [13,15,17]. The sizes of these genes are 32, 9.5 and at least 14 kb respectively. It is noteworthy that the introns in both the *PKL* and *PKM* genes interrupt the protein-coding sequences of the four isoenzymes at identical positions. It can be assumed from the viewpoint of evolution that these genes are derived via gene duplication from an ancestral gene. Thereafter, each gene acquired the systems of expression of two isoforms from a single gene, such as, for example, the tissue-specific promoter or tissue-specific mutually exclusive splicing.

NUTRITIONAL AND HORMONAL REGULATION OF L-PK GENE EXPRESSION

Liver

Hepatic pyruvate kinase activity, which is largely attributed to the L-type isoenzyme, decreases as a result of starvation or

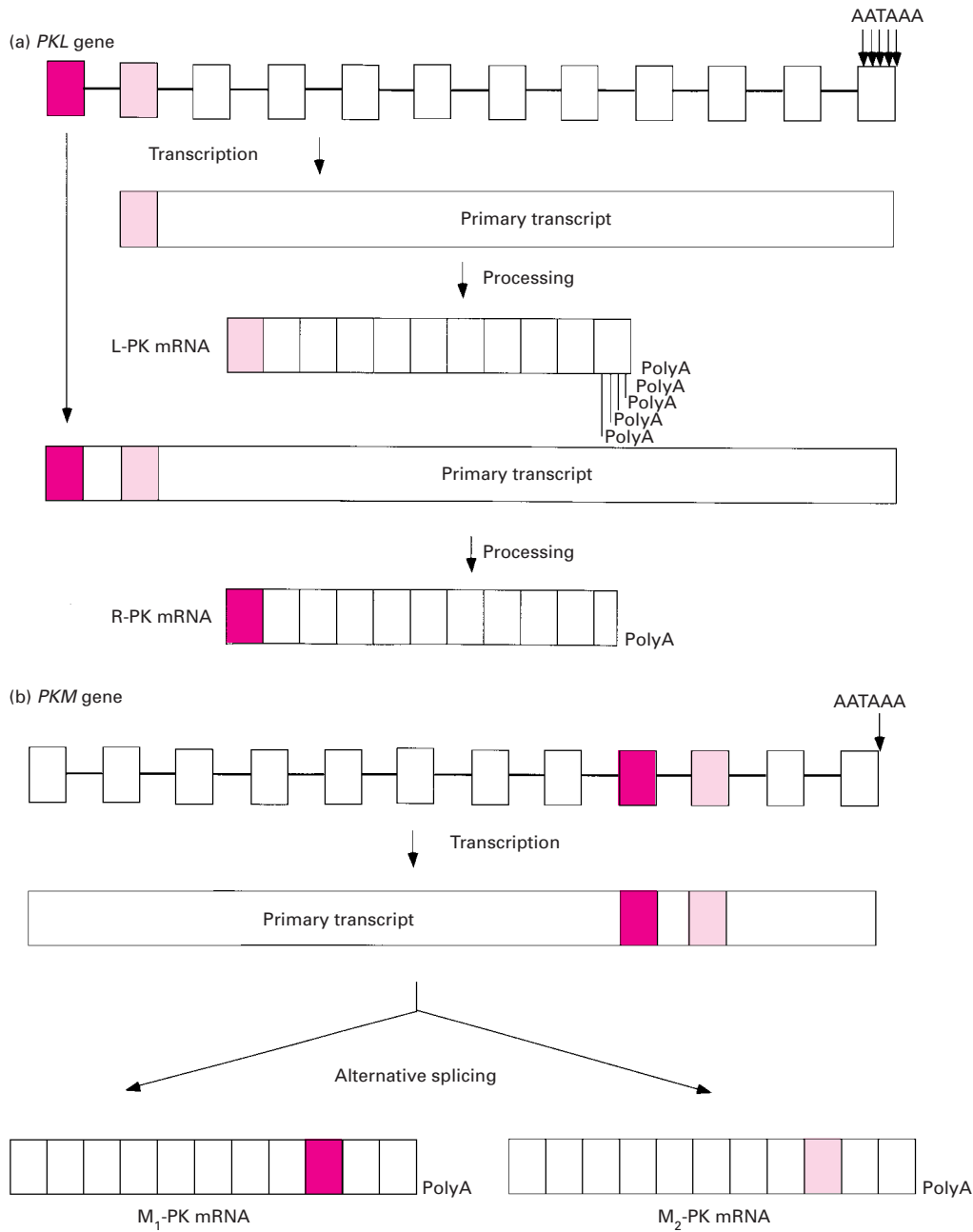


Figure 1 Schematic representation of the expression of R- and L-type PK mRNA from the rat *PKL* gene by the use of tissue-specific promoters (a) and of M₁- and M₂-PK mRNA from the rat *PKM* gene by mutually exclusive alternative splicing (b)

(a) Exons R and L are shown in red and pale pink respectively. (b) Exons M₁ and M₂ are shown in red and pale pink respectively.

diabetes and increases as the result of a high carbohydrate diet or by the administration of insulin to diabetic rats [18]. Glucagon via cAMP inhibits hepatic L-PK activity. Such regulation occurs at the post-translational as well as pre-translational levels. cAMP stimulates phosphorylation of the L-PK enzyme by PKA to inhibit its enzyme activity [19,20], while insulin activates L-PK by stimulating the dephosphorylation of the enzyme [21]. On the other hand, as shown in Table 2, the level of hepatic L-PK mRNA is also increased by feeding a high-glucose diet and is decreased in streptozotocin-induced diabetic rats which are fed a high glucose-diet or in fasted rats [22,23]. Insulin administration

to diabetic rats results in an increase in L-PK mRNA levels in liver. Increases in the level of the L-PK mRNA as the result of a high-glucose diet in normal rats or by insulin administration to diabetic rats are largely due to stimulation of gene transcription [24,25], although glucose in the presence of insulin modulates the stability of the L-PK mRNA in cultured hepatocytes [26]. The action of insulin on L-PK gene transcription in liver requires ongoing protein synthesis, since this insulin effect is blocked by cycloheximide [25]. Glucagon inhibits both the accumulation of L-PK mRNA and an increase in the L-PK gene transcription by insulin [25,26]. Diets containing fructose or glycerol also induce

Table 2 Dietary and hormonal regulation of L-PK mRNA in various tissues

ND, not determined.

	Liver	Kidney	Small intestine
Normal			
+ Glucose	Increase	No effect	Slightly increase
+ Fructose	Increase	Increase	Increase
+ Glycerol	Increase	Increase	No effect
+ PUFA	Decrease	ND	ND
Diabetes			
Or fasting	Decrease	No effect	Decrease
+ Insulin	Increase	No effect	ND
+ Insulin + Glucagon	Decrease	ND	ND
+ Glucose	No effect	No effect	No effect
+ Fructose	Increase	Increase	Increase
+ Glycerol	Increase	Increase	ND

the accumulation of the L-PK mRNA in rat liver [24,25,27], but other dietary carbohydrates, such as galactose, sorbitol, xylose and xylitol, do not [27]. The time course for the increase by fructose or glycerol is much faster than that of the increase induced by dietary glucose in normal rats or insulin in diabetic rats [25,27,28]. The mechanisms of the fructose-induced (and probably glycerol-induced) increase in the L-type PK mRNA are dependent upon plasma insulin levels. In both normal and diabetic rats, similar increases in the amount of L-PK mRNA are observed following an intake of dietary fructose [24,25,27,28]. However, the transcriptional rate of the L-PK gene in diabetic liver was found to be much lower than that in normal liver [25,27]. As a result, the expression of the L-PK gene by insulin and dietary carbohydrates in liver is regulated at both the transcriptional and post-transcriptional levels. The stability of L-PK mRNA is a critical step in regulation at the latter level.

In the liver of rats that have been fed a high-carbohydrate diet containing polyunsaturated fatty acids (PUFA), such as linoleic acid, eicosapentaenoic acid or docosahexaenoic acid, both the activity and the level of L-type PK mRNA is decreased by 60 and 70% respectively [29–31]. These effects are specific to PUFA, since oleic acid does not substitute for them. In addition, PUFA inhibited increases in both the activity and mRNA levels of L-PK by insulin and glucose in primary cultured hepatocytes [30].

Kidney

Dietary glucose and insulin have no effect on the expression of the L-PK gene in the kidney of both normal and diabetic rats [27]. However, both dietary fructose and glycerol were found to induce the accumulation of L-PK mRNA in this tissue of both rats. The L-PK mRNA levels in the kidney reached a maximum up to 8 h after fructose feeding. This time course is similar to that observed in the diabetic liver. Although the transcriptional rate of the L-PK gene is increased in the kidney of normal and diabetic rats which are fed a fructose-diet, the magnitudes of the increase were lower than those of the mRNA levels. Thus dietary fructose affects the stability of the mRNA as well as transcription of the gene in the normal and diabetic kidney.

Small intestine

Regulation of L-PK gene expression by dietary carbohydrates was also determined in the small intestine [27,32]. Both dietary glucose and fructose increased the L-PK mRNA level in this tissue, but the latter is a much stronger inducer of L-PK than the

former. The time course of induction of L-PK mRNA in the small intestine by dietary fructose is similar to those in the liver and kidney. In contrast, dietary glycerol had no effect on L-PK mRNA levels in the small intestine.

While the metabolism of fructose is catalysed by fructokinase expression in the liver, kidney and small intestine, that of glycerol is catalysed by glycerol kinase expression in the liver and kidney. Therefore dietary fructose and glycerol are capable of inducing increases in L-PK mRNA levels only in tissues in which they were metabolized. Thus it is hypothesized that metabolite(s) common to the metabolism of fructose and glycerol affect L-PK gene expression both at the transcriptional and post-transcriptional levels. Tissue-specific L-PK gene expression and its regulation by hormones and nutrients were further analysed using transgenic mice that harbour the L-PK gene promoter linked to a reporter gene, such as chloramphenicol acetyltransferase (CAT) gene, and transient expression assay of the fusion genes in cultured cells (see below).

REGULATORY ELEMENTS AND TRANS-ACTING FACTORS OF THE RAT L-PK GENE

Cell type-specific expression

As shown in Figure 2(a), both positive and negative regulatory elements in the rat L-PK gene promoter have been identified by the CAT reporter assay using cultured cells [33,34] or transgenic mice [35–39]. Three positive regulatory regions were identified [34] and designated as L-I, L-II and L-III. These regions were also identified and named as L1, L3 and L4 respectively by Kahn's group [40]. These elements are located in the regions –94 to –76, –149 to –126, and –170 to –150, respectively. In addition, Kahn's group has identified a nuclear factor-1 (NF-1)-binding site, named L2, from –116 to –99 by *in vitro* footprint analysis [40], that functions as a weak negative element [38]. When a single copy of L-I was linked upstream of the TATA box-containing CAT construct, transcriptional activity was observed. In contrast, neither L-II nor L-III had any independent effect when a single copy of each element was linked to the above construct. However, combinations of L-I + L-II and L-II + L-III, but not of L-I + L-III, showed synergistic transcriptional activities when oriented in the same direction. The inclusion of all three elements oriented in the same direction showed the maximal synergistic effect. These results suggest that these elements function as a unit. In fact, this unit enhanced transcription from heterologous as well as homologous promoters. The activity of the unit was not detected in HeLa cells or K562 erythroleukaemia cells, suggesting that it possesses cell-type specificity [34]. When an analysis of transgenic mice, which carry an enhancer unit between –189 and +37 linked to the CAT reporter plasmid was performed, the expression of the transgenes was observed only tissues in which the endogenous L-PK gene is expressed [37].

Within the unit, the L-I and L-II element contained the putative binding sequences of liver-enriched transcription factor of hepatocyte nuclear factor (HNF) 1 and 4, which are 5'-GTTATACTTTAAC-3' and 5'-TGGACTCTGGCCC-3', respectively [34,40]. In fact, it has been shown that HNF1 and HNF4 bind to the L-I and L-II elements respectively, as evidenced by an electrophoretic-mobility-shift assay (EMSA) [34,41,42]. We also purified heat-stable L-II element-binding proteins (L-IIBPs) from rat liver nuclear extracts and found that they are members of the NF-1 family, which includes such proteins as NF-1L and NF1/Red1 [41]. HNF4 and NF-1 family members bind to overlapping sequences of the L-II element. The co-transfection of the expression vector of these transcription factors with

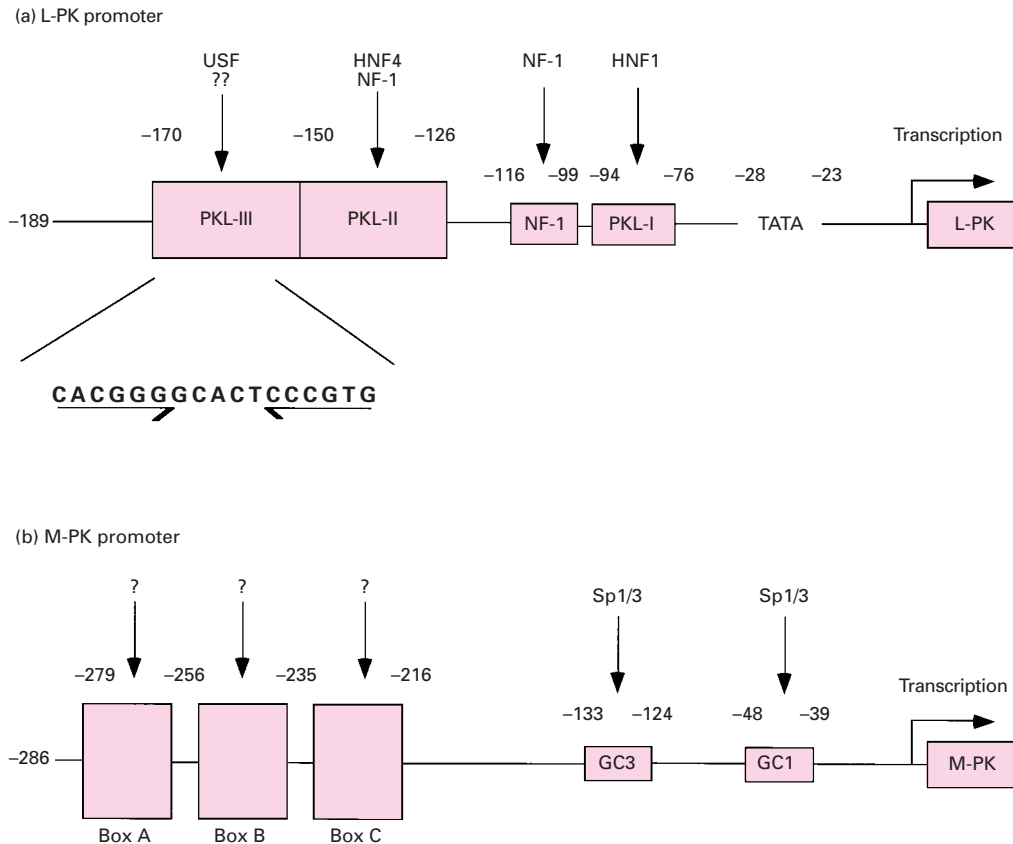


Figure 2 Schematic representation of the *cis*-acting elements and binding proteins of the *PKL* (a) and *PKM* (b) gene promoter

L-PK–CAT reporter plasmid revealed that HNF1 and HNF4 induced a CAT activity which was dependent upon the L-I and L-II elements respectively [42] and that both NF-1L and NF1/Red1 repressed HNF4-induced CAT activity [41]. In addition, it has been reported that the chicken ovalbumin upstream promoter–transcription factor (COUP-TF) binds weakly to the L-II element and that co-transfection of the COUP-TF expression vector represses CAT activity [42]. However, we and others failed to detect the binding of COUP-TF to the L-II element ([43]; K. Yamada, T. Tanaka and T. Noguchi, unpublished work).

The L-III element consists of two imperfect palindromic E box (5′-CCCGTG-3′) sequences separated by 5 bp [43]. The E box represents the binding site of the basic helix–loop–helix (bHLH) class of transcription factors. There are conflicting reports relative to the L-III-element-binding protein (L-IIIIBP). Kahn’s group has reported that the upstream stimulating factor (USF) binds to the L-III element [40,44] and that co-transfection of the USF expression plasmid stimulates the expression of the reporter gene which contains the L-III element [44]. However, we found that a major L-IIIIBP was an unknown protein, different from USF, as judged from the following observations. First, the binding of rat liver nuclear protein to the L-III element was not competitive with respect to the oligonucleotide-containing USF-binding site, and this protein did not react with a specific antibody raised against USF [34] (K. Yamada and T. Noguchi, unpublished work). Second, L-IIIIBP was inactivated by heat treatment at 60 °C for 5 min, whereas USF was heat-resistant (K. Yamada and T. Noguchi, unpublished work). Recently, Kaytor et al. also reported that an unknown protein which was

different from USF interacted with the L-III element as well as with the L-III like element of the rat Spot 14 (S_{14}) gene (see below) [45], although they previously demonstrated that the hepatic nuclear factor is indistinguishable from the USF which binds to this element [46]. However, it remains to be determined whether this protein is identical with that described above. The reason(s) for the discrepancy in these results is not known, but may be due to differences in experimental conditions that have not yet been identified.

Thus transcription factors which interact with the L-II and L-III elements have not been clarified completely. However, of the factors mentioned above, HNF1 and HNF4 may be the most important for the tissue-specific expression of the L-PK gene, since only these factors are expressed in a tissue-specific manner. In addition, an unidentified L-IIIIBP may also be required for cell type-specific expression.

Carbohydrate-response element (ChoRE)

The L-PK gene promoter up to position –189 containing an enhancer unit was carbohydrate-responsive in primary cultured hepatocytes and INS-1 cells, an insulinoma β -cell line [39,47–49]. This region was also responsive to dietary fructose as well as dietary glucose in transgenic mice [37]. The efforts, therefore, were focused on the identification of a *cis*-acting element which is responsive to carbohydrate and its *trans*-acting factor(s). In the context of a natural promoter, a synergistic interaction between the L-II and L-III elements is required for carbohydrate resp-

onsiveness as well as for cell type-specific expression [46,48]. A single copy of neither the L-II nor the L-III element conferred carbohydrate responsiveness to the heterologous promoter. However, when the multimerized L-III element, but not the L-II element, was linked to the thymidine kinase promoter-CAT construct, glucose responsiveness was conferred on the heterologous promoter [48]. In addition, a mutation in the L-III element in the context of natural promoter resulted in loss of carbohydrate responsiveness [46]. Therefore the L-III element itself possesses responsiveness to carbohydrate, and the L-II element functions as an accessory element.

Transcription of the rat S_{14} gene, which is believed to be involved in some aspects of lipid metabolism [50], is also stimulated by carbohydrates through a *cis*-acting element which is designated ChoRE and which contains sequences similar to the L-III element [51]. This ChoRE also contains two imperfect E box sequences (Table 3). As in the case of the L-PK gene, a full response to glucose in this gene requires both a ChoRE and an adjacent upstream accessory site [43]. Hereafter, the L-III and L-II elements of the L-PK gene are referred to as the ChoRE and accessory element respectively. The ChoRE of the L-PK gene has also been referred to as the glucose response element (GIRE) by others [48]. The nucleotide sequences of ChoRE and the accessory elements of both genes are compared in Table 3. In the ChoREs of both genes, replacement of the nucleotide sequence of either or both the two imperfect E boxes with a consensus E box sequence (5'-CACGTG-3') or alterations of the nucleotide sequence between the two imperfect E box sequences had no effect on glucose responsiveness [42,43]. However, changes in the length between the two E boxes using other sequences, which are either shorter or longer than 5 bp, result in a dramatic decrease in the carbohydrate-responsiveness [43]. Therefore, for the case of carbohydrate response, it is very critical that two E boxes are separated by precisely 5 bp.

As mentioned above, at least two types of nuclear proteins, USF and an unidentified heat-labile protein, have been reported to bind to the L-III element and also to the ChoRE of the S_{14} gene. USF belongs to the bHLH/leucine zipper (LZ) family, which is a class C of the bHLH protein [52]. USF has two isoforms, a 43 kDa form (USF1) and a 44 kDa form (USF2), both of which are expressed ubiquitously [53]. These isoforms can form a heterodimer as well as a homodimer [53–56]. A

USF1–USF2 heterodimer accounts for more than 65% of the USF-binding activity in the liver [56]. The DNA-binding activity and transactivating potential of USF are regulated in a redox-dependent manner [57]. However, USF binding to the L-III element is not altered in liver nuclear extracts from rat fed on a high-carbohydrate diet [40]. An analysis of USF2-deficient mice revealed that, although only 20% of these mice survived for more than a few hours after birth, the surviving mice had low levels of L-PK and S_{14} gene expression in liver and showed a delayed response to carbohydrates [58]. However, the glucose responsiveness of these genes was not impaired in USF1-deficient mice [59]. In addition, microinjection of anti-USF2 antibodies into the nucleus of pancreatic β -cells inhibited the induction of L-PK gene transcription by glucose [60]. These results do not necessarily mean that USF is directly involved in carbohydrate regulation of the L-PK and S_{14} genes, since some proteins which are involved in carbohydrate regulation may be regulated by USF. L-PK, S_{14} or other carbohydrate-regulatable genes are expressed and regulated in a tissue-specific manner, while USF is an ubiquitously expressed protein. Even though other genes that are expressed in liver include a USF-binding site in their functional promoter, many of these 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 [46]. Thus it appears that USF itself is not a carbohydrate response factor. In fact, Kaytor et al., on the basis of results obtained with dominant negative forms of USF and with ChoRE mutants [45], reported that USF was not involved in the glucose-mediated regulation of the L-PK gene.

As described above, NF-1 family members and HNF4 bind to the accessory element, L-II, of the L-PK gene. We demonstrated that the former, but not the latter, also interacted with the corresponding element of the S_{14} gene [41], although this element appears to contain only a half site of the NF-1-binding consensus sequence (TGGN₇CCA) (Table 3). These findings suggest that only NF-1 family members are involved in transcriptional stimulation by carbohydrates of the two genes. Although NF-1L and NF1/Red1 repressed the HNF4-induced expression of the reporter gene which contains the L-II element (see above), they may interact directly with L-IIIBP to mediate transcriptional stimulation by carbohydrates. Indeed, the interaction between USF and CTF/NF1, a member of the NF-1 family, contributes to the transcriptional activation of the type-I plasminogen-activator-inhibitor gene by transforming growth factor- β [61]. It has also been reported that USF physically interacts with CTF/NF1 and binds to the transforming growth factor β -responsive unit of the collagen $\alpha 2(I)$ gene [62]. Alternatively, NF-1 family members may interact indirectly with L-IIIBP via a third partner, such as a co-activator, to stimulate transcription of the L-PK and S_{14} genes. Since the distances between the L-II and L-III elements are independent of carbohydrate responsiveness [63], the latter possibility seems to be more likely.

Interestingly, increases in both activity and mRNA accumulation of L-PK have been observed in transgenic mouse livers that overexpress c-Myc protein [64], which is a member of the bHLH/LZ protein family [65]. In addition, these mice did not show diabetic alterations. Although c-Myc does not bind to the ChoRE *in vitro*, the overexpression of c-Myc could somehow mimic the function of the carbohydrate-responsive factor. Alternatively, since both the activity and mRNA levels of glucokinase are increased in these transgenic mice, the putative glucose signalling machinery may be stimulated to increase L-PK gene expression (see below). In this case, c-Myc might exert an indirect effect through the ChoRE of the L-PK gene promoter.

Table 3 Sequence comparison of ChoRE and accessory element between the rat PKL gene and the rat S_{14} gene

E boxes are shown in **bold**. Putative NF-1- and HNF4-binding sites are shown as by asterisks (*) and underlining respectively.

(a)

ChoRE	Position	Sequence	Position
PKL-III	–170	CGCC CACGGG GCAC TCCC GTGG	–150
S_{14}	–1442	TCT CACGTG GTGG CCTGTGC	–1422

(b)

Accessory element	Position	Sequence	Position
PKL-II	–149	TTCC <u>TGGACTCTGG</u> CC CC AGTGT	–126
S_{14}	–1467	GCAAGGGGAACACTCTGTTT <u>GG</u> CC CC AGTTCT	–1440

cAMP-response element

Although stimulation of reporter activity by glucose/insulin of a construct which contains the L-PK gene promoter up to position -189 is inhibited by cAMP, no binding site for the cAMP-response-element-binding protein (CREB) was found in this region [48]. Interestingly, the cAMP response element is mapped on both the L-II and L-III elements in transient transfection [48] and on the L-III element in transgenic mice [39]. Since the role of insulin in the stimulation of L-PK gene expression seems to be mainly the induction of glucokinase synthesis (see below), this inhibitory effect of cAMP can be explained by the inhibition of glucokinase induction by cAMP. In addition, L-II-element-binding proteins such as HNF4 and NF-1 family members or L-III-element-binding proteins such as USF and an unidentified protein could be regulated by cAMP. In addition to these factors, any other protein involved in carbohydrate responsiveness of the L-PK gene could also be regulated by PKA. In fact, it has been reported that HNF4, when phosphorylated by PKA, showed decreased DNA-binding and transcriptional activities [66].

The PUFA-response element

The transient transfection of L-PK-CAT fusion constructs in primary cultured hepatocytes revealed that the PUFA-response element was mapped on the L-II element [30]. Although the peroxisome-proliferator-activated receptor (PPAR) α is reported to be involved in PUFA-responsive gene expression in the liver and the retinoid X receptor (RXR)-PPAR heterodimer is able to recognize HNF4-binding sequence [67,68], the co-transfected PPAR α expression plasmid with or without RXR expression plasmid did not alter CAT expression from the L-PK-CAT reporter plasmid in HepG2 cells, a human hepatoma cell line (K. Yamada, T. Tanaka and T. Noguchi, unpublished work). In addition, PUFA-mediated suppression of L-PK gene expression is observed even in PPAR α -deficient mice [69]. Thus either HNF4 or NF-1 family members appear to be involved in this response. It has recently been reported that fatty acyl-CoA thioesters serve as ligands of HNF4- α [70]. Various fatty acids are capable of modulating HNF4 binding to its cognate sequences of the human apolipoprotein C-III gene promoter and to alter transcription from a C-III-CAT reporter plasmid. The degree of these changes are dependent upon the chain length and the degree of saturation of the fatty acids.

MECHANISMS OF TRANSCRIPTIONAL STIMULATION BY CARBOHYDRATES

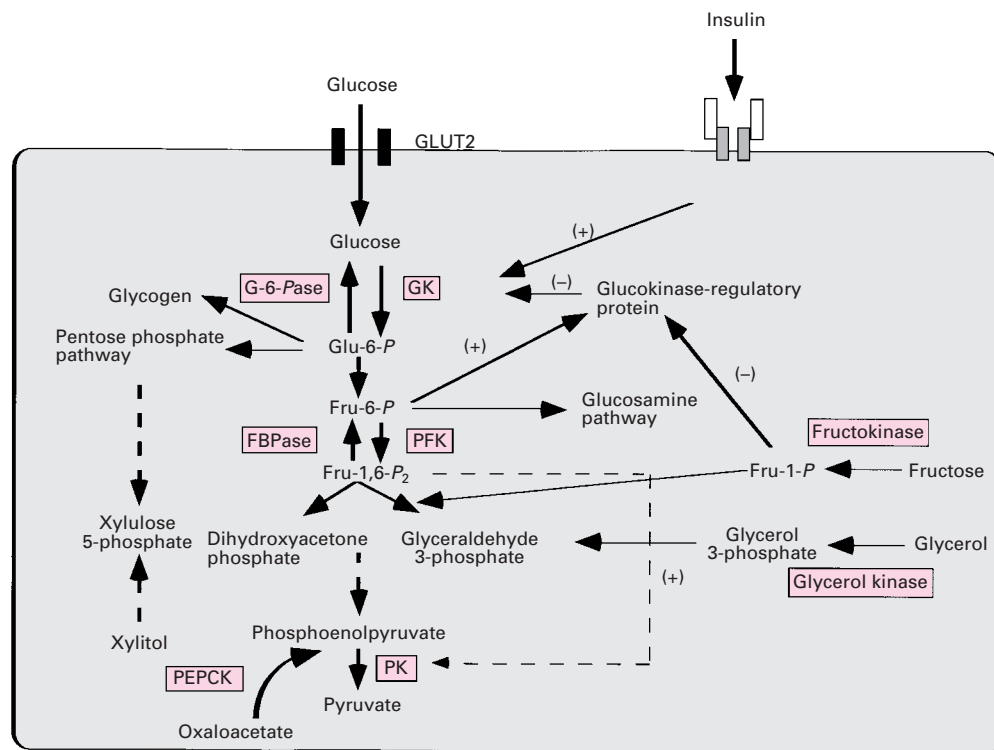
Schematic diagrams of glucose metabolism in the liver are shown in Scheme 1. Glucose initially enters the cells via facilitative glucose transporter GLUT2. While GLUT2 is expressed in hepatocytes and pancreatic β -cells, its activity is not regulated by insulin [71]. The kinetic properties of GLUT2 result in intracellular glucose levels similar to those in plasma. Accordingly, in hepatocytes and pancreatic β -cells, the conversion of glucose into glucose 6-phosphate by glucokinase is a key step for glucose-utilizing pathways such as glycolysis and glycogenesis. It has been reported that GLUT2 expression is required for the carbohydrate response of the L-PK gene in hepatocytes [72].

The regulation of the gene expression of L-PK by hormones and carbohydrates has been examined in rat primary cultured hepatocytes [26,73]. The presence of both insulin and glucose were required to stimulate L-PK gene expression in this system. The extent of accumulation of L-PK mRNA was shown to be dependent upon the concentrations of both insulin and glucose.

2-Deoxyglucose, a glucose analogue which is phosphorylated but not further metabolized, failed to substitute for glucose in the accumulation of L-PK mRNA, suggesting that glucose metabolism is essential for the induction of L-PK mRNA and that a metabolite of glucose is involved in this induction. The over-expression of glucokinase results in the induction of the activity of the co-transfected L-PK-CAT reporter gene in the absence of insulin in cultured hepatocytes [74]. Since insulin stimulates glucokinase gene expression in hepatocytes in the absence of glucose [73,75], it has been suggested that the role of insulin in L-PK gene stimulation is to induce glucokinase synthesis. This, in turn, causes stimulation of glucose metabolism and the production of metabolite(s), which may induce the transcription of the L-PK gene through the activation of a carbohydrate-responsive transcription factor.

Fructose and glycerol at 5 mM also increased the accumulation of L-PK mRNA only in the presence of 10 nM insulin, but the extent of these increases was much lower than that observed for insulin/glucose [73]. These results are in contrast with those observed *in vivo*, since dietary fructose and glycerol are strong inducers of L-PK even in diabetic rats [25,28,76]. The reason for this discrepancy is presently unknown. Whatever the reason is, however, these *in vitro* as well as *in vivo* studies suggest that fructose and glycerol also act on the L-PK gene through the same metabolite as glucose. Two mechanisms for the production of this metabolite have been proposed. It has been reported that low concentrations (0.2 mM) of fructose in the presence of 20 or 40 mM glucose stimulate the expression of the L-PK/CAT fusion gene in hepatocytes [74]. A glucokinase regulatory protein is co-expressed with glucokinase and regulates its activity by binding to the enzyme [77]. In the presence of fructose 6-phosphate, the glucokinase regulatory protein binds to glucokinase, thus inhibiting its activity [78]. This inhibition is counteracted by fructose 1-phosphate. Fructose is phosphorylated by fructokinase to produce fructose 1-phosphate (Scheme 1). Accordingly, it has been suggested that stimulation of L-PK/CAT gene expression by fructose in the liver is mediated by the inhibition of the glucokinase regulatory protein via fructose 1-phosphate, followed by the release of glucokinase. However, this is not the case in small intestine and kidney, since both glucokinase and its regulatory protein are not expressed in these tissues [77,79,80]. Thus the putative metabolite must be directly generated from fructose and glycerol in these tissues. We also consider it likely that this mechanism occurs in liver, since we detected no accumulation of L-PK gene transcript in hepatocytes cultured with 0.2 mM fructose in the presence of 30 mM glucose [81]. In addition, glycerol does not activate glucokinase by inhibiting the activity of its regulatory protein in hepatocytes [82].

Which metabolite(s) functions as signal molecule(s) for mediating the transcriptional stimulation of the L-PK gene by carbohydrates? It has been reported that at least four metabolites, including glucose 6-phosphate, xylulose 5-phosphate, 3-phosphoglycerate and phosphoenolpyruvate, may be involved in transcriptional stimulation by carbohydrates in primary cultured hepatocytes [74,81,83]. There are several reports which support glucose 6-phosphate as a candidate signal molecule. Foufelle et al. suggested that glucose 6-phosphate is involved in the stimulation of fatty acid synthase (FAS) gene expression in response to glucose in adipocytes, since 2-deoxyglucose had a similar effect on this gene and since a marked parallelism existed between the concentrations of glucose 6-phosphate and the FAS mRNA levels [84]. 2-Deoxyglucose also partially mimicked the effect of glucose on L-PK gene expression in INS-1 insulinoma cells [49], in contrast with hepatocytes, in which this glucose analogue



Scheme 1 Carbohydrate metabolism and its regulation in the liver

GK, glucokinase; PFK, phosphofructokinase; PK, pyruvate kinase; G-6-Pase, glucose-6-phosphatase; FBPase, fructose-1,6-bisphosphatase; PEPCK, phosphoenolpyruvate carboxykinase; GLUT2, glucose transporter 2; Glu-6-P, glucose 6-phosphate; Fru-6-P, fructose 6-phosphate; Fru-1,6- P_2 , fructose 1,6-bisphosphate; Fru-1-P, fructose 1-phosphate; (+), activation; (-), inhibition.

failed to serve as a substitute. The stimulation of S_{14} and FAS gene expression by glucose, xylitol, and dihydroxyacetone is also closely correlated with glucose 6-phosphate levels in primary cultured hepatocytes [85]. In cultured hepatocytes, in contrast, xylitol induced an increase in the L-PK mRNA levels, even in the absence of insulin and in the absence of accumulation of glucose 6-phosphate [83]. Since xylitol is converted into xylulose 5-phosphate by L-xylose dehydrogenase and xylulokinase in hepatocytes [86], xylulose 5-phosphate has been proposed as a candidate molecule [83]. Massillon et al. reported that xylulose 5-phosphate was also responsible for the glucose regulation of glucose-6-phosphatase and phosphoenolpyruvate carboxykinase gene expression in liver [87]. Interestingly, xylulose 5-phosphate has been shown to activate protein phosphatase 2A [88] and okadaic acid, a specific inhibitor of protein phosphatase 1 and 2A, has been reported to block the glucose-induced transcription of L-PK, S_{14} and FAS genes [89–91]. In addition, recent reports showed that AMP-activated protein kinase inhibited the transcriptional activation of L-PK and FAS genes in hepatocytes [89,91]. This protein kinase is the analogue of SNF1 [92], which is essential for the transcriptional activation of glucose-repressed gene in yeast [93]. Thus phosphorylation and dephosphorylation mechanisms are involved in the regulation of L-PK gene transcription as is the case in yeast.

NUTRITIONAL AND HORMONAL REGULATION OF PKM GENE EXPRESSION

M_2 -PK activity was decreased in the small intestine of hypothyroid rats, and the administration of thyroid hormone to these rats restored the enzyme activity [94]. T_3 also increases the

accumulation of M_1 -PK mRNA and stimulation of the transcription rate of the *PKM* gene in GH_3 cells, a rat pituitary cell line [95]. However, the mechanisms of such stimulations have not been studied in detail. Enzyme activity, protein and mRNA levels of M_2 -PK increased in proliferating thymocytes after stimulation with concanavalin A and interleukin-2 [96]. This increase coincides with the S-phase of the cell cycle. In addition, glucose is required for the proliferation of thymocytes and the induction of glycolytic enzymes [97]. Insulin has been shown to cause an increase in M_2 -PK activity in adipocytes when both glucose and glutamine were added to the medium [98]. This enhancement is due to an increase in the amount of the enzyme and is dependent upon ongoing mRNA synthesis. Glucosamine is capable of substituting for glucose and glutamine for this effect, suggesting that the hexosamine-biosynthetic pathway is involved in the increase in M_2 -PK activity. This is supported by the finding that the M_2 -PK induction by insulin/glucose/glutamine, but not by insulin/glucosamine, was inhibited by the addition of *O*-diazoacetyl-L-serine, an inhibitor of glutamine:fructose-6-phosphate amidotransferase, the enzyme which catalyses the formation of glucosamine 6-phosphate from glutamine and fructose 6-phosphate, and is a rate-limiting enzyme in hexosamine-biosynthetic pathway [98,99]. Thus it has been proposed that some hexosamine products are involved in transcriptional regulation of the *PKM* gene and that the role of insulin in this regulation is to increase the formation of hexosamine products by stimulating the uptake of glucose and glucosamine [98]. In addition, it has been reported that the hexosamine biosynthetic pathway mediates the effects of glucose on the expression of several genes such as glutamine:fructose-6-phosphate amidotransferase, transforming growth factor- α , trans-

forming growth factor- β and leptin [100–104]. However, this pathway is not involved in carbohydrate responsiveness of the L-PK gene [49].

REGULATORY ELEMENTS AND *TRANS*-ACTING FACTORS OF THE *PKM* GENE

Cell type-specific expression

Regulatory elements of the rat *PKM* gene were analysed by detection of DNase I-hypersensitive sites or a transient transfection assay [14,15,105,106]. In a rat hepatoma cell line, dRLh-84, in which the transcription of the *PKM* gene is active, two DNase I-hypersensitive sites, designated HS1 and HS2, were detected in the first intron and around the transcription initiation sites respectively [106]. These sites were not detected in rat hepatocytes, in which the *PKM* gene is not expressed. When various CAT constructs which contain the *PKM* gene promoter were transfected into dRLh-84 cells, the constructs which contained HS2 showed CAT activity. However, HS1 showed no activity, even in the presence of HS2. The upstream region up to -457 had promoter activity in dRLh-84 cells. Three *cis*-acting regions from -279 to -216, box A (-279 to -265), box B (-256 to -242) and box C (-235 to -216), have been identified in the *PKM* gene promoter region (Figure 2b) [106]. These regions had no independent effect, but the inclusion of all regions gave rise to synergistic transcriptional activity. EMSA revealed that unidentified nuclear proteins bind to all elements. The major binding protein for box C, but not box A or B, in nuclear extracts from dRLh-84 cells is different from that found in hepatocyte nuclear extracts. In the transient transfection analysis, all constructs which showed strong promoter activity in hepatoma cells were silent in hepatocytes, suggesting that this difference is dependent upon the box-C-binding protein. It remains to be determined which protein(s) binds to box C and stimulates the hepatoma cell-specific transcription of *PKM* gene.

In addition, three GC boxes, GC Box 1 (-48 to -39), GC Box 2 (-86 to -77), and GC Box 3 (-133 to -124), are located up to 133 bp upstream of the transcription initiation site of the *PKM* gene [105]. A mutation in either GC box 1 or 3, but not in GC box 2, resulted in a 50% decrease in promoter activity. EMSA using anti-Sp1 or anti-Sp3 antisera indicated that both Sp1 and Sp3 bind to these GC boxes. Co-transfection with the expression vectors of Sp1 or Sp3 revealed a strong stimulatory function for these proteins.

ChoRE

No two E boxes separated by 5 bp exist in the rat *PKM* gene promoter. Thus far, the ChoRE has not been determined precisely. However, a construct, which contains from -457 to +78 of the *PKM* gene promoter and linked to luciferase gene, showed glucose responsiveness in rat hepatoma FTO2B cells, which was repressed by treatment with okadaic acid [107]. As described above, this region contained boxes A, B and C and two functionally determined GC boxes 1 and 3 [105,106]. Treatment of FTO2B cells with glucose causes an increase in Sp1-binding to the GC boxes and this increase is inhibited by okadaic acid [107]. This suggests that glucose increases the amount of dephosphorylated Sp1 by stimulating protein phosphatase 1 or 2A and that this, in turn, stimulates transcription of the *PKM* gene. A similar mechanism has been proposed for the transcriptional stimulation of acetyl-CoA carboxylase gene by glucose in adipocytes [108,109]. In addition, it has been reported that Sp1 binding to the GC boxes of the *PKM* gene promoter is regulated

by redox status [107,110]. Nuclear extracts which had been treated with H₂O₂ decreased Sp1-binding activity, whereas dithioerythritol reversed this effect [110]. However, the physiological significance of this phenomenon has not been clarified.

FUTURE DIRECTIONS AND CONCLUSION

Much effort has focused on the identification of transcription factors and signal molecules in the glucose-signalling pathway, leading to the transcriptional stimulation of the PK genes. In spite of these studies, conclusive evidence has not yet been obtained.

Evidence for involvement of USF, especially the USF1/USF2 heterodimer, in the stimulation of the L-PK gene by carbohydrates has been presented by Kahn's group, while we and others do not agree with this. If the latter is the case, identification of an L-IIIBP (ChoRE-binding protein) other than USF would be very crucial in proving this hypothesis. This could be achieved by purification of L-IIIBP using standard biochemical techniques or by direct cloning of its cDNA using molecular biological techniques. If the former is the case, it is unlikely that USF is a direct carbohydrate-responsive protein. This is because USF is expressed ubiquitously and interacts with many genes, most of which are not under the control of carbohydrates, and substitution of the L-III element with USF binding sequences in the L-PK gene promoter abolishes carbohydrate-responsiveness. Thus a factor other than USF is required to explain specific gene regulation by carbohydrates. Two possibilities can be considered: one is that USF forms a heterodimer with a putative carbohydrate-responsive protein to bind the L-III element and the other is that this protein forms a carbohydrate-response complex with USF dimer bound to the L-III element. Thus far no evidence to support either possibility has been presented.

In any case, the carbohydrate-response complex probably contains L-IIIBP, since a synergistic interaction between the L-II and L-III elements is required for carbohydrate-responsiveness of the L-PK gene. We suggest that this L-IIIBP is member of the NF1 family. Until recently, HNF4 was believed to be involved in carbohydrate stimulation of the L-PK gene, since the co-transfection of HNF4 expression plasmid with the reporter plasmid containing the L-II element stimulates the expression of the reporter gene. However, this is unlikely since NF1, but not HNF4 proteins, binds to the accessory element of the S14 gene. Thus NF1 proteins may directly interact with the L-IIIBP complex or indirectly with the complex through a third partner to stimulate the L-PK gene transcription.

Members of NF-1 family contain NF-1L, NF1/Red1 and CTF/NF1, which are also referred to as NF1-A, NF1-B and NF1-C respectively, and NF1-X [111–115]. Although we purified NF-1L and NF1/Red1 as heat-stable L-IIIBPs from rat liver nuclear extracts, all four NF-1 isoforms are expressed in liver, kidney and small intestine. All NF-1 proteins contain a highly conserved N-terminal 220-amino-acid region, which mediates DNA binding and dimerization [116,117]. However, considerable variation occurs within the C-terminal regions of these proteins. Very recently, it has been reported that NF-1 isoforms differentially activate NF-1-responsive promoters [118,119]. Thus a question arises from these results as to which member of NF-1 family is involved in the carbohydrate regulation of the L-PK gene.

Whatever the putative carbohydrate-responsive protein is, the available evidence suggests that the carbohydrate-signalling pathway to the L-PK gene includes a metabolite of glucose as well as phosphorylation and dephosphorylation mechanisms. Thus the carbohydrate-responsive protein may be activated by either

phosphorylation or dephosphorylation to form the carbohydrate-response complex. Although the metabolites proposed include glucose 6-phosphate and xylulose 5-phosphate, no evidence to unambiguously support either metabolite has yet been presented. The candidate metabolite must be accumulated in liver as the result of feeding glucose, fructose or glycerol, in kidney by fructose or glycerol, and in the small intestine by glucose or fructose. In the case of liver, insulin is required for the stimulation of glucose metabolism and the resultant increase in the L-PK gene transcription in response to glucose. The role of insulin is mainly to stimulate synthesis of glucokinase. The metabolite must, in some way, specifically regulate the phosphorylation and dephosphorylation system. In this respect the report that xylulose 5-phosphate activates protein phosphatase 2A is quite interesting. This report, together with the one which concludes that AMP-activated protein kinase inhibited the stimulation of the L-PK gene, may provide a clue for the elucidation of the carbohydrate-signalling pathway.

As described above, HNF4 is probably involved in PUFA suppression of L-PK gene transcription. The question, then, is what is the mechanism for this suppression. It has been reported that the binding of acyl-CoA, derived from PUFA, to HNF4 either inhibits HNF4 binding to its cognate DNA response element or decreases the availability of the HNF4 dimer by shifting the HNF4 oligomeric ↔ dimeric equilibrium. If this is the case, it is difficult to explain the PUFA suppression of the L-PK gene transcription based upon a mechanism involving NF-1 proteins for the carbohydrate regulation described above. If the ligand-unbound form of HNF4 is included in the carbohydrate-response complex, PUFA repression can be explained by the above observations. Thus further studies are required to solve this problem.

The L-II and/or L-III elements are responsible for cAMP inhibition of L-PK gene transcription. Nevertheless, these elements do not contain a possible binding sequence for CREB. However, cAMP could inhibit the L-PK gene transcription by interfering with the carbohydrate-signalling pathway via PKA. Glucokinase is one such target, since the transcription of this gene is inhibited by cAMP. Any other protein in the signalling pathway is also a candidate target for cAMP. The elucidation of all factors involved in the pathway will help to clarify this point.

Glucose also stimulates the transcription of the *PKM* gene by a mechanism which probably involves a glucose metabolite, as well as phosphorylation and dephosphorylation. However, factors involved in the signalling pathway to the *PKM* gene may be different from the L-PK gene. It has been proposed that hexosamine derivatives, which are generated from glucose, somehow stimulate protein phosphatase 1 or 2A to dephosphorylate Sp1, which, in turn, stimulates transcription of the *PKM* gene. This hypothesis raises several questions. First, since Sp1 is expressed ubiquitously and interacts with many genes, most of which are not under control of glucose, the question arises as to how Sp1 stimulates the expression of a specific gene in response to glucose. Secondly, where is the glucose-response element in the *PKM* gene? In addition to two proximal GC boxes, Sp1 may also bind to other elements. Thirdly, what is the nature of the hexosamine derivative which is involved in gene stimulation? Fourthly, what is the nature of the protein phosphatase which is involved in the *PKM* gene regulation and is this phosphatase different from that involved in L-PK gene stimulation?

Finally, it has been reported that a number of genes are regulated by glucose via its metabolism at the transcriptional level. These include genes for *S₁₄*, FAS, acetyl-CoA carboxylase, ATP-citrate lyase [120], glucose-6-phosphatase, phosphoenolpyruvate carboxykinase, glutamine:fructose-6-phosphate ami-

dotransferase, transforming growth factor- α , transforming growth factor- β and leptin. It would be very interesting to determine whether these genes are regulated by mechanisms which are similar to those which regulate the PK genes.

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