Functional synergism in the carbohydrate-induced activation of liver-type pyruvate kinase gene expression

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Hepatic expression of the liver-type pyruvate kinase (L-PK) gene is induced at the transcriptional level by increased carbohydrate metabolism in the rat. The carbohydrate response of the L-PK gene requires sequences from −171 to −124, which encompass adjacent major late transcription factor (MLTF)-like and hepatic nuclear factor (HNF)-4 binding sites. Neither site alone is capable of conferring a response, prompting us to explore the mechanism of synergy between the MLTF-like factor and HNF-4. Spacing requirements between the two factor binding sites were tested by generating a series of mutations that altered the distance between these sites. Surprisingly, all of the constructs with spacing mutations were capable of responding to elevated glucose when introduced into primary hepatocytes. Thus the glucose response does not depend on the rigid phasing of the MLTF-like and HNF-4 factors, suggesting that the factors binding to these two sites do not interact directly with each other. Substitution or inversion of the PK HNF-4 site abrogated the response to glucose and also significantly suppressed the promoter activity under non-inducing conditions. We conclude that the MLTF-like factor and HNF-4 co-operate functionally to maintain the basal activity, as well as the carbohydrate responsiveness, of the L-PK gene. A mechanism other than co-operative DNA binding is responsible for the synergism.

INTRODUCTION

The L-type pyruvate kinase (L-PK) gene in rat liver encodes one of the key enzymes in glycolysis. Dietary carbohydrate and insulin induce L-PK gene expression, while glucagon represses it [1,2]. This pattern of regulation is shared with several other genes encoding enzymes involved in hepatic lipogenesis, including malic enzyme, acetyl-CoA carboxylase and fatty acid synthase (for reviews see [3,4]). Based on its response characteristics and tissue distribution, the S_{14} gene is also a member of this class [5,6]. This gene encodes a nuclear protein of unknown physiological significance that is induced rapidly following carbohydrate administration [7,8]. The induction of lipogenic gene expression following dietary carbohydrate appears to be an adaptive response of the organism to allow it to more effectively convert simple carbohydrates into triglyacylcerols, the preferred form for energy storage.

Regulation of expression of the L-PK gene by carbohydrate, insulin and glucagon occurs at the transcriptional level [1]. Transcriptional induction is also seen with several other members of the lipogenic gene family, including the S_{14} gene [9]. Although the signal transduction pathway involved in enhanced transcription of L-PK and other lipogenic genes is unknown, indirect evidence suggests that an intracellular signal generated from increased carbohydrate metabolism is responsible for stimulating the expression of these genes (for review see [10]). Culturing of primary rat hepatocytes in elevated glucose conditions in the presence of insulin mimics the response of the whole animal to a carbohydrate-rich diet [11]. On the other hand, insulin alone is unable to stimulate gene expression in cells cultured in low-glucose conditions. Thus the principal role of insulin appears to be to stimulate carbohydrate metabolism. Studies on isolated adipocytes and a pancreatic β-cell line suggested that glucose 6-phosphate may be the signal that triggers expression of these genes in response to glucose [12,13].

Transient transfection of primary hepatocytes has provided a valuable means to help delineate the critical DNA elements for the regulation of the L-PK gene. A short fragment from the 5′-flanking region of the L-PK gene (−279 to −63) has been found to be sufficient for directing liver-specific expression of a reporter gene in hepatocytes [14]. Thompson and Towle [15] demonstrated that sequences within this region between positions −183 and −96 are necessary and sufficient for supporting a response to elevated glucose in hepatocytes. Vaulont et al. [16] defined three binding sites for hepatic nuclear factors within this segment. The sequences of these binding sites are similar to consensus binding sites for the major late transcription factor (MLTF), hepatic nuclear factor (HNF)-4 and nuclear factor (NF)-1. MLTF and NF-1 are ubiquitous transcription factors, whereas HNF-4 is enriched in the liver. We have since shown that L-PK sequences from −171 to −124, which encompass the MLTF-like and HNF-4-like sites, are crucial for the glucose induction [17]. HNF-4 binds to the PK cognate site and participates in the regulation of the gene [17,18]. Although MLTF is capable of binding to the PK MLTF-like site, its binding is not capable of supporting the carbohydrate response. Thus another member of the c-myc family distinct from MLTF, or a complex involving MLTF together with additional hepatic factors, may be involved. The factor(s) binding to the MLTF-like site appears to play the primary role in receiving the signal from carbohydrate metabolism. When multiple copies of the MLTF-like site were linked directly to the PK promoter region (−96 to +12), a

Abbreviations used: L-PK, liver-type pyruvate kinase; MLTF, major late transcription factor; HNF-4, hepatic nuclear factor-4, NF-1, nuclear factor-1; EMSA, electrophoretic mobility shift assay; CAT, chloramphenicol acetyltransferase; C/EBP, CCAAT enhancer binding protein.

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robust response to glucose was observed [17–19]. Additionally, the PK MLTF-like site contains a sequence with 9 of 10 bp identity to the regulatory sequences of the S_{14} gene shown to be critical for carbohydrate regulation. On the other hand, the HNF-4 site could not elicit a response when present as either a single or multiple copies. In the present study, we have explored the mechanism for the synergy between the MLTF-like factor and HNF-4 in the activation of transcription of the L-PK gene by carbohydrate metabolism. In particular, we have asked whether a direct interaction between factors binding to the MLTF-like and HNF-4 sites is critical to the carbohydrate response of the L-PK gene.

MATERIALS AND METHODS

Plasmids

PK MLTF/HNF-4/(−96)CAT (where CAT is chloramphenicol acetyltransferase) was generated by ligating the PK MLTF-like oligonucleotide (−171/−145) to the 5′-end of PK HNF-4/(−96)CAT [17] by introduction of a *Sal*I site. The resulting construct contains a 10 bp insertion between the PK MLTF-like and HNF-4 sites and is denoted as SP(+10). Other mutations altering spacing between the two binding sites were prepared by digesting PK MLTF/HNF-4/(−96)CAT with *Sal*I followed by treatment with mung bean nuclease (Life Technologies, Inc.). The resulting blunt-ends were re-ligated and DNA sequencing was performed to determine the extent of deletion. Alternatively, the 5′-overhanging ends generated by *Sal*I cleavage were filled in with the Klenow fragment of DNA polymerase I (New England BioLab) prior to ligation to generate SP(+14).

To test mutations with altered spacing between MLTF-like and HNF-4 sites in the context of a heterologous promoter, fragments containing PK MLTF-like and HNF-4 sites with various spacing alterations were amplified by PCR from PK promoter constructs using Pfu polymerase (Stratagene). The products were purified and inserted into pS14(−4316/−2111)CAT(An)−290 [20] at the *Hin*dIII site.

Oligonucleotides corresponding to CCAAT enhancer binding protein (C/EBP) and HNF-3 sites were synthesized. They were ligated upstream to PK(−96)CAT and subsequently joined to the PK MLTF-like site, with a *Sal*I site in between (10 bp insertion). PK MLTF/inverted HNF-4/(−96)CAT was generated by fusing the oligonucleotides indicated below with PK(−96)CAT [15].

Sequences

Sequences of the PK MLTF-like and HNF-4 sites were described previously [17]. The C/EBP site from the mouse transthyretin enhancer between 1.96 and 1.86 kb [21] had the sequence:

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5′   TCGACGTTTTCCATCTTACTCAACATCCT 3′
3′        GCAAAAGGTAGAATGAGTTGTAGGAGATC 5′
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The sequence of the HNF-3 site from the human transthyretin promoter (−109/−86) [22] was:

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5′   TCGACTGACTAAGTCAATAATCAGAATCT 3′
3′        GACTGATTCAGTTATTAGTCTTAGAGATC 5′
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Other sequences were the single-stranded oligonucleotide encompassing the PK MLTF-like and inverted HNF-4 sites with natural spacing, 5′CGTCTAGAGACCTGAGACCGGGGGTCACAGAACCACGGGAGTGCCCCGTGCGCCC 3′; and the 5′ primer that anneals to the above oligonucleotide, 5′ GCAAGCTTGGGCGCACGGGG 3′.

Primary hepatocytes and transfection

The procedure described by Thompson and Towle was followed [15]. Briefly, primary hepatocytes were isolated from male Sprague–Dawley rats (160–240 g) maintained *ad libitum* on standard chow. After a 6 h attachment period, transfections were performed overnight in Williams E medium (lacking glucose and methyl linoleate) supplemented with 23 mM Hepes, 26 mM sodium bicarbonate, 2 mM glutamine, 0.617 μM insulin, 0.01 mM dexamethasone and 10 mM lactate using Lipofectin reagent (Life Technologies, Inc.). Cells were cultured for an additional 48 h in the presence of either 10 mM lactate or 27.5 mM glucose. Afterwards, cells were harvested and CAT assays were carried out as described. For transfections in the presence of Matrigel, 250 or 500 μg/ml Matrigel (Collaborative Biomedical Products) was added to the culture media following transfection.

Gel mobility shift assay

Nuclear extracts were prepared by the method of Gorski et al. [23]. The gel shift assay was performed essentially as described [17], except that the non-specific competitor used in the binding reactions was 100 μg/ml sonicated salmon sperm DNA. Fragments with variable spacing between the PK MLTF-like and HNF-4 sites were generated by digestion of the appropriate S_{14} plasmids with *Hin*dIII followed by agarose gel purification. The resulting products were 5′-end-labelled using the Klenow fragment of DNA polymerase I in the presence of [α-P^{32}]dCTP (du Pont/NEN). The reactions were incubated at 23 °C for 30 min and subsequently subjected to electrophoresis on a 4% non-denaturing polyacrylamide gel in 50 mM Tris, 384 mM glycine and 2 mM EDTA, pH 8.3.

RESULTS

Effects of extracellular matrix components on the response of the L-PK promoter to glucose

Previous studies from our laboratory examining the response of hepatocytes to glucose have used cells cultured directly on to plastic dishes. Under these conditions, increased expression of both the endogenous L-PK gene and the exogenous L-PK promoter constructs was observed in response to elevated glucose concentrations [15]. However, a significant variability occurred in the magnitude of the response between different preparations of hepatocytes. In order to try to minimize this variation, we tested the effect of adding extracellular matrix components to the hepatocyte culture. Matrigel is a solubilized basement membrane preparation extracted from the Engelbreth–Holm–Swarm mouse sarcoma, a tumour rich in extracellular matrix proteins. Its major components include laminin, collagen IV, heparan sulphate and proteoglycans. However, Matrigel has been reported to inhibit transfection efficiency [24]. Recently we found that Matrigel added to cultured hepatocytes following the transfection step (approx. 20 h after plating) can still stimulate cell responses (H.-H. Shih and H. C. Towle, unpublished work). Hepatocytes grown on plates with Matrigel under these conditions displayed a morphology more similar to that of natural hepatocytes compared with their counterparts without Matrigel, and remained firmly attached to the plates throughout the culture. When the glucose responsive construct [PK(−197)CAT] was tested, the CAT activity in 27.5 mM glucose was increased

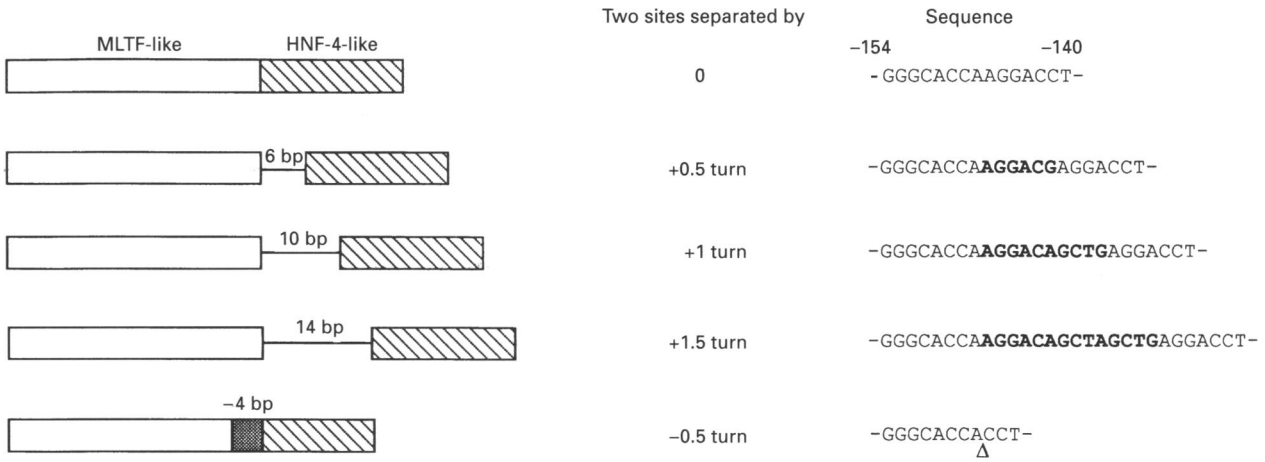

Figure 1 Diagram of the mutations containing various distances between the PK MLTF-like and HNF-4 sites

The sequences of the junction region between the two sites are shown on the right side of the figure. The additional sequences inserted compared with that of the wild-type are shown in bold letters. The location of the deletion is marked with △.

dramatically in the presence of Matrigel, whereas the activity in 10 mM lactate remained about the same. As a result, an enhanced induction was observed. For example, CAT activities from cells cultured in the presence of 0, 250 μg/ml and 500 μg/ml Matrigel in lactate were 0.725, 0.469 and 0.54 units respectively, while those in glucose were 2.75, 6.69 and 11.04 units. Therefore, the fold induction increased from 3.82 to 21.18. Thus Matrigel is useful in improving the morphology and glucose response of the primary hepatocytes, even when added after cells have been cultured for a period of time.

Spacing between the MLTF-like and HNF-4 sites is not critical for the response to glucose

The response of the L-PK gene to carbohydrate requires the presence of both the MLTF-like and HNF-4 binding sites. These sites lie directly adjacent to each other between −171 and −124 of the L-PK promoter. The close positioning of these two critical sites suggested that co-operative DNA binding might be important to the functional synergism observed for glucose activation. To test this possibility, we asked whether the distance between the two factor binding sites affected their binding activity for hepatic nuclear factors. A series of mutations that altered the distance between the two binding sites was generated, as shown in Figure 1. Constructs denoted as SP(+6), SP(+10) and SP(+14) represent mutations that contained 6, 10 and 14 bp insertions between the two sites compared with the natural sequence, whereas SP(−4) had a 4 bp deletion. Among these mutations, SP(+10) separated the two binding sites by one helical turn of DNA and should maintain the same angular orientation between the two sites. The remaining mutations varied the distance by non-integral numbers of turns and should result in the two sites being on opposite faces of the helix relative to the wild-type sequence. We first performed an electrophoretic mobility shift assay (EMSA) using fragments containing PK MLTF-like and HNF-4 sites with various spacing as probes. The end-labelled probes were incubated with rat liver nuclear extract. As shown in Figure 2, identical patterns were observed for oligonucleotides containing different spacing between the sites. In each case, two major protein–DNA complexes, with the lower complex more intense than the upper one, were seen. When an

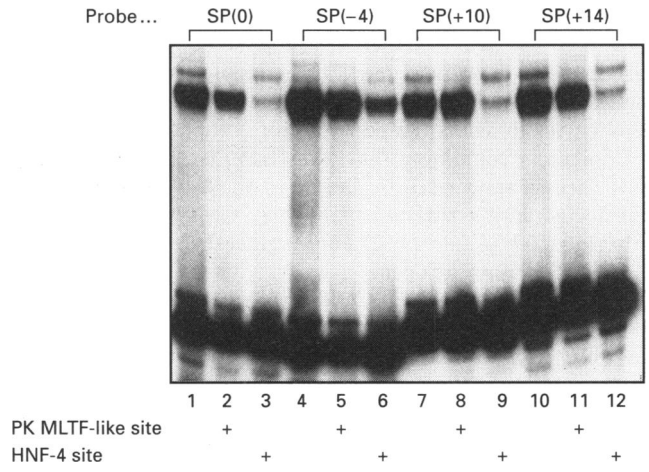

Figure 2 Fragments containing different spacings between the PK MLTF-like and HNF-4 sites exhibit identical binding activities

Rat liver nuclear extract was incubated with ^{32}P-labelled oligonucleotides containing PK MLTF-like and HNF-4 sites with different spacings between them. The different probes used are indicated. No competitor was included in the binding reactions for lanes 1, 4, 7 and 10; 100 ng of the unlabelled oligonucleotide corresponding to the PK MLTF-like site was included in the reactions for lanes 2, 5, 8 and 11; 100 ng of unlabelled oligonucleotide corresponding to the PK HNF-4 site was present in the reactions for lanes 3, 6, 9 and 12.

oligonucleotide corresponding to the PK HNF-4 site was included as a competitor, the lower band was displaced, whereas the upper complex remained unaltered. Incubating *in vitro* translated HNF-4 with the same oligonucleotide resulted in a complex that co-migrated with the lower dark band, confirming that the lower complex was due to HNF-4 binding (results not shown). The upper complex was competed by an unlabelled oligonucleotide containing the PK MLTF-like site. In addition, formation of the upper band was inhibited by antibody to the HeLa cell MLTF, supporting the conclusion that MLTF binds to this region (results not shown). These studies establish that the fragments containing spacing variations between the two sites retained their ability to bind MLTF and HNF-4, suggesting that

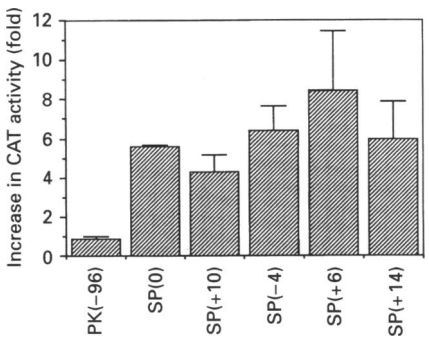

Figure 3 Constructs containing PK MLTF-like and HNF-4 sites with various spacings are capable of responding to glucose in the L-PK promoter

Plasmids containing PK MLTF-like and HNF-4 sites with various spacings were used to transfect primary hepatocytes. Cells were cultured in the presence of 10 mM lactate or 27.5 mM glucose for 48 h. CAT assays were performed. The fold increases in CAT activity were obtained by comparing the activity of cells in glucose media relative to that of cells in lactate media. Results represent the means ± S.E.M. of five experiments.

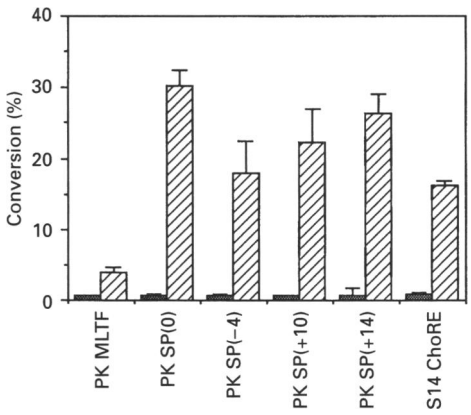

Figure 4 Constructs containing PK MLTF-like and HNF-4 sites with various spacings are capable of responding to glucose in the S_{14} promoter

Oligonucleotides containing the PK MLTF-like and HNF-4 sites with various spacings were fused to S14(−290) in the presence of upstream enhancer region (−4316 to −2111). The constructs were transfected into primary hepatocytes. PK MLTF-like site/S14 represents the construct containing the PK MLTF-like site alone in the context of the S_{14} promoter [20]. SP(0) denotes the construct containing the natural sequence of the PK MLTF-like and HNF-4 sites. The S_{14} ChoRE has been described previously [20]. CAT activities of each construct in lactate (■) or glucose (▨) are expressed as the percentage conversion to acetylated chloramphenicol. Each value represents the average of three independent experiments. Bars indicate the S.E.M. values.

co-operativity is not a critical factor in binding *in vitro*. However, since it is not clear whether MLTF is actually involved in the carbohydrate-dependent regulation, despite its avid binding to the PK site *in vitro* [17,25], we turned to functional assays using hepatocytes to assess the potential for co-operativity.

Constructs containing the PK MLTF-like and HNF-4 sites with various spacings were linked to the L-PK promoter and transfected into primary hepatocytes. Cells were cultured in either 10 mM lactate or 27.5 mM glucose for 48 h and then assayed for reporter gene activity. Surprisingly, all of the constructs containing these mutations were capable of responding to glucose stimulation to a similar degree (Figure 3). The result was observed in several independent preparations of hepatocytes. Some variation in the basal level of promoter activity was seen in constructs containing different spacings between the MLTF-like and HNF-4 sites. In these constructs, bases between −124 and −96 of the PK promoter were deleted. This resulted in closer positioning of the MLTF-like and HNF-4 sites to the HNF-1 site (−95 to −66) and to the basal promoter. Perhaps this positioning affected the basal promoter activity in these constructs.

To confirm the result that the spacing between the PK MLTF-like and HNF-4 sites was not critical for responsiveness to glucose, we tested these mutations in the context of a different liver-specific promoter. S_{14} is another gene studied in our laboratory whose expression is stimulated in response to increased carbohydrate metabolism [26,27]. The carbohydrate response elements of S_{14} gene lies between −1457 and −1428 [20]. A construct containing an upstream enhancer region (−4316 to −2111) linked to the S_{14} promoter (−290 to +18) is not capable of supporting a response to glucose. However, insertion of the L-PK segment from −183 to −96 in this construct resulted in a glucose response [20]. Oligonucleotides containing each of the mutations altering the distance between the MLTF-like and HNF-4 sites were inserted into the S_{14} promoter–enhancer context. Consistent with the results observed with the PK promoter, mutations with altered spacing between the MLTF-like and HNF-4 sites retained carbohydrate responsiveness compared with the wild-type spacing (Figure 4). In the context of the S_{14} promoter, there was little variation in either basal or glucose-induced promoter activity among mutations with different spacing. Thus the glucose response of the L-PK carbohydrate-responsive element does not depend on the rigid phasing of the MLTF-like and HNF-4 sites.

Replacing or inverting the HNF-4 binding site abolishes the response of the L-PK gene to glucose

These observations suggest that the factor binding to the MLTF-like site does not directly interact with HNF-4. Instead, each may interact independently with downstream factors such as HNF-1 or components of the basal transcription apparatus. Since the binding protein recognizing the MLTF-like site is the primary factor responsible for eliciting a response to carbohydrate, we asked whether the role of HNF-4 can be substituted by other hepatic activators. If the synergy for the carbohydrate response depends on a specific interaction between HNF-4 and the basal machinery, substitutions which disrupt this interaction would abolish the activity. On the other hand, if such an interaction is not specific, other hepatic transcription activators might be able to substitute functionally. Binding sites for the hepatic factors HNF-3 and C/EBP [21,22] were individually inserted in place of the HNF-4 site in the L-PK promoter. In both cases, the substitutions rendered the constructs unresponsive to glucose (Figure 5). Unexpectedly, the promoter activity of these mutants in non-inducing conditions was also significantly suppressed compared with the wild-type sequence in the same context [SP(+10)], despite the fact that HNF-3 and C/EBP have been reported to function as positive regulators for several hepatic genes (for review see [28]). Thus HNF-4 appears to be essential for maintaining the appropriate level of basal activity, as well as for enabling the carbohydrate responsive factor to transmit the signal generated by glucose metabolism. These results are consistent with our previous data from mutational analysis of the L-PK promoter, where we observed decreased basal activity in the mutations disrupting the HNF-4 binding site [17].

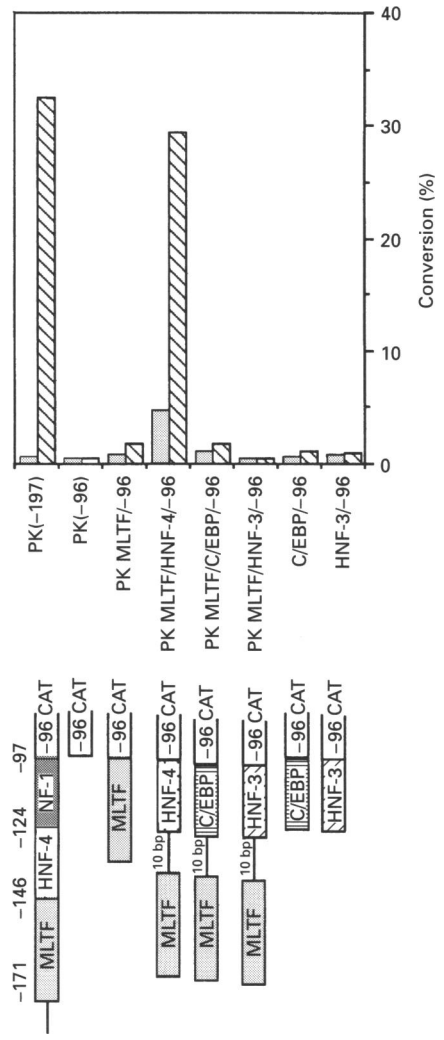

Figure 5 Replacing the HNF-4 site with binding sites of HNF-3 or C/EBP abolishes the response of the L-PK promoter to glucose

Various constructs were transfected into primary hepatocytes and cultured under conditions described in the legend to Figure 3. CAT activities of each construct in lactate (▨) or glucose (▧) are expressed as the percentage conversion to acetylated chloramphenicol. Construct PK MLTF/HNF-4/−96CAT is the same plasmid as SP(+10). PK MLTF/−96CAT is equivalent to PK(−171/−142)CAT, as described previously [17]. The data shown are representative of three independent experiments.

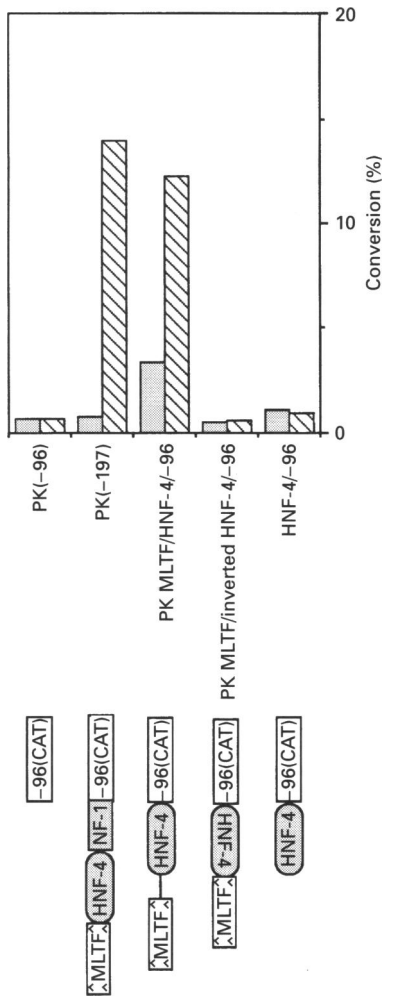

Figure 6 HNF-4 by itself is not sufficient to maintain basal and glucose-induced promoter activity

Constructs containing the HNF-4 site alone in front of PK(−96)CAT and the HNF-4 site inverted relative to the MLTF-like site were transfected into primary hepatocytes and their activities were compared with those of the wild-type sequences. The cells were cultured under conditions described in the legend to Figure 3. CAT activities of each construct in lactate (▨) or glucose (▧) are expressed as the percentage conversion to acetylated chloramphenicol. These data are representative of two separate experiments.

We next tested whether HNF-4 is solely responsible for maintaining the promoter activity in non-inducing conditions, or whether the MLTF-like site binding factor is also required. As seen in Figure 6, when the HNF-4 site alone was present in front of PK(−96), both the basal activity and the glucose-stimulated activity were very low, similar to that seen in the PK(−96)CAT construct. This observation indicates that the presence of the HNF-4 site alone was not sufficient to boost the basal promoter activity. The importance of the HNF-4 site was also analysed by preparing a construct in which this site was inverted relative to its native orientation. This alteration completely abolished the activity in both basal and induced states when compared with SP(+10), the construct with the natural orientation of the HNF-4 site. Taken together, these observations suggest that the role of HNF-4 in the regulation by carbohydrate of the L-PK promoter is specific.

DISCUSSION

Previous work demonstrated that the stimulation of L-PK gene expression by glucose requires the sequence from −171 to −124, which encompasses an MLTF-like (−171 to −145) and an HNF-4 (−144 to −124) binding site. A construct with a single copy of the MLTF-like site linked to the PK promoter could not support the glucose response, whereas a construct with three tandem repeats of this site was responsive [17,19]. In contrast, a construct with three copies of the HNF-4 site was unable to elicit a response. Based on these observations, we speculated that the role of HNF-4 might be to recruit the factor binding to the MLTF-site, which is probably the carbohydrate-responsive factor. If this model is correct, synergy between the two factors should require their direct interaction. In the current paper, we have described experiments indicating that the synergy of MLTF-like and HNF-4 site binding factors does not require rigid spacing between the two binding sites. The spacing mutations

that altered that distance between the two sites by inserting or deleting various numbers of bases still retained glucose responsiveness. Notably, changes that separated the two sites by non-integral numbers of DNA helical turns did not impair the ability to respond to glucose. These results were seen in the context of both PK and S_{14} promoters. Replacing the HNF-4 site with HNF-3 or C/EBP binding sites, which both bind positive transcription factors in hepatocytes, significantly suppressed basal as well as glucose-induced activity. A similar observation was made when the HNF-4 site was inverted in its orientation or when the HNF-4 site was present in the absence of the MLTF-like site.

Our interpretation from these results is that the MLTF-like and HNF-4 site binding factors do not interact directly with each other. However, functional co-operation between them is responsible for maintaining the basal activity and carbohydrate inducibility of the L-PK gene. Such functional interaction is specific, since mutations replacing or inverting the HNF-4 site rendered these constructs non-functional.

Synergism among transcription factors is commonly found. It allows particular combinations of activators to generate unique patterns of gene expression that impose developmental and tissue-specific control. Such 'combinatorial control' is believed to be one of the predominant mechanisms for transcriptional regulation in eukaryotic cells. Co-operative DNA binding of transcription activators to adjacent sites represents the simplest mechanism for transcriptional synergy. In several cases, co-operative DNA binding has been observed [29,30] *in vitro* and generally reflects specific protein–protein interactions. However, because of the highly specific nature of such interactions, this mechanism usually occurs between factors from the same family that bear structural similarities. Thus it is not likely to account for most of the synergistic actions that occur among transcription factors. An alternative mechanism, which was suggested by Ptashne [31], postulates that two eukaryotic activators can interact synergistically by simultaneously touching a third protein. The third protein could be a component of the basal transcription apparatus or an 'adaptor' protein. Indeed, synergistic activation has been observed *in vitro* under conditions in which the binding sites for a given activator protein are saturated, thus rendering co-operative binding irrelevant [32,33]. Further evidence supporting this notion came from studies showing that the acidic activation domains of the fusion protein Gal4–VP16 function by enhancing interaction between TATA-binding-protein-associated factors and TATA binding protein, instead of stimulating interaction of the TATA binding protein with DNA [34]. This 'simultaneous contact' model may explain the promiscuous nature of synergistic activation among many transcription factors. For example, the yeast transcription activator Gal4 works synergistically with a variety of mammalian transcription factors [35]. Recently, Herschlag and Johnson [36] presented some possible models for 'kinetic synergism'. In their hypotheses, transcriptional synergism does not have to involve direct or indirect physical interactions. Rather, there could be kinetic synergism, in which the activators are physically distinct in their action, but nonetheless give greater-than-additive stimulation when present together. This occurs by independent stimulation of sequential steps in the process of transcriptional initiation. Although examples have not yet been found, it is highly plausible that in some cases synergism is achieved through such a mechanism.

HNF-4, a member of the steroid–thyroid superfamily, has been shown to synergistically activate hepatic-specific gene expression in several cases. It binds to overlapping regions of the apolipoprotein B gene promoter with C/EBPα and synergistically activates transcription [37]. Similar to the case of the L-PK gene, HNF-4 and C/EBPα do not bind DNA co-operatively *in vitro*. C/EBP proteins belong to a family that possess a DNA binding domain composed of basic region and leucine zipper motifs [38]. Recently Nishiyori et al. [39] reported that functional co-operation between HNF-4 and C/EBPβ plays a critical role in the liver-specific activity of the ornithine transcarbamylase enhancer. Our data suggest that the carbohydrate response is mediated by a member of the c-myc family. This family bears structural characteristics of basic/helix-loop-helix/leucine zipper motifs. It is tempting to speculate that a similar mechanism of transcriptional synergy might account for activation of these genes.

Although our data suggest that the factor binding to the MLTF-like site does not interact directly with HNF-4, HNF-4 by itself is not capable of maintaining the basal activity. This was suggested by the suppressed promoter activity under the non-inducing conditions observed in the construct containing HNF-4 fused to PK(−96)CAT. Substitution of the HNF-4 site with binding sites for HNF-3 or C/EBP also resulted in low basal activity. Moreover, when the orientation of the HNF-4 site was inverted relative to the MLTF-like site, low activity was found in both basal and induced states. These data indicated that MLTF-like and HNF-4 sites form a functional unit in the PK promoter to support basal activity as well as the response to glucose.

It is perplexing that Bergot et al. [19] reported that insertion of a 14 bp linker between the MLTF-like and HNF-4 sites disrupted the response of the L-PK promoter to glucose. This difference might be due to the fact that this mutation was constructed in the context of the natural sequence; that is, sequences between −123 and −96 were not deleted. This would result in moving the MLTF-like site 14 bp upstream from the basal promoter and might have resulted in 'distance effect'. Since we observed the responsiveness of SP(+14) in the context of PK as well as S_{14} promoters, we were convinced that this mutant was capable of eliciting a response to glucose.

At present we have not been able to determine the identity of the carbohydrate responsive factor or resolve the issue of whether MLTF is involved in mediating the carbohydrate response of the L-PK gene. We cannot rule out the possibility that the carbohydrate responsive factor, which evades detection in binding assays, in fact binds DNA co-operatively with HNF-4. However, we do not favour this scenario, because co-operative DNA binding usually requires strict spatial arrangements.

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