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Cis-regulation of the L-type pyruvate kinase gene promoter by glucose, insulin and cyclic AMP

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ABSTRACT

The glucose/insulin response element of the L-pyruvate kinase gene is a perfect palindrome located from nt −168 to −144 with respect to the cap site. This element (L4) is partially homologous to MLTF binding sites. Its full efficiency requires cooperation with a contiguous binding site for HNF4, termed L3 and located from nt −145 to −125. In the presence of the L4 element contiguous to L3, cyclic AMP inhibits activity of the L-PK promoter while in its absence, or when the normal L4-L3 contiguity is modified, cyclic AMP behaves as a transcriptional activator that does not seem to be sequence-specific. Therefore, we propose that the mechanism of inhibition of the L-PK gene by cyclic AMP requires precise interactions between the nucleoprotein complex built up at sites L4 and L3 and other components of the L-PK transcription initiation complex.

INTRODUCTION

The L-type pyruvate kinase (L-PK) gene, encoding a liver-specific glycolytic enzyme, is transcriptionally regulated, positively by glucose and insulin and negatively by glucagon, through its second messenger, cyclic AMP [1,2]. This regulation has been studied *in vivo* [1−3] and *ex vivo* in hepatocytes in primary culture [4], but is lost in established, differentiated cell lines and, *in vitro*, in a transcription cell-free system [5,6]. Consequently, determination of DNA elements responsible for response of the L-PK gene to carbohydrates and hormones requires to use either transgenic mice or hepatocytes in primary culture. Though we and others have shown that the rat L-PK gene is fully active and controlled in transgenic mice [7,8], our first attempts at reproducing regulation of L-PK constructs by glucose, insulin and cyclic AMP after introduction of DNA into hepatocytes by the calcium phosphate coprecipitation technique, were not successful, probably because of interference between calcium and the glucose/insulin signalization pathway [9]. Nevertheless it was recently demonstrated that L-PK constructs driven by at least 200 bp of 5′ flanking sequence were responsive to glucose and hormones after hepatocyte lipofection [10]. We have previously established that this proximal promoter fragment of the L-PK gene contains binding sites (boxes L1 to L4) for at least four proteins: from 3′ to 5′, hepatocyte nuclear factor 1 (HNF1 α), nuclear factor 1 (NF1), hepatocyte nuclear factor 4 (HNF4) and a factor with similar binding properties to major late transcriptional factor (MLTF) [11]. Upstream of this region, we have also identified another DNAse 1 footprint, the L5 box, located around position −265 and binding a ubiquitous factor [9 and N. Puzenat et al., in preparation]. The role of these DNA elements in the response of the L-PK gene to glucose and hormones has therefore been reevaluated using electroporation as a method of gene transfer. Electroporation has been demonstrated to be efficient in hepatocytes in primary culture [12] and, in particular, has been used to analyze the L-PK promoter [13]. We have found that the L4 element, able to bind partially purified MLTF [11], is the glucose/insulin response element and is also required for the inhibition by cyclic AMP. This element functions in close cooperation with the contiguous L3 element binding HNF4. In addition, full responsiveness to carbohydrates and cyclic AMP seems to require some interaction between the L4/L3 binding proteins and more proximal factors.

MATERIALS AND METHODS

Plasmids construction

All plasmids were constructed by standard DNA cloning procedures [14].

L-PK homologous promoter constructs. The series of constructs containing the 5′ deletions of the L-PK gene promoter have already been described in Cognet et al [9] , except for the 119PK/CAT and 150PK/CAT constructs. To prepare these plasmids, two DNA fragments were synthesized by polymerase chain reaction (PCR), using the 3193PK/CAT construct as

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template and the following oligonucleotides as primers: L3 and a CAT-specific primer to obtain the fragment 150/CAT, and L2 and the same CAT primer to obtain the fragment 119/CAT. The L2, L3 and CAT oligonucleotides are described in table 1. 119/CAT and 150/CAT were digested by XhoI and ligated into the 183PK/CAT vector previously digested by SmaI and XhoI. A set of oligonucleotides corresponding to the wild type L3 and L4 binding sites or containing single or double point mutations were synthesized; they are listed in Table 1. Using several pairs of these oligonucleotides as primers, six DNA fragments were prepared by PCR: L4L3, L4miL3, L4meL3, L4L3mi, L4L3me and L4meL3me. They were inserted into the blunt-ended BamHI restriction site of the 119 PK/CAT plasmid to obtain the following constructs: L4L3-119PK/CAT, L4miL3-119PK/CAT, L4meL3-119PK/CAT, L4L3mi-119PK/CAT,L4L3me-119PK/CAT and L4meL3me-119PK/CAT. Likewise, the plasmids L4L3-96PK/CAT and L3L4-96PK/CAT were prepared by ligation of the L4L3 PCR fragment into the blunt-ended BamHI restriction site of the 96 PK/CAT vector. To create the L4 = 150PK/CAT and L4-119PK/CAT plasmids, a double stranded oligonucleotide L4 with the same sequence as that described in Table 1 was inserted into the BamHI site of the 150 PK/CAT and 119PK/CAT vectors, respectively. To prepare the $(L4)_4$-96PK/CAT construct, a polymer of four double stranded oligonucleotide L4 was synthesized and ligated into the XbaI site of the 96PK/CAT vector, previously blunt-ended with Klenow DNA polymerase.

Thymidine kinase (TK) heterologous promoter constructs. All the fragments used in these constructs (L4L3L2L1, L4L3L1, L3L1, L4, L3, $(L4)_4$) were inserted into the XbaI site, previously blunt ended with Klenow DNA polymerase, of the $pBLCAT_2$ vector (Schütz et al. [15]) containing the TK promoter spanning from nt −105 to +1 in front of the CAT gene. To create the L4L3L2L1-TK/CAT plasmid, a L-PK fragment spanning from −172bp to −60bp (L4L3L2L1) was synthesized by PCR with the 183PK/CAT vector as template. The fragments L4L3L1 and L3L1 were synthesized by PCR using a plasmid in which the deletions had been previously introduced by PCR in a whole vector 183PK (Vaulont et al. [11]), using the oligonucleotides L3 and L1 as primers.

All the constructs were checked by DNA sequencing.

Cell culture and electroporation conditions

Hepatocytes from male Sprague−Dawley rats (180−250g) were isolated as described in Cognet et al. [9]. Freshly isolated hepatocytes were resuspended in Dulbecco's PBS, Ca^{2+} and Mg^{2+} free at a concentration of 5.10^6 cells/ml. 800 μl of this suspension, 20 μg of plasmid DNA and 100 μg of sonicated salmon sperm DNA were put into an electroporation cuvette and kept on ice for 15 min. Cells were then exposed to a voltage pulse of 300V at 960μF with the Gene Pulser system from Biorad, and kept on ice for a further 15 min. Hepatocytes suspensions were plated on 6 cm dishes in a final volume of 4 ml of 199 medium supplemented with 1μM triiodothyronine (T3), 2nM insulin, 25 mM glucose and 10% fetal calf serum. 3 hours later, the medium was removed and replaced with 199 medium containing 1μM triiodothyronine (T3), 1μM dexamethasone, 2nM insulin, 3% fetal calf serum and supplemented with either 10mM lactate or 25 mM glucose or 25mM glucose, 0.5mM 8-Bromo-cyclic-AMP (8-Br-cAMP) and 0.1mM 8-4-chlorophenylthio-cAMP (8-4-CPT-cAMP). 24 hours later, the medium was replaced with the above medium less fetal calf serum . During culture, the cells were maintained at 37°C under 7% CO_2.

Table 1. Nucleotide sequences of the single and double-stranded oligonucleotides used in this study; bold nucleotides correspond to the point mutations. (me) corresponds to external mutations and (mi) to internal mutations.

L2	−126	TGTACAAGGCTTCCGTTGGCAAGAGAGATGC ACATGTTCCGAAGGCAACCGTTCTCTCTACG	−96
L3	−150	TGGTTCCTGGACTCTGGCCCCCAGTGTAC ACCAAGGACCTGAGACCGGGGGTCACATG	−122
L4	−172	ATGGGCGCACGGGGCACTCCCGTGGTTCCT TACCCGCGTGCCCCGTGAGGGCACCAAGGA	−143
L4mi	−172	ATGGGCGCACGGGGCACTCCC**T**TGGTTCC	−144
L4me	−172	ATGGGCG**AAA**GGGGCACT	−154
L3mi	−122	GTACACTGGGGGCCAGAGT**AA**AGGAAC	−148
L3me	−122	GTACACTGGGGGC**G**AGAGTCC	−142
CAT	+51	CAACGGTGGTATATCCAGTG	+71

CAT assays

48 hours after electroporation, the cells were scraped, pelleted and resuspended in 100 μl of 100mM KH_2PO_4 pH 7.8, 1mM dithiothreitol and disrupted by three cycles of freezing and thawing. Cellular extract protein concentration was measured as described by Bradford et al. [16]. CAT activity was assayed essentially as described by Gorman et al. [17], using 100μg of protein extracts. Extracts were heated for 10 min at 65°C. The reaction was performed at 37°C for 180 min. The ^{14}C labelled substrate and reaction products were separated by thin layer chromatography with a mixture of chloroform and methyl alcohol (95/5). The chloramphenicol 3-acetylated spot was cut from the thin layer and counted.

Preparation of nuclear extracts and gel shift assays

Nuclear extracts from adult rat liver were purified according to Gorski et al. [18].

The PCR fragments containing the binding site for L4 and L3 described earlier were synthesized with the same pairs of oligonucleotides previously (γ-^{32}P)ATP 5′ end labeled with T4 polynucleotide kinase. Gel shift assays were performed as described by Raymondjean et al. [19] and 20 ng of oligonucleotides were added in competition experiment.

RESULTS

Functional 5′ deletion analysis of various L-PK constructs shows that the L4 element is indispensable to both stimulation by glucose/insulin and inhibition via cyclic AMP/glucagon

Fig. 1A shows the CAT activity of various L-PK constructs in electroporated hepatocytes cultured without glucose, with glucose or with glucose and cyclic AMP, insulin being present in all media. Without glucose, the activity was very low when the CAT gene was driven by a minimal promoter (50PK/CAT) and was gradually stimulated by adding the boxes L1 (96PK/CAT), L2 (119PK/CAT) and L3 (150PK/CAT). In contrast, adding the L4 box (183PK/CAT) resulted in a 50% inhibition and adding the L5 box (283PK/CAT) in a supplementary 25% inhibition. The construct with 3.2 kb of 5′ flanking sequence was expressed to the same extent as 283PK/CAT construct. Activity of constructs up to position −150 was similar both with and without glucose;

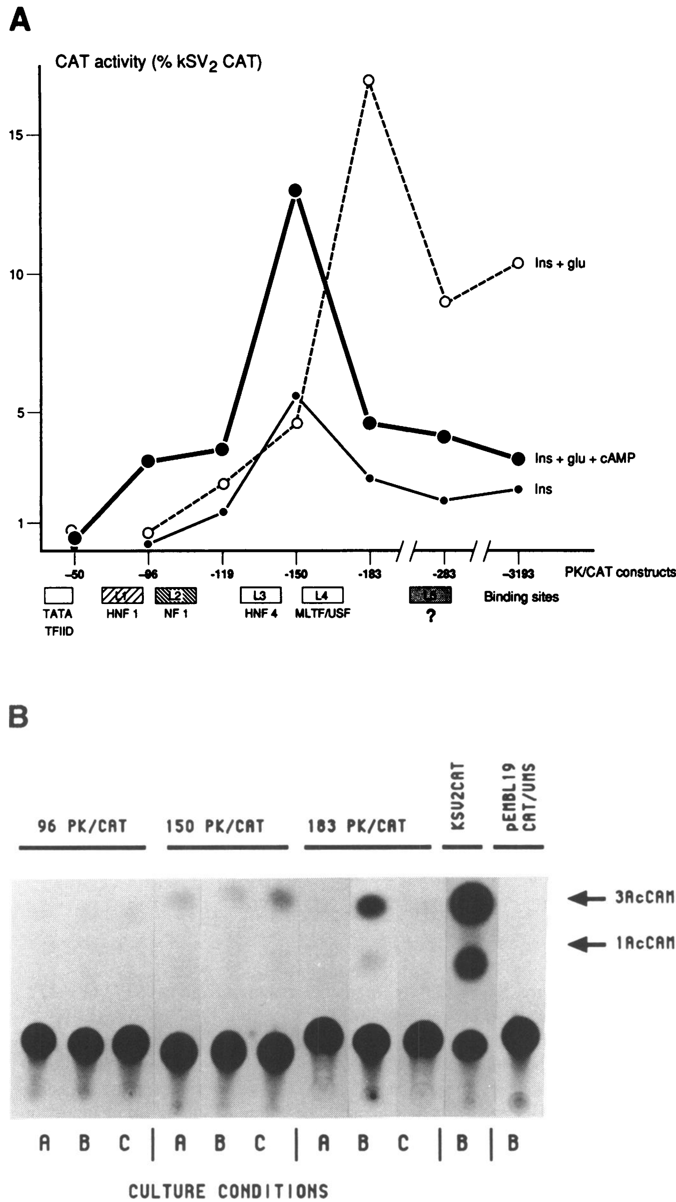

Fig 1. CAT activities generated by various 5′ deleted L-PK/CAT gene constructs in hepatocytes: effect of glucose and cAMP. The 5′ deletions of the L-PK gene constructs were introduced by electroporation into freshly isolated rat hepatocytes. Cells were cultured for 48 hours with 199 medium supplemented with 2nM insulin and either 10mM lactate (thin line), or 25mM glucose (dotted line) or 25mM glucose, 0.5mM 8-Br-cAMP and 0.1mM 8-4-CPT-cAMP (thick line). The cells were scraped and CAT activities were assayed as described earlier with 100μg of cell extract protein, for 180 min. **(A)**: CAT activities of the 5′ deletions in the three metabolic conditions. The activities are expressed as a percentage of the kSV_2CAT activity, in arbitrary units. The kSV_2CAT expression is similar in all conditions. The values are the mean of five to six experiments. For each one, the promoterless vector plasmid pEMBL19, CAT activity has been subtracted. **(B)**: representative CAT assay experiment performed on extracts of hepatocytes transfected with 20μg of 96, 150, 183PK/CAT, promoterless CAT/UMS pEMBL 19 (Cognet et al. [9]) and kSV_2CAT plasmids. Culture conditions are: A: 2nM insulin and 10mM lactate, B: 2nM insulin and 25mM glucose, C: 2nM insulin, 25mM glucose, 0.5mM 8-Br-cAMP and 0.1mM 8-4-CPT-cAMP.

addition of the L4 element resulted in a more than 6-fold glucose stimulation. The 283PK/CAT and 3193PK/CAT constructs were less active than the 183PK/CAT construct, but their glucose regulation was conserved. Action of cyclic AMP was biphasic: up to position −150, it stimulated activity of the PK/CAT constructs by about 3 fold but, when the L4 box was present, it inhibited this activity by about 3 fold. In fact, the curve of activity of the constructs in the presence of insulin, glucose and cyclic AMP was parallel to that without glucose, as if cAMP suppressed the stimulatory effect of glucose. Indeed,the stimulatory effect of cyclic AMP observed for the constructs up to −150 was similar both with and without glucose [data not shown]. We verified that the effect of cyclic AMP, especially stimulation of constructs devoid of L4, was not an artefact due to the cyclic AMP analogues added to the media by using glucagon or different types of cyclic AMP analogues (dibutyryl-cyclic AMP, 8-bromo-cyclic AMP, 8-4-chlorophenylthio-cyclic AMP). All had approximately the same influence on CAT activity. Finally, the respective influence of glucose and insulin was checked in some experiments by culturing hepatocytes with glucose and without insulin: these experiments confirmed that both glucose and insulin together were indispensable for stimulating activity of constructs containing the L4 element ([4] and data not shown).

A close cooperation between the L4 and L3 elements is needed to confer a full responsiveness of the L-PK promoter to glucose/insulin and cyclic AMP

5′ deletion analysis allow us to establish that the L4 element is necessary for the response to modulators but does not determine whether or not cooperation with more downstream element(s) is required for full activation. Although binding of L4 and L3-binding factors is independent, it generates a unique fused DNase 1 footprint that can only be separated into two parts by selective titration with excess of either L4 or L3 oligonucleotides [11]. We can therefore assume that, due to their contiguity when bound, functional interaction exists between proteins binding to the L4 and L3 elements. To address this question, we have investigated various point mutations of the L4 and L3 elements and evaluated the importance of their contiguity for a normal response to glucose/insulin and cyclic AMP. The four groups of point mutations were chosen as a result of previous determination by methylation interference of contact points between DNA and bound factors [11]. Fig. 2B shows location and nature of these mutations, termed L4me (double mutant), L4mi (single mutant), L3mi (double mutant) and L3me (single mutant). The ability of these different mutants to bind factors on L4 and L3 elements was investigated by a gel-shift assay using −172/−122 PK fragments as probes (Fig. 3). Specific-binding to the L4 element was greatly weakened with L4me and abolished with L4mi. Binding to the L3 element was totally abolished with L3mi and not affected with L3me. Specificity of the two retarded L4 and L3 complexes was systematically checked by titration with excess of either L4 or L3 oligonucleotides. Fig. 2A represents the activity of different constructs that differ from the wild plasmid 183PK/CAT by a 8 bp linker added between box L2 and fragment L4L3 (nt −172/ −122). A normal response to glucose and cyclic AMP was found with the wild type L4L3 fragment. In contrast, the groups of mutations affecting binding to L4 (L4me and L4mi) and L3 (L3mi) markedly reduced stimulation by glucose and suppressed or greatly reduced inhibition by cyclic AMP. The L3me mutant that still bound HNF4 conserved a good stimulation by glucose (× 9) and a significant inhibition by cyclic AMP (:2.3). Then, we tested the importance of the respective position of the L4 and L3 boxes and of their location with respect to the L1 site, (Fig 2A): spacing

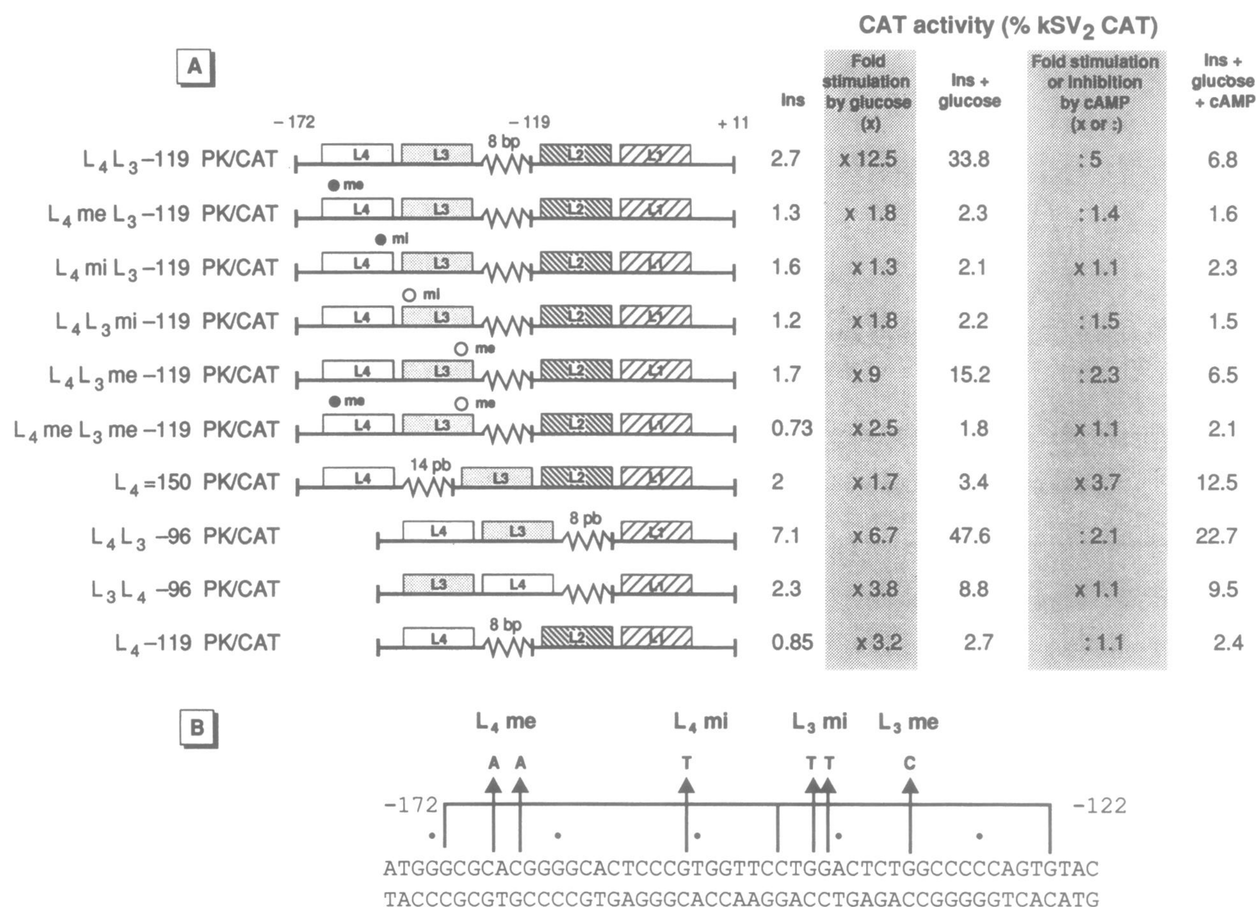

Fig 2. Functional consequences of L4 and L3 binding site modifications on L-PK/CAT constructs activity. Several DNA fragments spanning from nt −172 to −122, each containing or not containing single or double point mutations of the L4 or L3 binding sites, were inserted in front of the 119PK/CAT (L4L3 to L4meL3me-119PK/CAT). The wild type sequence was ligated in both orientations into the 96PK/CAT plasmid (L4L3 and L3L4-96PK/CAT). Finally box L4 was moved away from box L3 (L4=150PK/CAT) and box L3 was deleted (L4-119PK/CAT). Broken lines between the boxes represent the 8bp or 14bp linkers introduced during the preparation of the constructs. **(A)** : CAT activity generated by the constructs described above in primary hepatocytes. Activities are expressed relative to that of kSV_2CAT (100%). The extent of induction by glucose is the ratio between CAT activities with and without glucose. The extent of inhibition or induction by cAMP is the ratio between CAT activities with glucose and with or without cAMP. Each value represents the mean of six experiments. **(B):** Fragment spanning from nt −172 to −122 and binding sites L4and L3; the point mutations introduced into the fragment and their designations are indicated by arrows.

out the L4 and L3 boxes by inserting a 14 bp linker sequence between them, totally suppressed the normal response to glucose and cyclic AMP. Replacing the L2 element (NF1 binding site) by a 8 bp linker resulted in a construct that still responded positively to glucose (× 6.7) and negatively to cyclic AMP (:2.1). It should be observed that although the L2 element has been deleted, activity of this construct with or without glucose is high. We can speculate that putting L3, a strong activating element (see Fig. 1), in closer proximity to the HNF1 binding site and TATA box can compensate for the effect of NF1 binding site deletion on activation of the L-PK promoter. More interesting are the results of a construct in which the orientation of the L3L4 fragment has been reversed (Fig 2A). Consistent with the hypothesis that the distance between the L3 and proximal elements is an important parameter for L-PK promoter activity, maximal activity of this construct was reduced by 5 fold with respect to plasmid with L4 and L3 in the usual orientation. In addition, stimulation by glucose, although still significant, was reduced and inhibition by cyclic AMP was suppressed. The importance of the L3 element in L-PK promoter activity was also attested by the low activity of a construct in which L3 has been deleted. This plasmid, in which L4 is slightly closer to L1 compared to its usual position (a 13 bp shift) seemed to conserve a diminished but significant response to glucose, but no clear response to cyclic AMP. This latter result suggested that, although cooperating with L3 in its normal position, L4 could nevertheless be, by itself, a glucose-response element when moved closer to other proximal elements.

Element L4 is, by itself, a glucose-response element

To test the hypothesis that, in some conditions, L4 by itself can confer glucose responsiveness, we investigated activity and response to glucose (and also, to cyclic AMP) of four L4 elements ligated in direct repeats in front of either 96PK/CAT or TK/CAT constructs (this latter one driven by a 105bp fragment of the Herpes Simplex Virus Thymidine Kinase [15]). Fig 4 shows that adding four L4 elements to the 96PK/CAT plasmid resulted in a 3.4-fold positive response to glucose (instead of 1.3-fold without tetramer of L4) and also in a 2.1-fold inhibition by cyclic AMP (instead of a 2.3-fold stimulation). A tetramer of the L4 element

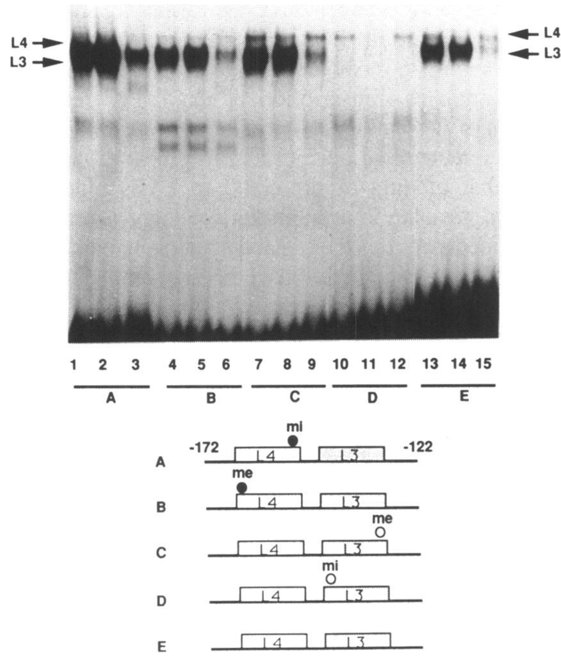

Fig 3. Gel mobility shift assays with labeled wild-type or various mutagenized L4L3 DNA fragments. 0.1 ng of the 5′ end-labeled fragments A (L4miL3), B (L4meL3), C (L4L3me), D (L4L3mi) and E (L4L3) were incubated with 2.5μg of liver nuclear extracts, in the presence of 3μg of poly-(dIdC), without competitor oligonucleotides (lanes 1,4,7,10,13), or with 20ng of non-labeled oligonucleotides L4 (lanes 2,5,8,11,14) or L3 (lanes 3,6,9,12,15).

Table 2. Response to glucose and cyclic AMP of hybrid PK/TK promoter constructs. The entire L-PK fragment spanning from boxes L1 to L4 (nt −170 to −60) or some parts of it and a were cloned in front of the TK/CAT fusion gene and introduced into hepatocytes by electroporation. CAT activities and induction or inhibition factor were expressed as shown in FIG 2A for three separate experiments.

	CAT activity (% of kSV2CAT)				
	Ins.	Fold stimulation by glucose	Ins. and glucose	fold stimulation by cAMP	Ins., glucose and cAMP
(L4L3L2L1)-TK/CAT	29.4	1.95	57	≈1	55
(L4L3L1)-TK/CAT	22.8	2.2	49.75	≈1	51
(L3L1)-TK/CAT	15	1.4	21	1.5	31
(L3)-TK/CAT	6.3	≈1	6.8	2.9	20
(L4)-TK/CAT	2.6	2.5	6.5	1.65	10.7
$(L4)_4$-TK/CAT	5.1	2.4	12.2	1.46	17.9
TK/CAT	4.4	1.2	5.3	3.1	16.4

ligated upstream of the TK/CAT promoter induced similary a significant 2.5-fold stimulation by glucose, compared to 1.2-fold for the construct without $(L4)_4$ (Table 2).

L4 (L3)-dependent repression by cyclic AMP and stimulation by glucose depends also on the nature of the promoter

The oligomerized L4 element was unable to totally reverse cyclic AMP-dependent activation of the TK promoter (Table 2),

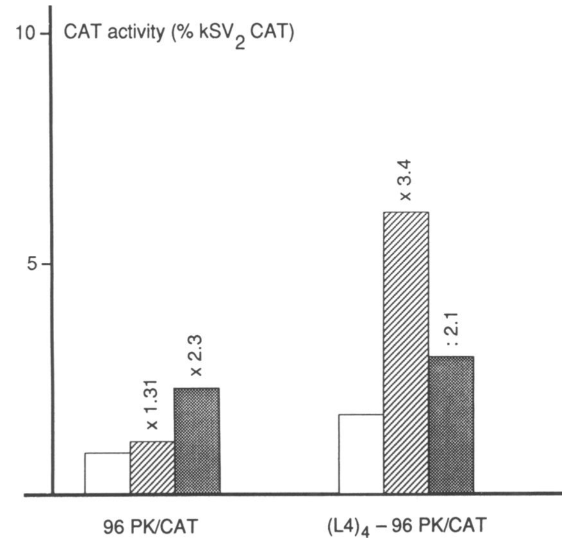

Fig 4. Effect of the L4 element on glucose and cyclic AMP responsivness of a minimal PK promoter. The 96PK/CAT construct carrying or not carrying a tetramer of the L4 element was transfected into hepatocytes; the CAT activities were analyzed under the three metabolic conditions described in Fig.1A: 2nM insulin and either 10mM lactate (empty boxes), or 25mM glucose (hatched boxes) or 25mM glucose, 0.5mM 8-Br-cAMP and 0.1mM 8-4-CPT-cAMP (shaded boxes); activities are expressed as a percentage of that of kSV_2CAT (100%). All values represent the mean of six separate experiments.

Table 3. Sequence of various insulin response elements (IRE) and of the consensus cyclic AMP response element (CRE) compared to the L-PK element L4. The underlined sequence in the GAPDH IRE-A represents the minimal active element; the homologous heptamer in the glucagon IRE is also underlined. The arrows on the sequence of element L4 indicate the perfect palindromic motifs and the black spots indicate the contact points detected by methylation interference.

Elements	Response	Genes	sequences	Ref.
CArG box	+	(c-fos immediate early genes)	$CC(A/T)_6GG$	(31)
IRE	−	(PEPCK)	−416 TGGTGTTTTGACAAC −402	(32)
	+	(GAPDH)	−480 AACTTTTCCCGCCTCTCAGCCGAAAG −435	(33,34)
	−	(Glucagon)	−265 GTTTTTCACGCCTGACTGAGATTGA −240	(35)
	+	(Amylase)	−167 CAGTTTATTTTGCGTGAGAGTTTCTAAAA −138	(36)
	+	(∂1 crystallin)	−78 CCCGCCCCC −70	(37)
CRE	+	(consensus)	CTGACGT $^{A}_{C}$(AG)	(47)
L4	+	(L-PK)	−168 GCGCACGGGGCACTCCCGTGGTTCC −144 CGCGTGCCCCGTGAGGGCACCAAGG	(11)

whereas it conferred slight negative responsiveness on the 96 PK promoter (Fig. 4). The L4-dependent glucose responsiveness was also stronger with the PK than the TK promoter. These results suggested that cyclic AMP inhibition and glucose stimulation could be more or less promoter-dependent. However, it remained possible that, in the context of the TK promoter, repression by cyclic AMP or activation by glucose and insulin imperatively required cooperation of L4 with L3. Therefore, we constructed various hybrid PK-TK promoters in which different DNA elements of the PK promoter were ligated upstream of the TK/CAT construct. CAT activity generated by these plasmids in hepatocytes is shown in Table 2. While the activating influence of the L1, L2 and L3 elements is evident from these results, the different plasmids were only slightly stimulated by glucose when

L4 was present (about 2 fold). All these constructs were either not responsive or stimulated by cyclic AMP, none of them being inhibited. The effect of the L4-L3 cooperation in promoting cyclic AMP-dependent transcription inhibition and strong glucose-dependent stimulation seems therefore to be lost in the context of the TK promoter.

DISCUSSION

Transcriptional regulation of genes by insulin and glucose has been until very recently a mysterious phenomenon... Indeed, once insulin has bound to its specific receptor, it results in activation of various signalization pathways, some dependent on and others independent of the presence of glucose [20], some requiring ongoing protein synthesis and others not [3,21–23], some acting very early on gene expression [21–25] and others acting after a long period [3,20,26–30]. Various types of insulin response elements (IREs) have been described in the last months [31–37].

Table 3 shows the sequences of these various insulin response elements and their location with respect to cap sites, compared to the L-PK L4 element, i.e. the L-pyruvate kinase glucose/insulin response element. This element globally has a structure very different from that of other IREs described to date. It is composed of two 6 bp perfect hemipalindromes (CACGGG) separated by a 5 bp spacer (GCACT) and is able to bind a protein with similar binding properties to MLTF [11]. Methylation interference experiments showed that this protein contacts symmetrically both G of the GTG trimers at the boundaries of the element on the lower (5′ end) and upper (3′ end) strands [11]. This and other reports concerning MLTF suggests that the L4 element binds a bipartite factor, possibly a homo or heterodimer whose subunits interfere with DNA on the same face of the double helix, one turn of the helix away. Since MLTF itself, a helix-loop-helix (HLH) protein [38], is not likely to mediate glucose response, the functional factor bound to L4 may be a related homodimeric protein, or a heterodimer (including MLTF), or an unrelated factor whose binding site overlaps that of MLTF. In this case MLTF might conceivably play a regulatory role by interfering with binding of the 'glucose response element binding protein'.

Although oligomerized L4 is able to confer glucose responsiveness by itself and, at least in the context of L-PK promoter, cyclic AMP-dependent inhibition, L4 in its normal position absolutely requires the presence of contiguous L3 to be functional. This is reminiscent of the close cooperation between a cyclic AMP response element and a binding site for a tissue-specific factor in a regulatory region of the tyrosine aminotransferase gene [39], or of the phosphoenolpyruvate carboxykinase gene 'metabolic regulation complex' that consists of intricated binding sites for several regulatory factors, in particular the IRE-binding protein and HNF4 [40–43]. The α amylase gene promoter also presents an IRE and a contiguous binding site for a specific pancreas transcriptional factor, PTF1 [29]. However, in contrast to Thompson's results [10] obtained by lipofection, we found by electroporation that L4L3 cooperation seems to be active only in the context of the L-PK promoter, and not when both elements are ligated upstream of the TK promoter, even when the L2 and L1 elements are also present. This suggests that the role of the L3 binding protein (that is to say, probably HNF4) could be to interact with downstream elements to stabilize the interaction between the L4-binding factor and the transcription initiation complex. According to this hypothesis, any displacement of the L-PK elements away from the TATA box, or intercalation of supplementary binding sites for different proteins between the L-PK elements and TATA box, is expected to disturb the hypothesized normal interactions, and hence the response to modulators. Refeeding rats a carbohydrate-rich diet stimulates transcription of the L-PK gene, without modification of L4-binding activity [2,11] or the *in vivo* footprint on the L4 element (S. Lopez et al., in preparation). It seems, therefore, that binding of protein(s) to L4 is independent of transcription stimulation or inhibition. Rather, we can speculate that the L4-mediated transcriptional activation by glucose involves a post-translational modification of the L4-binding factor bound to its cognate element. Consistent with this hypothesis is the fact that in the absence of glucose, L4 behaves as an inhibitory element. The effect of glucose/insulin is therefore double: to abolish the L4-mediated inhibition and to transform it into activation. Several examples of transcriptional factors that, depending on post-translational modifications, behave as transcriptional activators or inhibitors are known, e.g. c-ErbA. This is one of the thyroid hormone receptors that bind to TRE (thyroid response element) and is inhibitory in the absence of binding and strongly activating when bound to the hormone[44,45]. CREB is another example which binds to a CRE element of the *c-jun* gene regardless of its protein kinase A-dependent phosphorylation state, and inhibits transcription when dephosphorylated and activates it when phosphorylated [46].

The L4 element is not only the glucose/insulin response element, it is also required for cyclic AMP-dependent transcriptional inhibition. However, while glucose has no effect without L4, cyclic AMP has a strong and paradoxical effect under these conditions: it induces transcriptional activation. This activation is observed with the different constructs generated by the 5′ deletional analysis as well as with the TK/CAT construct. The fact that cyclic AMP-mediated activation is independent of promoter sequences suggests that it could be due to cyclic AMP-induced phosphorylation of some non-specific transcription factor(s). To explain reversal of cyclic AMP-mediated activation to inhibition when L4 is in close contact with L3 (in the context of the L-PK promoter), we can propose that this inhibition requires a special contact of the L4 binding factors with the putative phosphorylated factor and that this contact is stabilized through interaction of L4/L3 binding factors with downstream factors on the L-PK promoter, perhaps HNF1. The results obtained with the $(L4)_4$-96 PK/CAT construct suggest, however, that in the context of the L-PK promoter, oligomerized L4 can partially replace the L4/L3 cooperation to mediate inhibition by cyclic AMP.

In conclusion, we have been able to identify the sequence responsible for response of the L-PK promoter to glucose and insulin. It is a palindromic element capable of binding MLTF, a HLH factor, and which, in the absence of glucose (or of insulin), behaves as a negative regulatory element. Good activation by glucose/insulin requires, in the context of the L-PK promoter, close interaction of the L4 element with the L3 element, a HNF4 binding site. This cooperativity does not function in the context of another promoter. The multimerized L4 elements can partially replace the L4/L3 cooperation in conferring glucose responsiveness. The inhibition by cyclic AMP seems to be a still more complex phenomenon requiring very precise interactions between the L4, L3 and downstream element(s) on the L-PK promoter. Any disturbance of this precise arrangement as well as L4 deletion reverse the effect of cyclic AMP from inhibitory to stimulatory. Specific interactions

between phosphorylated general transcription factor(s) and the L4/L3 nucleoprotein complex could be the molecular basis of the cyclic AMP effect on transcription of the L-PK gene.

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