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Analysis by Cell-Free Transcription of the Liver-Specific Pyruvate Kinase Gene Promoter

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A DNA fragment spanning nucleotides −183 to −4 with respect to the cap site of the rat L-type pyruvate kinase (L-PK) gene contains at least four binding sites for putative transcriptional factors: hepatocyte nuclear factor 1 (HNF1), liver factor A1 (LF-A1), nuclear factor 1 (NF1), and major late transcription factor (MLTF). This fragment was used to direct transcription of a reporter sequence (a G-free cassette) in cell extracts. This L-PK promoter was active in liver nuclear extracts, but not in extracts from nonhepatic tissues. A reduction of 50% of the activity was obtained with a deleted L-PK promoter containing only the HNF1-binding site. In contrast, deletion of the HNF1-binding site inactivated the promoter by more than 90%. These results were confirmed by titration experiments with synthetic oligonucleotides. Titration of HNF1 resulted in an 85% decrease of transcriptional activity, while titration of LF-A1 resulted in only a 40% decrease. The influence of NF1 and MLTF seemed to be marginal in this system. The proximal 5′-flanking sequence of the L-PK gene therefore appears to function in vitro as an efficient liver-specific promoter which requires the binding of the liver factor HNF1 and which is also stimulated by the binding of another liver-specific factor, LF-A1.

The expression of the L-type pyruvate kinase (PK) gene is highly specific to liver and is accurately controlled by nutrients and hormones; transcription of the gene is stimulated by glucose and insulin and inhibited by glucagon and its second messenger, cyclic AMP (8, 36).

We have recently identified four DNA-binding proteins interacting with the promoter of the L-PK gene (S. Vaulont et al., J. Mol. Biol., in press). The target sites of these proteins are referred to as L1 (from −66 to −95), L2 (from −97 to −114), L3 (from −126 to −144), and L4 (from −145 to −168). Two of these proteins are liver specific, hepatocyte nuclear factor 1 (HNF1) (1, 6, 11, 12, 18, 19, 22, 29, 35), binding to site L1, and liver factor A1 (LF-A1) (19, 21), binding to site L3, while the other two are ubiquitous and probably identical to nuclear factor 1 (NF1) (14, 17, 21, 27, 32), binding to site L2, and major late transcription factor (MLTF) (5, 23, 26, 30, 33), binding to site L4.

To obtain a first insight into the specific role of each protein binding to the L-PK promoter and to assess their relative functional importance in conferring liver specificity and hormonal response, we used the in vitro transcription system from rat liver described by Gorski et al. (15). To allow a rapid analysis of in vitro-synthesized transcripts, we used the G-free cassette system initially described by Sawadogo and Roeder (34).

We report here that the first 183 base pairs (bp) of the 5′-flanking region of the L-PK gene are sufficient to direct an efficient tissue-specific RNA polymerase II-dependent in vitro transcription of the G-free reporter cassette. However, this transcription remains independent of the hormonal and dietary status of the animal from which liver nuclear extracts were prepared. Among the four factors interacting with the L-PK-proximal promoter, the ubiquitous NF1 and MLTF proteins do not seem to be required for L-PK in vitro transcription. In contrast, efficient L-PK transcription is largely dependent on the presence of the two liver-specific proteins HNF1 and LF-A1, HNF1 being the predominant transcriptional effector.

MATERIALS AND METHODS

Plasmid constructions. In the transcription studies, all the DNA templates containing the G-free cassette were derived from plasmids p(C_2AT) and pML(C_2AT) kindly provided by Sawadogo and Roeder (34).

As several nucleotides downstream of the L-PK cap site are G residues, we decided to substitute the first nucleotides of the mouse albumin gene (from −4 to +14), which lack G residues for those of the PK gene. This procedure was chosen, rather than subcloning the G-free cassette at position −1, since the exact role of the sequences downstream of the cap site is not known. To this end, we synthesized two oligonucleotides. Oligonucleotide I was a 33-mer whose first 16 3′ nucleotides were complementary to residues −19 to −4 of the L-PK coding strand and the 17 additional 5′ residues were complementary to residues −3 to +14 of the coding strand of the mouse albumin gene (including, therefore, the cap site of this latter gene), 3′-GTCTGGGTGTCTGTGTT AGTGGAAAGGATAGTT-5′. Oligonucleotide II was a 16-mer, complementary to the L-PK noncoding strand from −102 to −87, 5′-GAGATGCTAGCTGGTT-3′. These oligonucleotides were used as primers to amplify a fragment of 116 bp by the polymerase chain reaction method (31). The reaction was performed in a final volume of 50 μl containing 10 ng of L-PK template (a recombinant single-stranded M13 phage containing a PK insert spanning nucleotides −183 to +75), 500 ng of each oligonucleotide, and 2 mM all four deoxynucleoside triphosphates in 67 mM Tris hydrochloride (pH 8.8)–6.7 mM $MgCl_2$–16.6 mM ammonium sulfate–10% (vol/vol) dimethyl sulfoxide–10 mM β-mercaptoethanol–150 mM NaCl. After 5 min at 90°C and 5 min at room temperature, 2.5 U of *Taq* (*Thermus aquaticus*) DNA polymerase was added, and synthesis was performed for 5 min at 55°C. This step was followed by 12 cycles of amplification (denaturation, 1 min at 90°C; annealing, 1 min at 25°C; extension, 2 min at 68°C). The amplified products were separated by

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electrophoresis on 10% (wt/vol) polyacrylamide gels, and the 116-bp fragment, identified after ethidium bromide staining, was eluted, purified by ethanol precipitation, and then treated with kinase prior to the cloning. This fragment was first cloned into the blunted *Sst*I site of p(C_2AT) and then subcloned along with the 380-bp G-free cassette into the Bluescript vector. This construct, referred to as −102PK(380), was 3′ deleted with *Bal*31 to yield a shortened G-free cassette of 270 bp, the −102PK(270) construct. The −183PK(380) and −1170PK(380) constructs were obtained by insertion of additional PK sequences at position −96 of the −102PK(380) construct, into an *Nhe*I restriction site.

The two other PK constructs, −25PK(380) and −42PK (380), were obtained by 5′ deletion with *Bal*31 of the −102PK(380) construct.

All PK constructs were sequenced to eliminate any polymerase chain reaction and/or cloning artifacts.

The 5′-flanking region of the major late adenovirus (from −404 to +10) along with shortened G-free cassettes of 270 or 170 bp were also subcloned into the Bluescript vector. These constructs containing the major late adenovirus promoter (AdML), which is known to be active in most cell types, were used as internal controls.

To improve the efficiency of transcription termination, all the templates were cut at a *Hin*dIII site in the vector polylinker directly downstream of the G-free cassette. No notable difference in transcriptional efficiency was observed between these linearized templates and their circular counterparts.

Extract preparation. Liver and spleen nuclear extracts from 3-month-old male Wistar rats were prepared by the method of Gorski et al. (15). For dietary and hormonal studies, liver extracts were prepared from rats either starved for 72 h or starved for 72 h and then refed a carbohydrate-rich diet or treated with rapid glucagon (500 μg/kg) before sacrifice.

Whole cell extracts (WCE) from HeLa cells were a generous gift from V. Moncollin.

In vitro transcription assays. In vitro transcription reactions were performed as described by Gorski et al. (15) with 200 ng of PK(380) constructs together with 100 ng of AdML(270). For competition experiments with L4 oligonucleotide, the −183PK(380) construct was transcribed together with 100 ng of −102PK(270) plus 160 ng of AdML(170). Each reaction mix contained 26 μg of nuclear extract (liver or spleen).

Phosphorylation of the nuclear proteins by protein kinase A was done by incubating 26 μg of nuclear extract with 10 μg of the purified catalytic subunit of cyclic AMP-dependent protein kinase in the presence of 0.01 mM ATP and 7.5 mM $MgCl_2$ for 10 min at 25°C. Activity of the catalytic subunit was checked by measuring transfer of radiophosphate from [γ-^{32}P]ATP to histones (28). Phosphorylated extracts were used as described above.

Transcriptional activity of the various constructs was determined by densitometry of the bands with either AdML or −102PK(270) signals as references.

The sequence (upper strand) of the oligonucleotides containing the L-PK promoter elements L1 to L4 was as follows: L1 (from −106 to −60), 5′-AAGAGAGATGCT AGCTGGTTATACTTTAACCAGGACTCATCTCATCT-3′; L2 (from −123 to −93), 5′-ACAAGGCTTCCGTTGGCAAG AGAGATGCTAG-3′; L3 (from −150 to −121), 5′-TGGTT CCTGGACTCTGGCCCCCAGTGTACA-3′; L4 (from −172 to −143), 5′-ATGGGCGCACGGGGCACTCCCGTGGTTC CT-3′.

RESULTS

Transcriptional efficiency of the different L-PK constructs with liver nuclear extracts. L-PK constructs containing different lengths of 5′-flanking region (−25, −42, −102, −183, and −1170 relative to the cap site) were tested in vitro for their transcriptional efficiency with liver nuclear extracts (Fig. 1). The 5′ regulatory L-PK sequences were linked to an artificial DNA fragment that does not contain G residues on the RNA-like strand (the 380-bp G-free cassette initially described by Sawadogo and Roeder [34]). Each PK construct contains the mouse G-less albumin sequences (−4 to +14) upstream of the G-free cassette since the L-PK sequences downstream of the cap site contain several G residues (see Materials and Methods). As shown in Fig. 1B, the size of transcripts generated from L-PK constructs was approximately 395 nucleotides (nt), as determined from the molecular weight markers and the 400-nt transcript synthesized from the albumin promoter (construct Alb400, a gift from U. Schibler [15]). This is in agreement with the expected start site of transcription at +1 of the inserted albumin fragment. The internal control was the AdML promoter (34) driving the transcription of a shortened G-free cassette leading to a 280-nt transcript.

Maximum transcription from the L-PK promoter was obtained with the −183PK construct (Fig. 1A). This template contains the four L1 to L4 binding sites (Vaulont et al., in press) whose location is schematically presented in Fig. 1C.

With additional PK sequences (−1170PK), about the same transcriptional signal was observed. With the −102PK, containing only the L1 box, binding site for HNF1, 50% of the maximal −183PK signal was obtained. With the −42PK construct, containing only the TATA box, transcription was reduced to 7% of the maximum. Finally, without an intact TATA box (−25PK), the transcription from the PK promoter was completely abolished. The intensity of the signal obtained with the −183PK construct was comparable to that obtained with the 650 bp of the albumin promoter (Alb400 construct; Fig. 1B). At 2 μg/ml, the RNA polymerase inhibitor α-amanitin completely abolished L-PK transcription, indicating that in vitro transcripts are synthesized by RNA polymerase II (not shown).

Tissue specificity of the L-PK promoter activity in vitro. In nuclear extracts prepared from spleen, a tissue that does not express the L-PK gene in vivo, transcription from −183PK was barely detectable while the AdML promoter was as efficient as in the liver (Fig. 2). Thus, the 183 bp upstream of the L-PK cap site were able to reproduce in vitro the tissue specificity of the gene expression observed in vivo. When an equal amount of liver extracts was added to these spleen extracts, transcription from the L-PK promoter was restored, suggesting a deficiency (or a loss of activity) of activating factors rather than the presence of a repressor(s) in spleen extracts. The same results were found with WCE from HeLa cells (kindly provided by V. Moncollin).

Titration experiments with L1 to L4 oligonucleotides. In order to evaluate the respective roles of the L1 to L4 binding proteins in the transcriptional activation of the L-PK promoter by liver nuclear extracts, we performed competition experiments with L1 to L4 oligonucleotides in the in vitro transcription assays.

As shown in Fig. 3A, with the two oligonucleotides binding liver-specific proteins (L1 binding HNF1 and L3 binding LF-A1), the transcriptional activity of the L-PK promoter was reduced by more than 80% for L1 and by

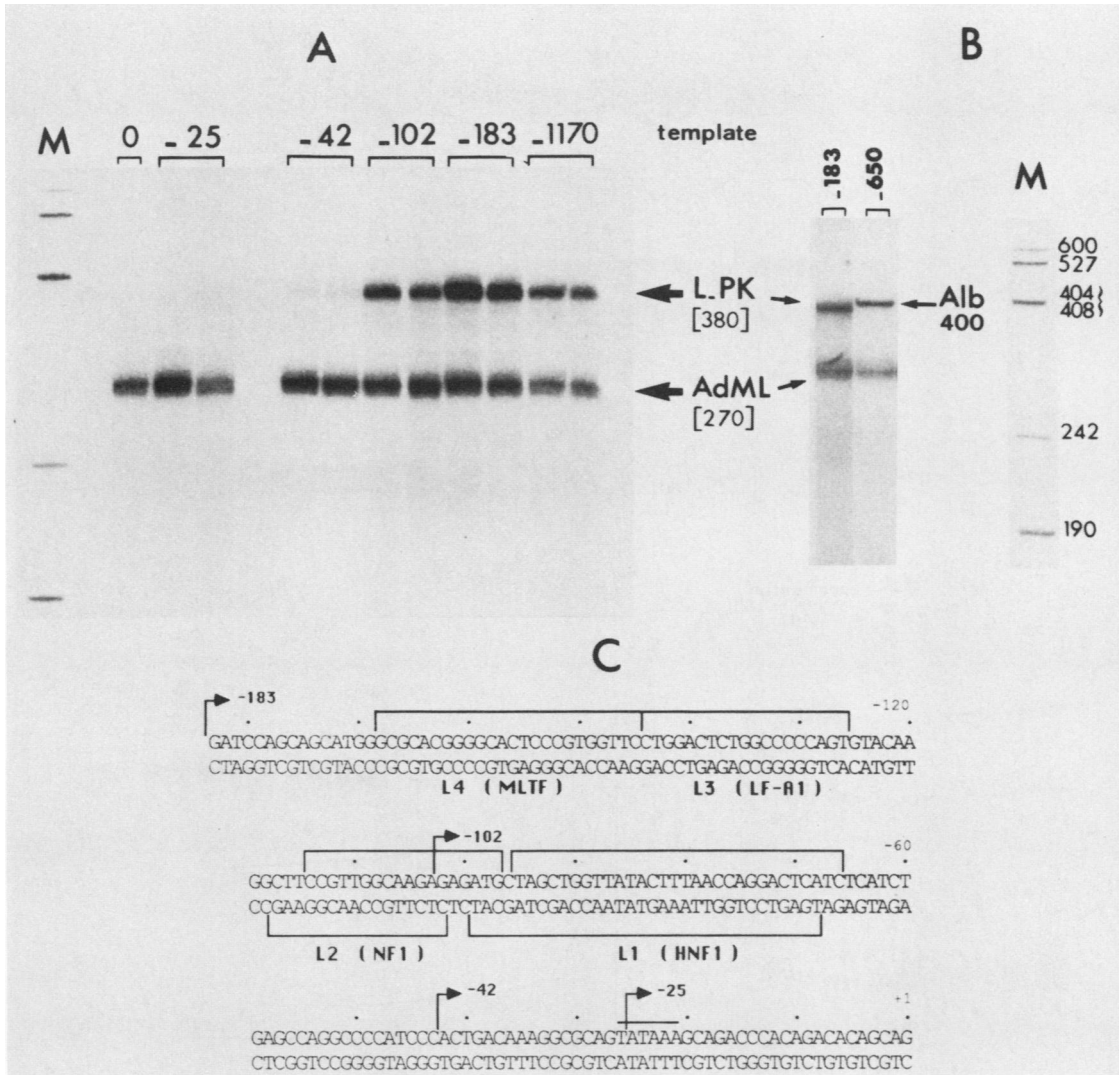

FIG. 1. In vitro transcription of the different L-PK constructs with liver nuclear extracts. (A) In vitro transcription was performed in the presence of 26 μg of liver nuclear extract with 200 ng of each PK(380) template (−25, −42, −102, −183, and −1170) together with 100 ng of AdML(270). Duplicate lanes correspond to two independent experiments. Lane 0 corresponds to the Bluescript vector containing the G-free cassette without promoter, and lanes M are size markers (pSV_2CAT digested with *Hpa*II) (in base pairs). (B) Comparison of transcripts synthesized either from the −183PK(380) construct or from the albumin gene promoter (Alb400; a kind gift from U. Schibler [15]) leading to a 400-nt transcript. The quantitated results were expressed as a percentage of the maximum value (obtained with the −183PK construct) with AdML as the standard. (C) Schematic presentation of the DNA-binding proteins interacting with the L-PK gene promoter. Arrows indicate endpoints of the different L-PK constructs.

about 40% for L3. With L2 oligonucleotide, binding site for NF1, no noticeable difference was observed. These results were calculated with AdML(270) used as the internal control, assuming that the transcriptional signals from this promoter were not modified by an excess of L1, L2, or L3 oligonucleotides in the reaction mix. For L4 oligonucleotide, we always observed a strong inhibition of the internal control AdML. This finding was in good agreement with the well-established data that MLTF, the L4-related binding protein, is a transcriptional activator, essential for the activity of the AdML promoter. Thus, to determine the effect of MLTF on L-PK promoter activity in vitro, run-off assays were performed with the −102PK construct (containing only the L1 box) driving the synthesis of a 270-nt transcript. The AdML construct, driving the synthesis of a 170-nt transcript, was also included in these reactions. As shown in Fig. 3B, while transcription from the AdML promoter was again

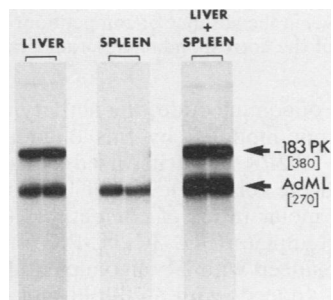

FIG. 2. In vitro transcription of the −183PK construct with liver and spleen nuclear extracts. In vitro transcription was performed with 200 ng of −183PK(380) and 100 ng of AdML(270) constructs in the presence of 26 μg of either liver or spleen nuclear extract or with a mixture containing 13 μg of both extracts. Duplicate lanes correspond to two independent experiments.

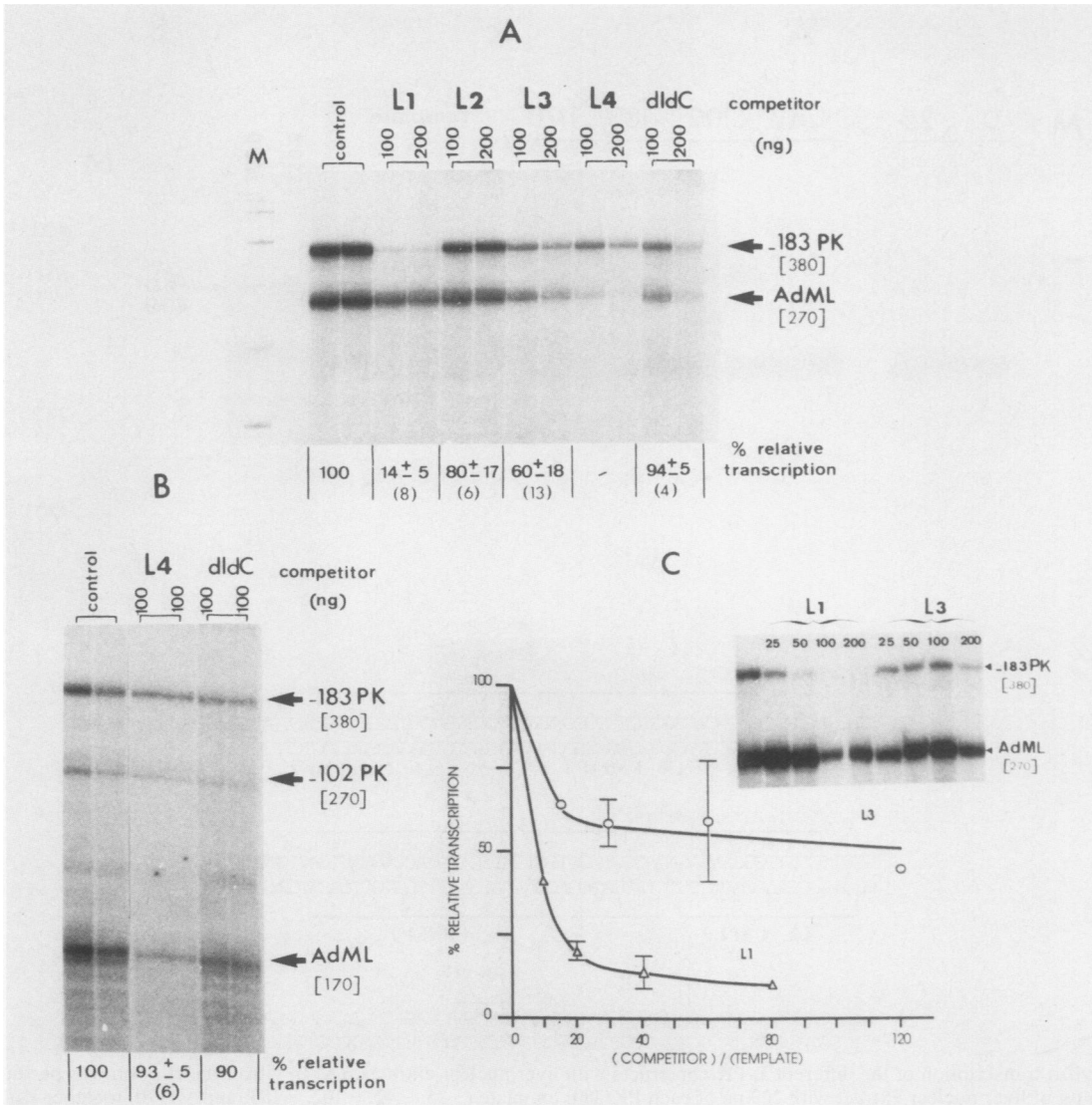

FIG. 3. Competition experiment with the L1, L2, L3, and L4 oligonucleotides. In vitro transcription was performed in the presence of 26 μg of liver nuclear extracts (A) with 200 ng of −183PK(380) plus 100 ng of AdML(270) or (B) with 200 ng of −183PK(380) together with −102PK(270) and AdML(170) constructs. The indicated amounts of competitor oligonucleotide were included in the in vitro transcription reaction mixes. Poly(dI-dC) was used as an unrelated competitor. The quantitative signal variations given under the autoradiograms were measured by using the AdML(270) signal as the standard for L1, L2, and L3 oligonucleotides. For L4 oligonucleotide (B), the −102PK(270) signal was used as the standard. The results were expressed as a percentage of the value obtained with the −183PK construct (control, 100%) and were averaged over several independent experiments (number indicated in parentheses). (C) The relative transcription of the −183PK template was plotted as a function of increasing competitor (L1 [△] or L3 [○])-template molar ratios and expressed as a percentage of its transcriptional level in the absence of competition. Error bars represent ±1 SD of the means calculated from four independent measures. The inset shows one of the corresponding autoradiograms with L1 and L3 oligonucleotides.

inhibited by L4 oligonucleotide, the activity of the −183PK construct was not modified by this oligonucleotide, with respect to the −102PK construct used as the internal control. We plotted the relative levels of L-PK transcriptional activity against molar ratios of competitors (L1 and L3) to −183PK DNA template (Fig. 3C). L-PK transcription was more strongly reduced with L1 oligonucleotide than with L3 oligonucleotide. Indeed, with a 20-fold molar ratio of both oligonucleotides to template, a plateau was reached corresponding to a reduction of about 50% for L3 and more than 90% for L1.

Effect of hormonal and nutritional modifications on L-PK in vitro transcription. In an attempt to delineate the hormone-responsive elements on the 5′-flanking region of the L-PK gene, we tested the efficiency of the three L-PK constructs (−102, −183, and −1170) with different types of liver nuclear extracts. With this aim, we purified liver nuclear extracts from carbohydrate-refed rats which actively expressed the L-PK gene and nuclear extracts from rats either starved or treated with glucagon, two metabolic conditions in which L-PK gene expression is strongly inhibited in vivo (37). As already shown in Fig. 1, with liver nuclear extracts from rats fed a regular diet, the maximum activity was obtained with −183PK, and this activity did not seem to be strongly affected by additional 5′ PK sequences, as suggested by the transcription from the −1170PK template. In contrast, the −102PK construct was active at 50% of the −183PK level. However, as illustrated in Fig. 4, this relative transcriptional

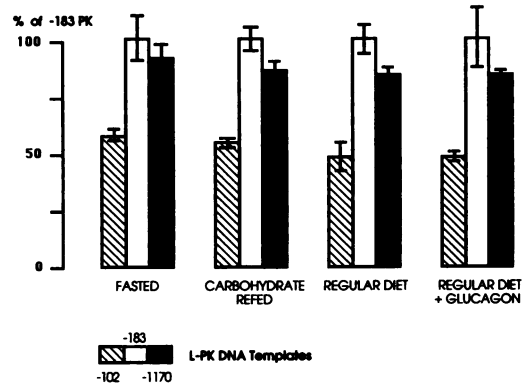

FIG. 4. Transcriptional efficiency of the L-PK constructs in the presence of liver nuclear extracts prepared from rats subjected to various nutritional and hormonal conditions. Liver nuclear extracts were prepared from rats either starved for 72 h or starved for 72 h and then refed a carbohydrate-rich diet or from rats fed a regular diet treated or not with glucagon. In vitro transcription was performed with 200 ng of the different PK(380) constructs together with −102PK(270) plus AdML(170) as standards. The results obtained with respect to both standards were the same; they are expressed as a percentage of the value obtained with the −183PK construct (100%). Error bars represent ±1 SD of the means calculated from at least four independent measures.

efficiency from the three L-PK templates was not modified by the type of liver nuclear extract tested. This result could signify that the sequences involved in the hormonal control are not part of the proximal promoter. Alternatively, it could be that the hormone response requires a special chromatin organization which is obviously lacking in run-off assays or that a required protein was lost during preparation of the nuclear extracts. An additional easily testable hypothesis is that part of the hormone response was due to phosphorylation of some transcriptional factor(s) and that this phosphorylation was lost during purification of the nuclear extracts. In order to test this latter hypothesis, we treated the liver nuclear extracts with the purified catalytic subunit of the cyclic AMP-dependent protein kinase in vitro prior to the in vitro transcription. No relative modification in transcriptional efficiency of the three L-PK construct was noted with or without treatment with the cyclic AMP-dependent protein kinase (data not shown).

DISCUSSION

The available recent data suggest that transcriptional regulation of gene expression occurs by a combination of a limited number of factors with defined *cis*-acting DNA elements. In many cases, the DNA sequences involved in tissue specificity as well as those involved in hormonal response have been identified in the vicinity of the start site of gene transcription. The question thus arises of the nature and the mechanisms of action of the nuclear factors operating through the *cis*-acting DNA elements and their cooperation with each other and with other regulatory proteins in generating a unique and specific promoter. From this viewpoint, the in vitro transcriptional assay is a useful system for trying to answer these questions and for assessing the functional role of *trans*-acting factors. It is, however, noteworthy that in this system, while working with purified DNA templates, the chromatin structure is disrupted.

In this study we analyzed the transcriptional efficiency of the promoter of the rat L-type pyruvate kinase gene whose expression in vivo is mainly confined to liver and is, in this tissue, accurately controlled by hormones and nutrients (36). Four nuclear factors interacting specifically with the L-PK promoter (L1 to L4 DNA elements) have been identified previously (Vaulont et al., in press). L1 binds HNF1 (1, 6, 11, 12, 18, 19, 22, 29, 35); L2 binds the ubiquitous NF1 (14, 17, 21, 27, 32); L3 binds LF-A1 (19, 22); and L4 binds the ubiquitous MLTF (5, 23, 26, 30, 33).

In order to establish the functional role of these nuclear DNA-binding proteins in promoter activity, we first performed in vitro transcription with PK promoter fragments of different lengths that drive the synthesis of the G-free reporter cassette (Fig. 1).

With liver nuclear extracts, the transcription from a −42PK construct containing only the TATA box was barely detectable, while the additional presence of only the HNF1 binding site (L1 box, −102PK construct) gave rise to an important increase in the transcriptional signal. However, maximum L-PK-specific transcription was obtained with 183 bp of L-PK promoter, a fragment containing L1 to L4 binding sites. These results suggest that the L1 box interacts with a strong transcriptional activator necessary but not sufficient to account for the maximum transcription obtained with the −183PK and thus that this latter construct may contain additional DNA elements binding *trans*-activating factors. The transcriptional activity of a construct containing the 1,170 bp of the PK 5′-flanking region was not significantly different from that of the −183PK construct. This could be due to the fact that it is very difficult to reproduce distal effects in an in vitro transcriptional experiment.

In order to define more precisely the respective role of each factor in conferring liver specificity and hormonal response, we then performed titration experiments with an excess of oligonucleotides—reproducing L1 to L4 binding sites—in the transcriptional reaction (Fig. 3). In these experiments with the −183PK construct as the template, we observed a difference in transcriptional efficiency of the template only with L1 and L3 oligonucleotides. Titration of the two liver nuclear factors binding to L1 and L3 sites, HNF1 and LF-A1, respectively, provokes more than 80% and about 40% L-PK transcriptional reduction, respectively, while titration of the two other ubiquitous factors does not seem to modify in vitro transcription of the L-PK construct (Fig. 3B). These results confirm that HNF1 is a strong transcriptional activator whose binding to the L1 site is essential for efficient transcription and suggest that LF-A1 could correspond to another transcriptional activator acting on the L-PK promoter.

As mentioned above, a construct containing only the L1 binding site was transcribed at about 50% of the maximum. This result is in good correlation with the 40% reduction obtained on the −183PK template by titration of L3 factor, assuming that residual transcription is due to HNF1 and that L2 and L4 factors (NF1 and MLTF) are not essential in this system.

Titration of L1 factor on the -183PK construct leads to a residual transcription of about 10% of the maximum. The same transcriptional inhibition was obtained by titrating L1 factor on the −102PK construct (not shown), suggesting that if L1 factor is missing, L3 factor is without any effect on PK promoter activity. The residual transcriptional activity, after competition with L1 oligonucleotide, may thus correspond to the basal activity of the promoter with the TATA box factors, as suggested by the same basal activity observed with the −42PK construct.

The functional role of HNF1 as a positive transcriptional

factor present exclusively in hepatic cells and its requirement for efficient transcription in liver have already been reported by other groups for different liver-specific genes, including the albumin (6, 18, 20, 29, 35), β fibrinogen (12), and α1-antitrypsin (19, 22) genes.

Furthermore, synthetic oligonucleotides, reproducing the specific HNF1-binding site from these three promoters, are able to confer increased in vitro transcription in nuclear extracts from rat liver (18, 19). Our finding of the requirement of this nuclear factor for L-PK gene transcription reinforces the idea that the same transcriptional *trans*-acting factor controls the expression of several liver-specific genes.

The high transcriptional level of the −102PK template which contains only the HNF1-binding site is in good agreement with the ability of HNF1 alone to induce liver-specific expression from either homologous (18, 35) or heterologous (22) promoters. However, the efficiency of HNF1 in activating transcription may be a function of its combination with other regulatory proteins, as suggested by the fact that 100% transcription was never achieved with the HNF1-binding site alone. Maximal activity of the PK promoter was obtained with the binding of both HNF1 and LF-A1 factors. The positive role of LF-A1 in transcriptional activation of a liver-specific gene has already been shown by Monaci et al.; the addition of the LF-A1-binding site (from α1-antitrypsin promoter) to the 80-bp TK promoter is able to induce liver-specific expression from this heterologous promoter (22). However, LF-A1 per se, when HNF1 is depleted, does not seem to have any effect on L-PK transcription in our system. This suggests a nonadditive functional cooperation, either direct or indirect, between LF-A1 and HNF1 to activate L-PK transcription.

In addition to HNF1 and LF-A1, other liver-specific proteins have been reported to bind to promoters or enhancers of liver genes, e.g., HNF3 and HNF4 (10, 16). The transcriptional role of these factors, suggested from transient transfection assays, has not yet been directly investigated in a cell-free system. Anyway, we found no footprints (Vaulont et al., in press) and no sequence homologies which could indicate that these factors bind to the PK promoter.

The PK promoter, like most of the other liver-specific promoters so far analyzed, binds both liver-specific and ubiquitous proteins. Here, the ubiquitous proteins are NF1 and MLTF, which, in the context of other promoters, seem to play a role in the activation of transcription (7, 24, 33). While these factors may be instrumental in the context of nuclear chromatin, they have little if any activation potential in our cell-free system.

The in vitro L-PK promoter activity is specific to the liver, since with nuclear extracts prepared from spleen, a nonexpressing tissue, no transcripts from L-PK promoter were detected (Fig. 2). These extracts were nevertheless transcriptionally competent, since the activity of the AdML promoter was as efficient as in the liver. However, when both spleen and liver nuclear extracts were added to the transcriptional reaction mix, the PK transcriptional signal was comparable to the one obtained with liver nuclear extracts alone. These data, taken together with the known lack of HNF1- and LF-A1-binding activities in spleen nuclear extracts (Vaulont et al., in press) suggest that it is probably the absence of tissue-specific activators that is responsible for the nonexpression of the L-PK construct in spleen extracts. This assumption of the lack of specific activators in nonexpressing cells has been confirmed recently by Lichsteiner and Schibler, who demonstrated that addition of purified HNF1 to spleen nuclear extracts can restore transcription activity to the albumin promoter (19a).

While the endogenous PK gene as well as transgenes containing 3.2 kb of 5′-flanking sequences are regulated in vivo by hormones and diet (Tremp et al., J. Biol. Chem., in press), we were unable to reproduce these effects in our cell-free transcription system. A first explanation for these negative results could be that the DNA elements responsible for the responses to hormones and diet are not included in the promoter region analyzed, i.e., in the 1,170 bp upstream of the L-PK cap site.

This interpretation should however be considered with caution. If, indeed, the possibility of reproducing the in vivo tissue specificity of a promoter in cell-free transcription systems is well established (2–4, 13, 15, 22), there are, to date, only a few examples of hormonal response obtained in these systems. Such an in vitro transcriptional response to hormones has recently been reported for the estrogen-dependent transcription of the vitellogenin gene in liver nuclear extracts (9). In addition, the catalytic subunit of protein kinase A has been shown to stimulate the transcription of the urokinase-type plasminogen activator gene in epithelial WCE (25) and that of the somatostatin gene in brain nuclear extracts (37). In all these examples, the hormone-responsive elements were in the first 300 bp upstream of the cap sites, even in the first 150 bp in the case of the promoters responding to cyclic AMP. It is therefore conceivable that most of the hormone-responsive elements need to be proximal to the cap site to be active in a cell-free system. It could also be that, in come cases, hormone response is lost in the absence of chromatin organization, so that, in fact, a negative result of in vitro hormone response does not definitely preclude the presence of a hormone response element in the DNA fragment investigated. Our data that in vitro phosphorylation of the liver nuclear extracts does not modify their transcriptional efficiency on the L-PK templates contrast with the data on the promoters of the urokinase-type plasminogen activator and somatostatin genes and suggest, in any case, that a similar mechanism of direct phosphorylation of transcriptional factors by protein kinase A is not operating in our experiments.

In conclusion, while run-off transcriptional assay of the L-PK gene promoter has allowed us to define the DNA elements and binding proteins required for its tissue-specific expression, it could be that precise dissection of the various hormone and nutrient response elements needs the combined use of transgenic mice and transfection experiments in different cultured cells to be successful. These investigations are currently in progress in our laboratory.

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