Communication

Exploration of a Liver-specific, Glucose/Insulin-responsive Promoter in Transgenic Mice*

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The functional role of the different sites binding transcriptional factors on the tissue-specific, glucose-responsive promoter of the L type pyruvate kinase gene (L-PK) has been investigated in transgenic mice. These sites are able to bind, from 3' to 5', HNF1, NF1, HNF4, and MLTF/USF, respectively. We have compared the level of chloramphenicol acetyltransferase reporter transgene expression when driven by a L-PK promoter fragment of either -96 base pairs (bp) (containing only the HNF1 binding site) or -150 bp (lacking the MLTF/USF binding site) or driven by a -183-bp L-PK promoter fragment with or without the NF1 binding site. Our results demonstrate that: 1) HNF1 alone is not sufficient to promote an efficient L-PK gene transcription in vivo; 2) with only binding sites for HNF1, NF1, and HNF4, though the tissue-specific pattern of expression is respected, the level of the gene transcription is low and the hormonal control is lost; 3) the MLTF/USF binding site is the target of the hormonal control, required for both positive response to carbohydrates and negative response to glucagon; 4) the role of NF1 in the promoter activity could be to negatively modulate the L-PK gene expression in the different tissues, without interfering with the glucose and hormone responsiveness.

Pyrurate kinase, a glycolytic enzyme, has four isozymes in mammals. The L type enzyme (L-PK)3 is expressed primarily in liver and is also present in kidney and small intestine as a minor form (1, 2). Its expression in rat liver is under dietary and hormonal control; expression is high when animals are reared a carbohydrate-rich diet in the presence of insulin, while it is undetectable when animals are fasted or treated with glucagon (3). This process occurs at two levels, a short-term control, modifying the enzyme activity, and a long-term control, modifying the enzyme synthesis. This latter control has been shown to occur mainly at the transcriptional level (3).

In order to gain insight into the transcriptional control of the L-PK gene expression, we have developed ex vivo analyses by transient expression in hepatocytes in primary culture (4, 5). In good agreement with the studies of Thompson and Towle (6), the transcriptional response to carbohydrates and hormones has been ascribed to a -150 to -180 bp sequence, termed GIRE for "glucose-responsive element" (5). This site is characterized by two degenerated E boxes able to bind in vitro the major late transcriptional factor (7), which belongs to the b-HLH-LZ transcriptional factor family. As discussed in an earlier paper (5), the L-PK GIRE is clearly different from other insulin response elements described for several genes. However, interestingly, this element seems closely related to the carbohydrate response element recently described by Shih and Towle (7) in the regulatory region of the gene encoding the S14 protein specific to adipocytes. This carbohydrate response element and the L-PK GIRE were both demonstrated to belong to a DNase I-hypersensitive region, whose intensity was reduced in fasted animals and induced upon carbohydrate refeeding (8, 9). Thus, it could be that a common element, located in a hypersensitive region and characterized by its ability to bind members of the MLTF/USF family, is involved in the transcriptional control of different genes in response to carbohydrate metabolism. Although further investigations are needed to reveal its mode of function, we wanted to reexamine the potential hormonal regulatory activity of this element in vivo in the whole animal. Furthermore, as we have previously shown that -183 bp are sufficient to reproduce the tissue-specific and hormone-regulated pattern of the gene expression in transgenic mice (10), we wanted to assess the role of the other binding sites detected in the L-PK promoter, i.e. from 3' to 5' for HNF1, NF1, and HNF4 (11), in building up this specific L-PK promoter. We have therefore undertaken a study in transgenic mice using hybrid genes containing the CAT reporter gene under the control of various deleted L-PK promoter fragments.

MATERIALS AND METHODS

Construction of Hybrid Genes—Plasmid DNAs were digested by SauI (which cuts in 5', in pEMBL8+ linker) and Nael (which cuts in 3', in pEMBL8+ sequences thus leading to a 600-bp plasmid fragment in 3' of each microinjected construct). The restriction fragments containing the chimeric genes were purified from agarose gels and microinjected into fertilized mouse eggs by the methods described previously (10, 13). DNA was isolated from tails of 2-week-old mice, digested by Xhol enzyme, and analyzed on Southern blot by hybridization with a CAT probe (a NcoI fragment from pBSV, CAT vector, i.e. from -107 to +551 bp). We selected the first two positive founders, and these F0 mice were outbred to generate heterozygous mice. All subsequent studies were performed on F1 or F2 mice.

Production and Identification of Transgenic Mice—Plasmid DNAs were digested by SauI (which cuts in 5', in pEMBL8+ linker) and Nael (which cuts in 3', in pEMBL8+ sequences thus leading to a 600-bp plasmid fragment in 3' of each microinjected construct). The restriction fragments containing the chimeric genes were purified from agarase gels and microinjected into fertilized mouse eggs by the methods described previously (10, 13). DNA was isolated from tails of 2-week-old mice, digested by Xhol enzyme, and analyzed on Southern blot by hybridization with a CAT probe (a NcoI fragment from pBSV, CAT vector, i.e. from -107 to +551 bp). We selected the first two positive founders, and these F0 mice were outbred to generate heterozygous mice. All subsequent studies were performed on F1 or F2 mice.

Nutritional and Hormonal Treatment—6-8-week-old F1 or F2 animals were submitted to different nutritional and hormonal treatments. To maximally lower the level of CAT activity before the onset of the experiment, animals were previously fed with a high protein diet for

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about 2 weeks and fasted for 24 h. Then animals were separated into three groups. For the first group, fasting was pursued for an additional 10-h period ("fasted conditions"). For the second group, animals were refed during this 10-h period with a high carbohydrate diet ("refed conditions"). For the third group, animals were also refed the high carbohydrate diet but with a glucagon treatment throughout the refeeding period: 1 mg of zinc-glucagon/kg of body weight injected subcutaneously every 3 h ("glucagon conditions"). Mice were sacrificed at 10 p.m. and the tissue samples stored at −80 °C.

**Assay for CAT Enzyme**—Individual tissue samples were homogenized in 500 μl of 250 mM Tris-HCl (pH 7.5), 5 mM EDTA with Eppendorf micropesctubes directly in the tube. Homogenates were microcentrifuged at 4 °C for 10 min, and the concentration of soluble proteins in the supernatant was determined by the Bio-Rad protein assay. After an 8-min incubation at 65 °C, tissue extracts were assayed for CAT activity according to standard methods (12); 500–1000 pg of proteins were incubated for 1.5 h at 37 °C with 1.2 μCi of [14C]chloramphenicol and 140 μg of acetyl coenzyme A. The products were separated by thin-layer chromatography. Acetylated forms of [14C]chloramphenicol were excised individually and counted in a scintillation counter. For quantification, assays were demonstrated to be within linear range for incubation time and enzyme concentration.

**Isolation of Total RNA and Northern Blot Analyses**—Up to 100 mg of frozen tissue were homogenized, and RNAs were purified by a modified guanidinium chloride procedure (5). Northern blot analysis was conducted as described (3), except that electrophoresis was performed in formaldehyde-containing gel. For the detection of mouse L-PK-specific mRNAs, a rat cDNA clone complementary to the coding sequence was used at low stringency. For the detection of CAT transcripts, a CAT probe was used (a Neo fragment from pSv2CAT vector). Specific bands were quantified by scanning the autoradiographs using a Shimadzu densitometer.

## RESULTS

**Organization of the Constructs and Generation of Transgenic Mice**—To produce transgenic mice, L-PK/CAT constructs were microinjected into the pronuclei of fertilized mouse eggs as previously described (13). The constructs were hybrid genes containing the CAT reporter gene driven by an L-PK promoter fragment of progressive deleted length: either a −183-bp fragment encompassing the four binding sites L1-L4 that interact, from 3' to 5', with HNF1, NF1, HNF4, and MLT/FNSF, respectively (11); a −150-bp fragment lacking the MLT/FNSF binding site; a −96-bp fragment containing only the HNF1 binding site; or a −183-bp fragment with an internal deletion from nt −96 to nt −122, i.e. deleted for NF1 binding site (Fig. 1A).

In all cases, a 400-bp fragment (corresponding to a distal L-PK activating region located between nt −2500 and nt −2900) was added to the transgenes in order to obtain a high expression level. This fragment, indeed, as previously shown (10), may be required for the full activation of the L-PK gene and is devoid of any effect on both hormonal regulation and tissue specificity.

The genomic DNA from each mouse tail was analyzed by Southern blotting and probed with a CAT-specific fragment. Twelve to three transgenic lines were established for each construct. Fig. 1B shows Southern analyses of XhoI-digested DNA of F1 positive offspring. As XhoI cuts once in each injected molecule, a band of the same length as the injected fragment was detected, indicating that a head-to-tail array has been formed in each case. The estimated copy number of each line, given at the bottom of Fig. 1B, was evaluated by rehybridizing the Southern blot with a murine c-myc probe that reveals an endogenous single-copy gene.

**CAT Activity and CAT Transcripts as Measured in Various Tissues of Transgenic Animals; an in Vivo Exploration of the L-PK Promoter**—CAT activity was measured in several tissues of the transgenic mice. Generally, CAT assay was performed on three to four independent animals, and results were subsequently quantified by counting the radioactivity. An arrangement of a typical experiment for each line is shown in Fig. 2. No significant CAT activity could be detected in any tissue of mice made transgenic with the −96 construct. For constructs −150 and −183, in agreement with the known tissues expressing the endogenous L-PK gene, CAT activity was detected especially in liver, but also to a lesser degree, in kidney and in small intestine. The CAT activity is low and at a comparable level in liver of both R43 and R73 mouse lines. This activity is enhanced 3–4-fold in liver of line Q20. The exceptionally elevated CAT activity level observed with line Q5 could be explained by the very high level of copies integrated in the genome of this mouse. Surprisingly, CAT activity level was especially elevated in mice carrying the −183ΔL2 construct. In these mice, CAT expression becomes much more marked in the small intestine than in the liver and, in addition, expression in the kidney is as high as in...
the liver. This result is probably independent of the influence of the integration site since the two S34 and S57 lines exhibit exactly the same pattern of expression.

Finally, it is noteworthy that for each tested transgenic mouse, we have always observed CAT activity in restricted L-PK expressing tissues and never in any of the other analyzed tissues.

These data should be compared with the Northern blot presented in Fig. 3, showing the level of CAT transcripts in the brain and in the liver of these mice. It can be noticed that CAT transcripts are undetectable for the three lines of the -96 construct. In contrast, CAT transcripts are detected for the constructs -150 and -183, with relative amounts that correlate very well with CAT activity level; line Q5 overexpresses the CAT transgene, and line Q20 expresses the transgene at a level 3-4-fold higher than lines R43 and R73. Mice from the two S34 and S57 lines produce about 15-fold more CAT RNAs in their liver than line Q20 and practically the same amount as the high copy number Q5 line. Finally, no CAT transcripts are detected in negative tissues such as the brain. Controls were established by loading, from left to right, RNAs from a -392-bp L-PK/CAT transgenic mouse, RNAs from a transgenic mouse containing the whole regulatory region of ~3200 bp, and RNAs from a non-transgenic mouse. Finally, we present at the bottom of the figure the amount of endogenous L-PK RNAs in each mouse liver, as obtained after rehybridizing the filter with a L-PK probe.

Taken altogether, these data suggest that (i) HNF1 factor binding alone on the L-PK promoter is not sufficient to promote an efficient in vivo transcription of the reporter gene, even in the presence of the 400-bp upstream activating fragment, (ii) the additional presence of NF1 and HNF4 is sufficient to allow the L-PK/CAT gene to be transcribed in the appropriate tissues at a level that is enhanced 3-4-fold by the binding of MLTF/USF on box L4 (see line Q20 as compared with line R43 and R73), and (iii) the NF1 factor binding to box L2 could play a negative modulator role since its deletion permits the promoter to be more active in liver and, above all, in kidney and small intestine.

Hormonal Response Elements Are Located between -150 and -183 bp in the L-PK Promoter—Hormonal regulation of the L-PK/CAT transgenes was studied, as previously described (10), under three metabolic conditions: animals were either fasted, fasted and refed with a high carbohydrate diet, or refed a high carbohydrate diet associated with glucagon treatment. At least two to three animals were studied for each metabolic condition. The abundance of CAT transcripts was examined by Northern blot analysis in liver, small intestine, and kidney (Fig. 4).

The amount of endogenous L-PK RNAs was also checked and is shown in the lower part for each line. It varies as expected according to the metabolic status: very low in fasted and glucagon-treated animals, and increased 40-100-fold by carbohydrate refeeding (3).

Fig. 4 shows analysis of L-PK and CAT transcripts in the liver of transgenic mice. In the lines carrying the -183 construct and the lines carrying the -183ΔL2 construct, variations in the amount of CAT transcripts mimic exactly those of the endogenous L-PK RNAs, with low amounts in the liver of fasted or glucagon-treated animals and a pronounced accumulation in the liver of refed animals. In contrast, for the two lines bearing the -150 construct, CAT expression is comparable in the liver of animals whether fasted, refed, or refed and treated with glucagon.

Northern blot analysis was also performed with RNAs purified from small intestine and kidney of mice made transgenic with the -183ΔL2 construct (line S57), the only construct that permits detection of a significant level of CAT RNAs in these tissues. As for the liver, we confirmed that the hormonal regulation is maintained in the small intestine with only -183 bp of the promoter and that the NF1 binding site is not implicated in this regulation. In kidney, the dietary regulation was also maintained, with low transcript abundance in fasted animals and accumulation in refed animals (not shown). These results establish that the hormone- and diet-responsive element effective in vivo in the L-PK expressing tissue is located between -150 and -183 bp on the L-PK promoter, a sequence corresponding to the L4 box, which has been identified ex vivo as the L-PK GIRE able to bind in vitro the MLTF/USF factor.

**DISCUSSION**

We have studied the level of various L-PK/CAT transgenes with respect to their tissue-specific expression and hormonal

**Fig. 3. Northern blot analysis of L-PK and CAT transcripts in liver and brain of transgenic mice.** Northern blot was performed with 10 μg of RNAs purified from liver (L) or brain (B) of carbohydrate-refed animals, as described under "Materials and Methods." On the left, control RNAs were purified from liver of previously reported mice (10) made transgenic with either a -392 L-PK/CAT construct or with a -5200 L-PK/CAT construct, or purified from a non-transgenic mouse fed a regular diet (NTM). The upper part shows hybridization with the CAT probe and the lower part, hybridization with the L-PK probe.

**Fig. 4. Northern blot analysis of CAT and L-PK transcripts as a function of hormonal and nutritional conditions.** Northern blot was performed with 10 μg of RNAs purified from liver (and from small intestine for line S57) of animals either fasted (F), or refed a carbohydrate-rich diet (R), or refed and treated with glucagon (G). The upper part shows hybridization with the CAT probe and the lower part, hybridization with the L-PK probe.
regulation. We confirm in this paper our previous results, namely that the proximal -183 bp of the L-PK promoter is sufficient to confer tissue-specific expression and contains all necessary information for dietary and hormonal control of the gene expression in vivo (10). A similar result has been very recently reported by Noguchi et al. (14). In this region, we were unable to assess a functional role in vitro for the NF1 binding site (15, 16), although we know by in vivo footprinting experiments that this site is indeed occupied.2,3 We thus tested in transgenic mice the effect of its deletion within the -183-bp promoter. Our results clearly establish that this site is not implicated in the hormonal response of the gene. It could, however, be involved in the tissue-specific level of the gene expression as a negative modulator. Its deletion provokes enhancement of the gene expression by about 15-fold in the liver and permits us to detect in the kidney and small intestine a large amount of CAT RNAs. It is noteworthy that an activation (although less effective) of the CAT transcription level was also observed when this -183-bp construct was transfected in hepatocytes in primary culture (5). Since the -183-bp construct contains a 30-bp deletion, we cannot rule out the possibility that this activation could be due, at least in part, to the closer proximity of L4L3 elements to the NF1 binding site. However, such an explanation does not account for the considerable difference in the relative activity of this construct in the liver, kidney, and small intestine compared with other constructs retaining the NF1 binding site.

There is to date no well characterized example of a repressive role of NF1 proteins on transcription. An NF1-like binding site has been described in the distal repressor of the retinol binding protein. Nevertheless, the construct containing these three NF1 promoter is sufficient to constitute a weak tissue-specific transgenic mouse.

Furthermore, a single L4 element was shown to be able to stimulate 8-fold in vitro transcription from a -80-bp TK promoter (16). However, this potential activating role of NF1 per se is also not reproduced in hepatocytes transfected with the -96 construct (5), suggesting the need for NF1 to cooperate with additional transcription factors to be fully active in vivo and ex vivo.

The addition of NF1 and NF4 binding sites to this minimal NF1 promoter is sufficient to constitute a weak tissue-specific promoter. Nevertheless, the construct containing these three binding sites is no longer responsive to glucose and hormones, emphasizing the requirement of the last MLTF/USF binding site for this response. Thus, consistent with results obtained by transfection of hepatocytes in primary cultures (5), we confirm here that the L4 element is indeed the target of the hormonal regulation and is not only involved in the glucose/insulin response but is also required for glucagon-dependent transcriptional inhibition.

This L4 element consists of two degenerated E boxes, in vitro binding sites for MLTF/USF. Experiments performed ex vivo in hepatocytes have yielded some additional information concerning its function.4 Although interacting with MLTF/USF in vitro, cotransfection with a MLTF expression vector leads to an inhibition of the L-PK promoter. Furthermore, function of the L-PK GIRE is impaired by increasing the affinity for MLTF/USF of the proximal, but not of the distal, E box of the L4 element. Finally, promoters carrying mutations suppressing any binding in one of the two E boxes are completely inactive.

In conclusion, although the exact mechanisms of its action deserve further investigation, our data establish that element L4 is, in vivo as well as in cultured hepatocytes, the L-PK GIRE, required for both gene stimulation by carbohydrates and inhibition by glucagon and cAMP. The GIRE is therefore the first element of this type, conferring positive response to glucose and insulin and negative response to cAMP, whose effect has been demonstrated in both transgenic mice and cultured cells (5). In addition, our investigation has allowed us to point out a new possible role for NF1, namely a tissue-specific negative modulation of gene expression.

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