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Elements Responsible for Hormonal Control and Tissue Specificity of L-Type Pyruvate Kinase Gene Expression in Transgenic Mice

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L-type pyruvate kinase (L-PK) is a key enzyme of the glycolytic pathway specifically expressed in the liver and, to a lesser degree, in the small intestine and kidney. One important characteristic of L-PK gene expression is its strong activation by glucose and insulin and its complete inhibition by fasting or glucagon treatment. Having previously established that the entire rat L-PK gene plus 3.2 kbp of 5′-flanking region functions in mice in a tissue-specific and hormonally regulated manner, various deletions of these 3.2 kbp of 5′-flanking regions were tested in transgenic animals to map the *cis*-acting elements involved in transcriptional gene regulation. Our experiments indicate that the proximal region between −183 and +11 confers tissue specificity and contains all the information necessary for dietary and hormonal control of L-PK gene expression in vivo. We found, however, that the transcriptional activity generated by this proximal promoter fragment can be modulated by distal sequences in a tissue-specific manner. (i) Sequences between bp −183 and −392 seem to play a dual role in the liver and small intestine; they induce L-PK expression in the liver but repress it in the small intestine. (ii) Sequences from bp −392 up to −1170 do not seem to have any additional effect on promoter activity. (iii) Between bp −1170 and −2080, we found a putative extinguisher whose transcriptional inhibitory effect is much more marked in the small intestine than in the liver. (iv) Finally, between bp −2080 and −3200, we identified an activating sequence required for full expression of the gene in the liver.

L-type pyruvate kinase (L-PK) is a glycolytic enzyme (ATP:pyruvate O_2-phosphotransferase; EC 2.7.1.40) which plays a central role in hepatic glucose metabolism. The rat L-PK gene contains two alternative promoters located 500 bp apart. The distal one is specific to erythroid cells (L′ promoter), and the proximal one is specific to the liver (L promoter). The expression of the liver form is accurately controlled by dietary factors and by hormones. Prolonged fasting or administration of glucagon to animals blocks gene expression, while refeeding a high-carbohydrate diet in the presence of endogenous insulin stimulates gene expression. This regulation occurs mainly at the level of gene transcription (9, 38).

The liver isoform is present not only in the liver but also, to a lesser extent, in the kidney and small intestine (27). The long-term goal of our laboratory is to understand the molecular mechanisms underlying two major features of L-PK gene expression, its tissue-restricted distribution and its accurate hormonal control.

A 13-kbp fragment encompassing the entire pyruvate kinase gene and 3.2 kbp of 5′-flanking sequences (7) is expressed in transgenic mice in the same manner as the endogenous gene (36). The 3.2-kbp upstream region is sufficient to confer this tissue-specific and hormone- and diet-regulated expression on reporter genes (4). The potential regulatory properties of these 3.2 kbp of 5′ sequences have been further studied by various combined approaches. We have previously established the following features. The proximal promoter (nucleotides [nt] −183 to +11 with respect to the start site of transcription) corresponds, in vivo, to a strong transcription-related DNase I-hypersensitive region, HSS1 (3). It contains various binding sites termed, from 3′ to 5′, boxes L1 to L5. The L1 and L3 boxes bind the liver-specific factors HNF1 and HNF4, respectively, while the L2 and L4 boxes bind the ubiquitous factors NF1 and MLTF, respectively (40). An additional upstream binding site, the L5 box, binds a not-yet-identified L5 binding factor (L5BF), which is partially homologous to HNF4 and differs in the liver and small intestine (31). In vitro, the 183-bp promoter fragment is able to direct efficient liver-specific cell-free transcription, largely dependent on the presence of the two liver-specific proteins HNF1 and HNF4 (39). This promoter is also sufficient to direct ex vivo the tissue-specific expression of a reporter gene, as shown by transient chloramphenicol acetyltransferase (CAT) expression assays in hepatocytes in primary culture and in hepatoma cells (6). Finally, this 183-bp proximal promoter fragment has very recently been shown to be able to confer glucose, insulin, and glucagon responsiveness on the CAT reporter gene in hepatocytes transfected by lipofection (35) and electroporation (2). In addition, the activity of the L-PK promoter in hepatoma cells is modulated by upstream sequences, an extinguisher (nt −1170 to −2082) and an activator (nt −2082 to −3192) corresponding to another in vivo liver-specific DNase I-hypersensitive site, HSS2 (3, 6).

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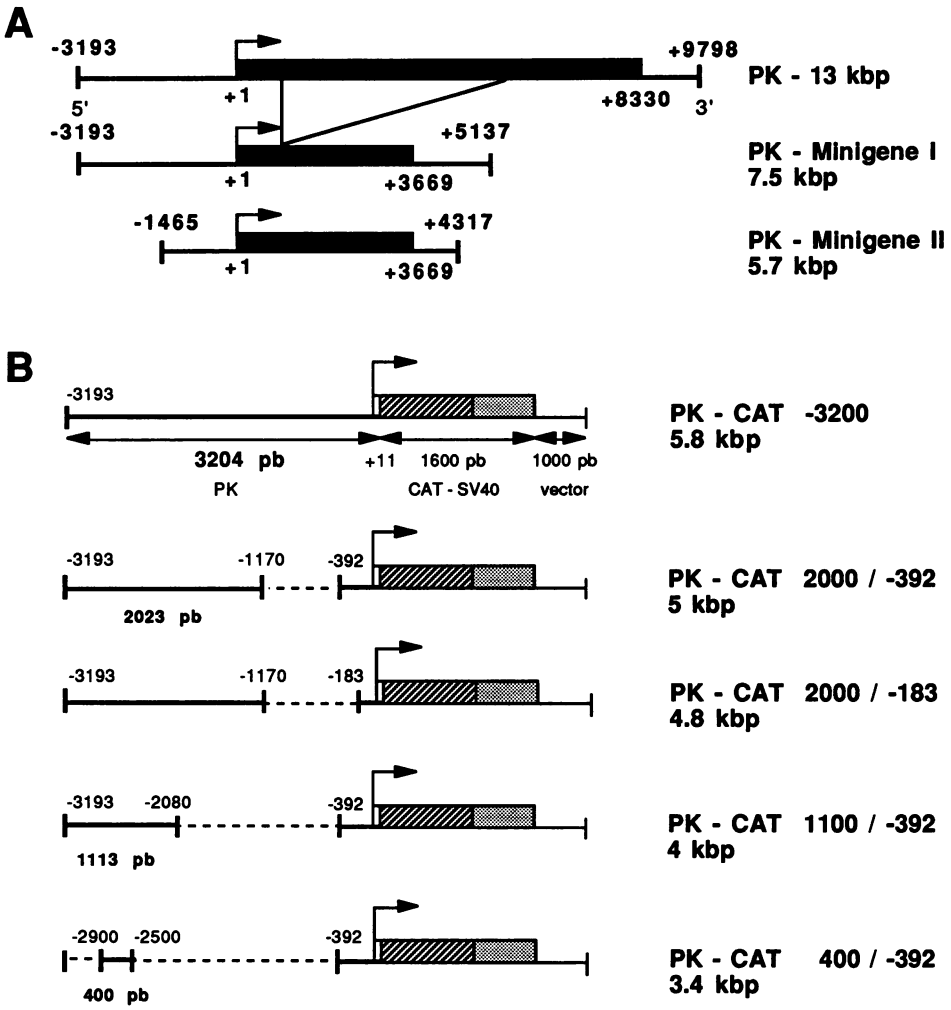

FIG. 1. Maps of minigenes and PK/CAT hybrid genes transferred to transgenic mice. (A) The PK 13-kbp clone consists of the entire gene (solid rectangle) plus its 5'- and 3'-flanking regions of 3.2 and 1.4 kbp, respectively (36). PK minigene I was created by excision of 4.7 kbp between the second and ninth introns of the PK 13-kbp clone by *Bgl*II digestion. PK minigene II was derived from PK minigene I by *Kpn*I cleavage, leading to a shortened 5'-flanking region of only 1.5 kbp. The arrow points to the liver transcriptional start site of the L-PK gene (+1). (B) Structures of the PK/CAT hybrid genes. The CAT structural gene-SV40 poly(A) cassette (hatched and dotted rectangles, respectively) was fused to PK 5' regions at nt +11 in all constructs. Solid lines indicate PK 5' sequences included in fusion genes, and dashed lines indicate PK 5' sequences absent from fusion genes. The name of each construct and its length are given on the right.

However, the various ex vivo-cultured cells do not represent perfect models of the in vivo situation, especially as the results listed above were all obtained in transient-transfection assays. These models do not permit the influence of the chromatin structure on gene expression and hormone response to be analyzed. We therefore undertook an investigation of the *cis*-acting elements regulating expression of the L-PK gene in transgenic mice.

We present in this article transgene expression analysis in the liver and in small intestine of animals harboring either L-PK minigenes or L-PK/CAT constructs with various 5' deletions. This comparative analysis allowed us to confirm that the 183-bp proximal sequence, in vivo as well as ex vivo, the main determinant of the response of the L-PK gene to diet and hormones. In addition, this study has permitted a modulatory function (negative or positive) specifically in the liver and small intestine to be ascribed to various 5' L-PK regulatory sequences.

MATERIALS AND METHODS

Construction of hybrid genes. A pEMBL8+ plasmid containing the rat L-type PK gene plus 3.2 and 1.4 kbp of the 5'- and 3'-flanking regions, respectively, was the source of all constructs (7). The constructions used for microinjection have been published elsewhere (3, 6, 36). They belong to two groups, depicted in Fig. 1.

In the first group (Fig. 1A), the two microinjected constructs are minigenes derived from our previous L-PK 13-kbp clone (7), whose expression in transgenic mice has been reported previously (36). L-PK minigene I contains a 4.7-kbp internal deletion between the second and ninth introns (36). L-PK minigene II was derived from L-PK minigene I by *Kpn*I cleavage, which eliminates 1.5 kbp at the 5' end and 0.8 kbp at the 3' end (3).

The second group of constructs (Fig. 1B) correspond to five L-PK/CAT hybrid genes (6): 3200/CAT, a hybrid gene containing all the 5' PK regulatory elements; 2000/−392, a

hybrid gene containing an internal 5′ deletion between bp −392 and −1170; 2000/−183, a hybrid gene containing an internal 5′ deletion between bp −183 and −1170; 1100/−392, a hybrid gene containing an internal 5′ deletion between bp −392 and −2080; and 400/−392, a hybrid gene containing one deletion between bp −392 and −2500 and another between bp −2900 and −3193.

In our construct terminology, the number on the left corresponds to the approximate length (in base pairs) of the distal region, and the number on the right refers to the 5′-most base pair of the proximal region. The bacterial CAT gene (12) and the simian virus 40 (SV40) early polyadenylation site, contained in an SV40 fragment which includes the small t intron, were fused to PK 5′ regions at nt +11, eliminating the ATG codon located at nt +18.

Production and identification of transgenic mice. Plasmid DNAs were digested with either *Sal*I plus *Cla*I (for the five PK/CAT constructs) or *Eco*RI (for PK minigene II). The restriction fragments containing the chimeric genes were purified from agarose gels and microinjected into fertilized mouse eggs by the methods described previously (36). DNA was isolated from the tails of 2-week-old mice, digested with appropriate restriction enzymes, and analyzed on a Southern blot by hybridization with either a specific rat L-PK probe (a fragment from bp +73 to +701), for minigene II detection, or a CAT probe (an *Nco*I fragment from the pSV_2CAT vector, from bp −107 to +551), for L-PK/CAT hybrid DNA detection. Positive founder F_0 mice were outbred to generate heterozygous mice. All subsequent studies were performed on F_1 mice or on F_2 mice when the F_0 founders were found to be mosaics. For one strain, female 74 (−3200/CAT), the transgene has probably integrated in the X chromosome; a positive F_1 male transmits the transgene only to female offspring.

Nutritional and hormonal treatment. F_1 or F_2 animals (6 to 8 weeks old) were subjected to different nutritional and hormonal treatments. To maximally lower the level of CAT activity before the onset of the experiment, animals were fed with a high-protein diet for about 2 weeks and then fasted for 36 h. The animals were separated into three groups. For the first group, fasting was continued for an additional 15-h period (fasted conditions). For the second group, animals were refed during this 15-h period with a high-carbohydrate diet (refed conditions). For the third group, animals were also refed the high-carbohydrate diet but with glucagon treatment throughout the refeeding period, 1 μg of zinc-glucagon per g of body weight, injected subcutaneously every 3 h (glucagon conditions). Mice were killed at 10 p.m., and tissue samples were stored at −80°C.

Assay for CAT enzyme. Individual tissue samples were disrupted directly in the tube in 500 μl of 250 mM Tris-HCl (pH 7.5)–5 mM EDTA with Eppendorf micropestles. Homogenates were centrifuged in a microcentrifuge at 4°C for 15 min, and the concentration of soluble protein 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 by standard methods (12). Proteins (20 to 1,000 μg) were incubated for 1.5 or 2.25 h at 37°C with 1.2 μCi of [^{14}C]chloramphenicol and 140 or 210 μg, respectively, of acetyl coenzyme A. For each new line, the protein quantity was determined to obtain a fraction of acetylated chloramphenicol that was linear with respect to enzyme activity. We have determined that the assay was linear between 1 and 1,000 μg of proteins. The products were separated by thin-layer chromatography. Acetylated forms of [^{14}C]chloramphenicol were excised individually, and radioactivity was counted in a scintillation counter. Specific activities were determined as counts per micromole of substrate per minute of reaction time and per microgram of protein. We subtracted the value for the brain of each animal, a tissue that does not express the L-PK gene at all, from all values; this value, which has never exceeded 1% of that obtained in the liver or small intestine, was considered to reflect nonspecific background CAT activity.

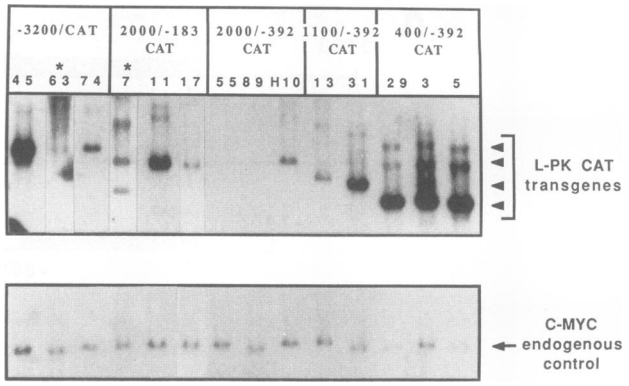

FIG. 2. Southern blot analysis of the different PK/CAT constructs in transgenic mice. *Xho*I-digested mouse tail DNA (positive F_1 offspring mice from founders) (10 μg) was loaded on a 0.8% agarose gel and transferred onto a Hybond N^+ membrane. The top panel shows hybridization with a CAT probe (an *Nco*I fragment from the pSV_2CAT vector). Arrows to the right indicate the positions of the various transgenes. The −3200/CAT construct is 5.8 kbp long; 2000/−183 is 4.8 kbp long; 2000/−392 is 5 kbp long; 1100/−392 is 4 kbp long; and 400/−392 is 3.4 kbp long. The numbers of the three lines obtained for each construct are indicated above each lane. The bottom panel shows rehybridization of the same filter with a murine *c-myc* probe, which reveals a unique band of about 3.8 kbp. Asterisks indicate threefold-longer autoradiograph exposure times.

Isolation of total RNA: Northern (RNA blot) and S1 nuclease protection analyses. Up to 100 mg of frozen tissue was homogenized, and RNAs were purified by a modified guanidium chloride procedure (38). Northern blot analysis was conducted as described before (38) except that electrophoresis was performed in a formaldehyde-containing gel. For the detection of mouse L-PK-specific mRNAs, a rat cDNA clone complementary to the coding sequence (clone G4 [21]) was used at low stringency. For the detection of CAT transcripts, a CAT probe was used (an *Nco*I fragment from the pSV_2CAT vector). Specific bands were quantified by scanning the autoradiographs with a Shimadzu densitometer. For S1 nuclease protection, an antisense single-stranded probe was synthesized by extension of a specific oligonucleotide complementary to nt +64 to +78, located in the first intron. Nuclease S1-resistant hybrids were detected as described before (3, 7).

RESULTS

Southern blot analysis and generation of transgenic mice. Transgenic founder mice were identified by Southern blot analysis throughout this study. The autoradiograph presented in Fig. 2 shows all the different mouse lines (generally, three lines for each construct) analyzed so far with the PK/CAT constructs: lines 45, 63, and 74 for the −3200/CAT construct; lines 7, 11, and 17 for the 2000/−183 construct; lines 55, 89, and H10 for the 2000/−392 construct; lines 13

TABLE 1. CAT activity in livers and small intestines of transgenic mice[a]

Construct	Mouse line	Copy no.	CAT sp act, cpm/μmol of substrate/min/μg of protein (no. of mice)			
			Liver	Liver, corrected for copy no.	Small intestine	Small intestine, corrected for copy no.
−3200/CAT	74	12	18,900 (9)	1,575	612 (9)	51
	45	38	262,600 (6)	6,910	750 (5)	20
	63	4	7,300 (4)	1,825	1,336 (3)	330
2000/−392	H10	6	20,000 (4)	3,333	142 (6)	24
	55	1	56 (5)		—[b]	
	89	1	8 (5)		—	
2000/−183	7	2	242 (5)	120	680 (9)	340
	11	23	725 (7)	32	7,500 (7)	326
	17	3	850 (3)	283	526 (3)	175
1100/−392	13	4	147,100 (6)	36,800	146,900 (6)	36,700
	31	23	244,200 (5)	10,300	113,200 (6)	4,900
400/−392	29	115	6,580 (3)	57	5,200 (4)	45
	3	68	6,200 (5)	91	7,030 (5)	103
	5	265	13,500 (6)	51	960 (5)	4

[a] Homogenates of liver and small intestine from high-carbohydrate-refed animals were assayed for CAT activity as described in Materials and Methods. Each value represents the mean of independent assays on different mice, the number of which is given in parentheses. The CAT activity value was divided by the number of integrated copies in the line to obtain the "corrected for copy number" values.

[b] —, not detectable.

and 31 for the 1100/−392 construct; and lines 29, 3, and 5 for the 400/−392 construct.

On each Southern blot, a given number of transgene copies were run beside the DNA to be tested. This allowed us to conclude that all the transgenes had integrated head to tail (except for lines 55 and 89) and without evident rearrangement and made it possible to estimate the number of integrated copies for each line. Because hybrid genes are present as a tandem head-to-tail array, digestion with *Xho*I, which cuts the hybrids once, gave rise to multiple copies of a restriction fragment whose length is the same as that of the PK/CAT transgene. We indeed detected, with a CAT probe, fragments of 5.8, 5, 4.8, 4, and 3.4 kbp for the −3200/CAT, 2000/−183, 2000/−392, 1100/−392, and 400/−392 constructs, respectively. The copy number computation was checked by rehybridizing the Southern blot in Fig. 2 with an endogenous single-copy gene, the murine c-*myc* gene, which reveals a unique band of about 3.8 kbp (Fig. 2, bottom). Estimated copy numbers are given in Table 1.

It is noteworthy that lines 55 and 89 have integrated only one copy of the transgene (indeed, the length of the restriction fragment does not correspond to that of the transgene but rather to that of the integration site within the host DNA). This accounts for the weak signal of these lines and explains why the fragments do not appear on the short-exposure blot shown.

For L-PK minigene II, the same procedure was used to reveal positive founders, and two lines have been analyzed so far: line K40, with about 40 integrated copies, and line K2, with about 2 integrated copies.

For L-PK minigene I, we used the previously presented B49 and B83 lines, harboring about 40 integrated copies (36).

For each line (minigene II and PK/CAT constructs), the transgene was stably inherited through the germ line, and most of the transgenic mice transmitted the transgenes to their offspring in a Mendelian manner.

Influence of the distal region: analysis of expression from mice made transgenic with minigene II. Having previously established that a distal fragment of the PK 5′-flanking region (nt −2080 to −3200), encompassing liver-specific hypersensitive site HSS2, was able to activate the minimal L-PK promoter in a transient-transfection assay (6), we wanted to analyze in vivo the real significance of this distal region in conferring a high level of expression. To this end, we studied expression from two minigene constructs (Fig. 1A) containing either 3.2 kbp (minigene I) or only 1.5 kbp (minigene II) of 5′ PK sequences. Figure 3 represents an S1 nuclease protection analysis of the nuclear precursors of L-PK mRNAs in transgenic mice harboring about 40 copies of minigene I or 40 copies of minigene II. Minigene II is expressed about 70-fold less than minigene I in the liver (average of two individual experiments). However, neither tissue specificity nor response to diet and hormones was modified; expression was clearly detectable in the liver, very low in the kidney and small intestine, and undetectable in the brain. In the liver, carbohydrate induction and glucagon repression were easily observed. The low level of minigene II transcription was confirmed with another line of mice harboring only about two copies of the integrated transgene (line K2). In refed mice, the signal generated by transgene transcripts was similar with 40 μg of RNAs from these K2 mice or 1 μg of RNAs from the high-copy-number K40 line (3). Considering that several lines of mice harboring either the entire L-PK gene or minigenes or oncogenes under the control of the 3.2 kbp of the L-PK regulatory region expressed the transgene at a level related to the number of integrated copies (3, 4, 36), these results support the hypothesis that the deleted fragment in minigene II is fundamental for the normal in vivo activation of the L-PK gene.

CAT activity in various transgenic-mouse tissues. In order to locate more precisely the elements involved in tissue specificity, various tissues (expressing or not expressing the endogenous L-PK gene) from positive F_1 mice were examined for CAT activity. Figure 4 presents an arrangement of typical experiments. The upper part shows the results for the three lines (74, 45, and 63) corresponding to the −3200/CAT construct, and the lower part shows the results for one line of each deleted construct.

For lines 74 and 45, exogenous CAT activity is strictly restricted to the tissues expressing the L-PK gene in vivo. Expression is high in the liver, weaker in the small intestine, and barely detectable in the kidney (probably at the limit of

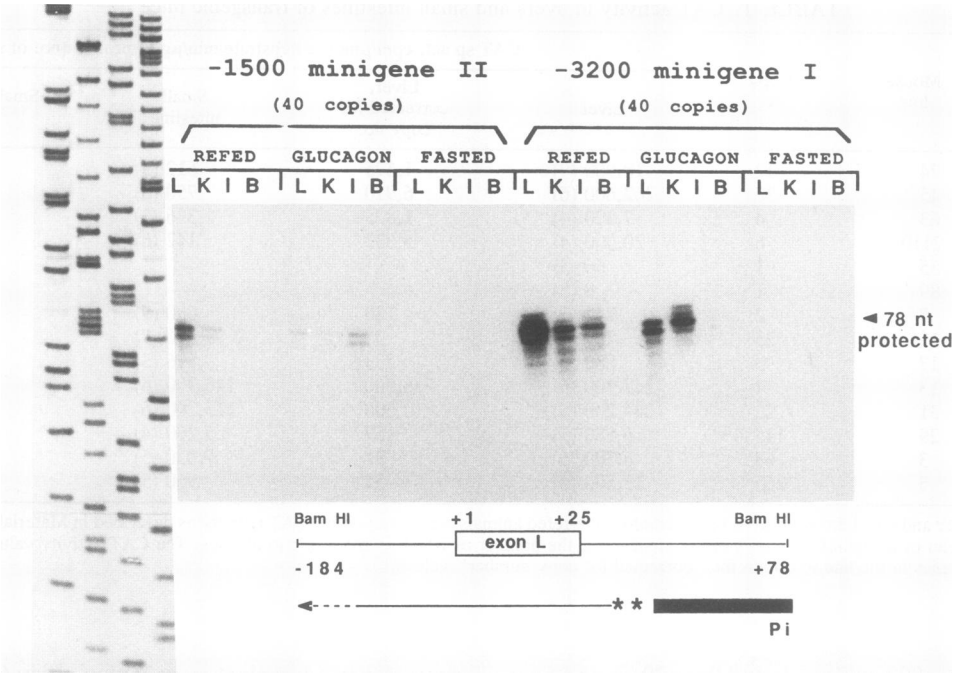

FIG. 3. S1 nuclease analysis of precursor minigene I and II transcripts in transgenic mice as a function of hormonal and nutritional conditions. For detection of the L-PK precursor RNAs, we synthesized a specific single-stranded probe (Pi) by extension of an oligonucleotide primer hybridizing with nt +64 to +78. The open rectangle symbolizes the position of the first L exon in the *Bam*HI-*Bam*HI template. The probe is represented by the arrow. The solid rectangle indicates the position of the primer, and stars show the labeled residues. The length of the protected fragment is 78 nt for precursor transcripts, as estimated by comparison with the sequence (ATCG reactions, right part). Total RNAs (40 μg) from the liver (L), kidney (K), intestine (I), or brain (B) of fasted, refed, or glucagon-treated animals made transgenic with minigene I (line B49) or minigene II (line K40) were tested.

the method's sensitivity). Line 63 behaves slightly differently, as expression is high in the three positive tissues. However, for the three lines, no significant CAT activity is detected in negative tissues such as brain, spleen, and muscle. For all the deleted constructs, tissue specificity is also maintained: CAT activity is detected mainly in the liver and small intestine, sometimes being even higher in the latter tissue (line 7, for example). Again, CAT activity either is not detected or is expressed at a very low level in all other tissues assayed. However, for line 5, there is a significant signal in muscle, but this signal was not reproducible with the other lines corresponding to the same construct. These results allowed us to confirm that the 3.2 kbp of 5′ PK sequences are involved in driving tissue-specific expression of the L-PK gene (36, 42) and to demonstrate that the smallest construct (400/−392) retains all the information needed to dictate this tissue specificity. As the distal sequence of 400 bp between bp −2500 and −2900 does not contain any information for tissue specificity (its deletion has no effect, as demonstrated with minigene II), tissue specificity can be ascribed to the proximal bp −392 sequences.

Differential expression of transgenes as measured in the liver and small intestine. The level of CAT activity was measured in the liver and small intestine of mice made transgenic with each construct (Table 1). The value shown is the mean of independent measurements obtained for several individual mice (the number of mice studied is shown). Several conclusions can be drawn from this table.

The three lines (74, 45, and 63) of the −3200/CAT construct express the transgene, although to a variable extent. However, when these values are divided by the corresponding copy number, the values are much more homogeneous, from 1,600 to 6,900 cpm in the liver and only 20 and 50 cpm in the small intestine for lines 45 and 74, respectively. Line 63 behaves differently, as expression is high in the small intestine but is still fourfold lower than in the liver.

For the next construct, 2000/−392, lines 55 and 89 express the transgene at a very low level in the liver. As these two lines contain only one copy of the integrated transgene, whose extremities could therefore be rearranged, we preferred not to take the values for these two lines into account. However, line H10 showed a level of CAT activity (about 3,300 cpm in the liver and 24 cpm in the small intestine) directly comparable to that observed with the preceding −3200/CAT construct. This first result supports the hypothesis that the sequences located between bp −392 and −1100, which do not seem to play any role ex vivo in transfected hepatocytes and hepatoma cells (2, 6), do not contain any important signal for the strength of the promoter in vivo in the liver and small intestine.

For the three lines (7, 11, and 17) corresponding to the 2000/−183 construct, CAT activity in the liver is reduced to between 32 and 233 cpm, compared with the range of 1,575 to 6,910 cpm in the four lines harboring the −3200/CAT and 2000/−392 constructs. In contrast, the level of CAT activity is 4- to 17-fold higher in the small intestine of these lines than in that of lines 74, 45, and H10. Although line 63 (3200/CAT) also exhibits high CAT activity in the small intestine, it is noteworthy that all three lines harboring the 2000/−183 construct have a CAT activity in small intestine that is higher than or approximately equal to that in the liver.

These results suggest that the sequence between bp −183

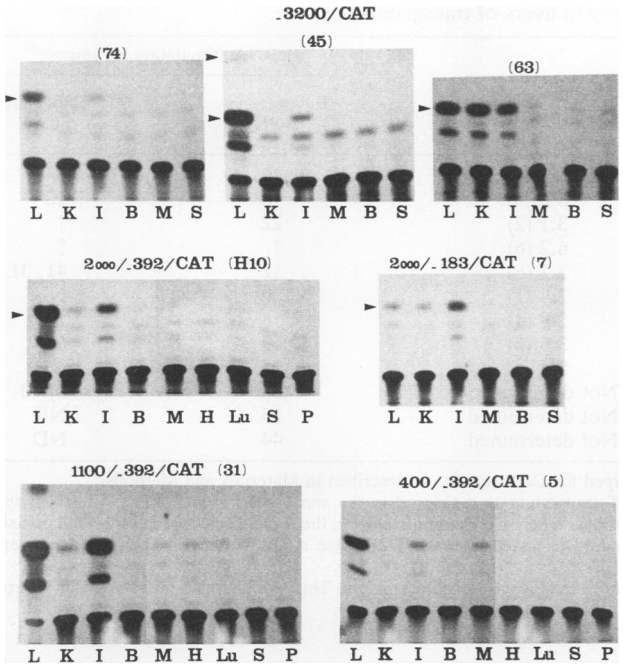

FIG. 4. Tissue-specific expression of L-PK/CAT constructs in transgenic mice. Samples from homogenates of the indicated organs were assayed for CAT activity. One thousand micrograms of proteins was used (except for line 45, 200 μg of proteins). The line number and the corresponding construct are indicated above each autoradiograph. The arrows indicate the radiolabeled acetylated chloramphenicol derivatives (CM-AC1 and CD-AC1,3) produced in CAT assays. L, liver; I, small intestine; K, kidney; B, brain; M, muscle; H, heart; Lu, lung; S, spleen; P, pancreas.

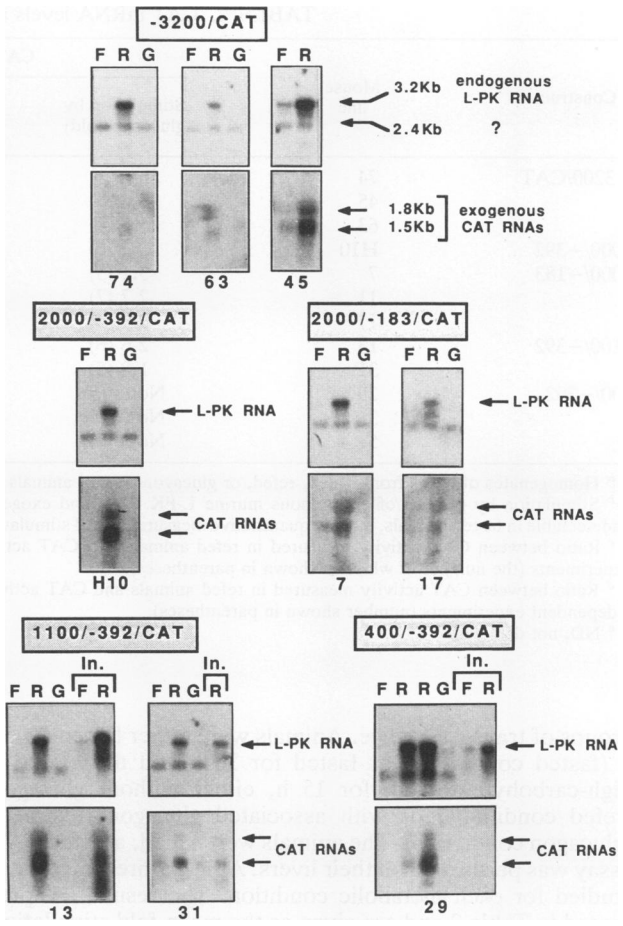

FIG. 5. Northern blot analysis of L-PK and CAT transcripts in livers of transgenic mice as a function of hormonal and nutritional conditions. RNA samples (10 μg) from the liver or small intestine (In) were separated by electrophoresis, transferred onto a Hybond N^+ membrane, and hybridized with the indicated probes. Mouse lines are indicated beneath the panels. F, fasted conditions; R, refed conditions; G, glucagon conditions (see Materials and Methods). With the L-PK probe, in addition to the 3.2-kb mRNA specific for mouse L-PK, we detected an mRNA species of 2.4 kb, corresponding to an unidentified messenger, the amount of which is not modified by nutritional and hormonal status.

and −392 plays a role in modulating tissue-specific L-PK gene expression; it seems to activate expression in the liver and repress it in the small intestine. Thus, its deletion in lines 7, 11, and 17 would be the cause of the low CAT activity in the liver and relatively high activity in the small intestine.

When the distal fragment (previously of 2,000 bp between bp −1170 and −3200 in the 2000/−392 construct) is reduced to 1,100 bp (between bp −2080 and −3193) and linked upstream of bp −392 of the promoter (construct 1100/−392), CAT activity in the liver is enhanced (10,300 and 36,800 cpm in lines 13 and 31, respectively, versus 1,575 to 6,920 cpm in lines 74, 45, 63, and H10). Thus, we not only recover the activation expected from the presence of the fragment from bp −183 to −392 but also observe a further activation that appears to be much more important in the small intestine than in the liver. Indeed, CAT activity in the small intestine is about 15- to 1,800-fold higher in lines 13 and 31 than in lines 74, 45, 63, and H10. These data suggest the presence of an inhibitory element, between bp −1170 and −2080, whose deletion would permit very strong expression in the small intestine and moderated activation in the liver. This deinhibition is observed only in these two tissues, as no ectopic reexpression was observed in the other tissues tested (Fig. 4).

The last construct studied, 400/−392, contains a shortened distal fragment of 400 bp (from bp −2500 to −2900) instead of the 1,100 bp of the previous construct. In this case, the CAT level is very low in the liver and small intestine of lines 29, 3, and 5. This could be the result of the high copy number of this construct integrated in each line, titrating some limiting factor important for expression of the L-PK gene. Alternatively, this low level of expression could reflect the disappearance of an important activating sequence present in the complete 1,100-bp fragment and lost in the 400 bp left in this construct. We strongly favor the second hypothesis for two main reasons. First, if the elevated copy number was the reason for the low level of expression, expression of the endogenous gene would also be altered. This is, in fact, not the case, as shown by the Northern blot analysis (Fig. 5). Second, the hypothesis that an activating sequence is located in this region has been proven by studying the expression of minigene II.

Hormonal regulation of the PK/CAT genes in transgenic mice. Expression of the PK gene is regulated positively by insulin and glucose and negatively by glucagon in the liver. To define the minimal sequences of the 5′ PK region needed for this specific dietary and hormonal responsiveness, we performed a series of metabolic analyses on the five different

TABLE 2. CAT mRNA levels and activity in livers of transgenic mice[a]

Construct	Mouse line	CAT activity		mRNA stimulation by glucose[b]	
		Stimulation by glucose[c] (fold)	Repression by glucagon[d] (fold)	Endogenous L-PK	Exogenous CAT
−3200/CAT	74	56 (5)	52 (5)	44	↑
	45	5.4 (5)	8 (2)	8	10
	63	5.6 (4)	3.1 (2)	22	↑
2000/−392	H10	3.2 (6)	6.2 (6)	↑	↑
2000/−183	7	3.1 (5)	3.1 (5)	12	41, 31
	11	2.7 (7)	2.7 (7)	100	ND[e]
	17	2.9 (4)	2.9 (4)	56	↑
1100/−392	13	2.6 (6)	3.6 (6)	67	65
	31	1.8 (5)	3.4 (4)	25	6
400/−392	29	None (5)	Not determined	68	5, 40
	3	None (5)	Not determined	15	ND
	5	None (6)	Not determined	44	ND

[a] Homogenates of livers from fasted, refed, or glucagon-treated animals were assayed for CAT activity as described in Materials and Methods.
[b] Stimulation by glucose of endogenous murine L-PK RNA and exogenous CAT transcripts as measured in the same refed animal. ↑, CAT RNA was undetectable in fasted animals, making quantitative measurement of stimulation impossible. When two values are shown, the second is for a different refed mouse.
[c] Ratio between CAT activity measured in refed animals and CAT activity measured in fasted animals. This value is an average of several independent experiments (the number of which is shown in parentheses).
[d] Ratio between CAT activity measured in refed animals and CAT activity measured in glucagon-treated animals. This value is also an average of several independent experiments (number shown in parentheses).
[e] ND, not detected.

groups of transgenic mice. Animals were either fasted for 51 h (fasted conditions) or fasted for 36 h and then refed a high-carbohydrate diet for 15 h, either without glucagon (refed conditions) or with associated glucagon treatment (glucagon conditions). The animals were killed, and the CAT assay was performed on their livers. At least three mice were studied for each metabolic condition. The results are presented in Table 2 and are given as the mean fold stimulation by glucose (ratio between CAT activity in livers from refed animals and fasted animals) and as the mean fold repression by glucagon (ratio between CAT activity in livers from refed animals and glucagon-treated animals). With the lines corresponding to the four first PK/CAT constructs, we noticed a significant and reproducible, although variable, effect of glucose refeeding and glucagon treatment: there is at least a twofold stimulation by glucose and a glucagon repression of about the same magnitude. For the three lines made transgenic with construct 400/−392, however, the level of CAT activity was identical in fasted and in refed animals.

Since CAT activity is only a reflection of CAT RNA abundance and gene transcription and also depends on the stability of the CAT enzyme, we decided to compare directly the abundance of CAT transcripts in the livers of the animals in the three groups for all mouse lines. This was done by Northern blot analysis, as shown in Fig. 5. The quantitated results (quantification of the intensity of specific bands, either endogenous L-PK transcripts or exogenous CAT transcripts) appear in Table 2; the fold stimulation by glucose is given for endogenous L-PK RNA and for exogenous CAT RNA.

As visualized on the Northern blot (Fig. 5), the amount of endogenous L-PK RNA varied, as expected, according to the metabolic status: it was not detectable or at a very low concentration during fasting and glucagon treatment and induced by glucose refeeding. This is true whatever transgene was integrated in the line and whatever its copy number, suggesting the absence of interference between endogenous and exogenous gene expression. The endogenous mouse gene is induced, on average, between 8- and 100-fold (depending on the refed animal; Table 2), which is in good agreement with the known induction in rat (38). As shown in the Northern blot (Fig. 5), CAT transcripts were also very well induced in refed animals. It appears, in fact, as two species of 1.5 and 1.8 kb, as already observed by other groups (20, 42). In contrast to the low stimulation of CAT activity by glucose, the stimulatory effect of glucose on CAT RNA is directly comparable to that observed for the endogenous gene. The fold stimulation is sometimes not even computable, since no CAT RNA was detectable on the blot for fasted animals. This discrepancy between the fold stimulation measured either by protein activity or by level of specific RNA could be explained if the half-life of the CAT protein is very long (longer than the 2 days of fasting).

However, what is important here is that induction by glucose and repression by glucagon (Fig. 5) are maintained in all lines. Even for line 29 (400/−392 construct), in which glucose-dependent CAT activity induction was not detectable, we measured a 5- and 40-fold stimulation in two different refed animals (and a strong inhibition in glucagon-treated animals; Fig. 5).

The influence of glucose refeeding and glucagon treatment in the small intestine was also measured. The hormonal response was conserved throughout the lines tested (not shown, except for line 13 on the Northern blot analysis, [Fig. 5]). For line 29, no CAT transcripts were detectable in the small intestine.

This study of CAT expression at the level of RNA transcripts allows us to conclude definitely that the sequences involved in positive and negative control of L-PK gene expression are present even in the smallest construct (400/−392).

DISCUSSION

Transgenic mice are an experimental tool that has proved very important in the study of gene regulation in a physiological context and that is, at present, the most rigorous system for characterizing regulatory DNA elements. Fur-

thermore, this method allows a comparison of the regulatory mechanisms characterized in vitro or in cultured cells with those operating in vivo. We previously described various transgenic mouse lines carrying either the entire rat L-PK gene (or a minigene; see Fig. 1) or the 3.2 kb of L-PK 5′-flanking region linked to reporter genes (4, 36). For each line, the transgene was tissue-specifically expressed (expression from the rat transgene promoter was strong in the liver and weaker in the small intestine) and properly controlled upon hormonal and nutritional adaptation (expression was decreased by fasting or by glucagon treatment and strongly stimulated by refeeding of a carbohydrate-rich diet). Moreover, we noticed that the level of expression of the transgenes was in good correlation with the number of copies integrated and seemed to be independent of the integration site, which suggests that the injected L-PK constructs contained control elements with a locus control region (LCR) effect (13).

In an attempt to define more precisely the regulatory sequences involved in the tissue-specific and hormone-regulated pattern of expression of the L-PK gene, we have analyzed expression from various deleted transgenes, either L-PK minigenes or L-PK/CAT hybrid genes, in detail.

With the procaryotic CAT reporter gene, the level of expression of the same transgene in different lines exhibited a variability that was imperfectly corrected after normalization to the integrated copy number. In fact, the copy number dependence of expression was much less evident with the CAT gene than, as reported previously (3, 4, 36), with other eucaryotic reporter sequences. Nevertheless, Table 1 shows that, whether normalized to the copy number or not, the different constructs can generally be distinguished by their pattern and level of expression. Because of the previously reported copy dependence conferred by the L-PK regulatory regions (3, 4, 36), and in spite of the persistent variability observed for the CAT constructs, it seemed to us more logical to reason mainly on normalized values.

In this article, we provide evidence that the proximal bp −183 fragment of the L-PK gene promoter was sufficient, in vivo, for transcriptional regulation of the L-PK gene by tissue-specific factors, diet, and hormones. This was deduced from the following data. (i) Expression from minigene II (with only 1.5 kbp of 5′-flanking sequences) is tissue-specifically and hormonally regulated. (ii) Within those 1.5 kbp, only the first 400 bp are essential, as judged from expression from the 2000/−392 and 1100/−392 constructs. (iii) Finally, within those 400 bp, only the first 183 bp are necessary, as judged from the results obtained with the 2000/−183 construct. Furthermore, we have demonstrated that the activity from this proximal promoter can be modulated, negatively and positively, by upstream sequences in a tissue-specific manner.

Only proximal sequences are needed for tissue-specific L-PK gene expression. In vivo, efficient tissue-specific transcription could be directed with −183 bp of the proximal L-PK promoter. Whatever line is studied, CAT activity is detected in the two main L-PK-expressing tissues, although at different levels according to the lines, and is very low or undetectable in tissues that do not express the endogenous L-PK gene.

The implication of such proximal sequences in correct tissue specificity in vivo has been described for several genes in various tissues: −191 bp for α sk actin gene expression in striated muscle (28); −208 bp for α-amylase gene expression in the pancreas (16); −323 bp for phosphoglycerate kinase 2 gene expression in testis (32); and many others. For other genes, such as the prolactin (8), major urinary protein (34), and albumin (30) genes, a clear cooperation between proximal and distal enhancer sequences has been described to ensure a strict cell type specificity. For the L-PK gene, we have no evidence for such cooperation between the distal activating element and the proximal region, since, although the expression is low without the distal activating element, it is always restricted to the appropriate tissues.

Finally, the pattern of expression observed for the various deleted L-PK transgenes in different tissues allowed us to conclude that the absence of L-PK expression in negative tissues is not due to the presence of a strong extinguisher in the 3.2 kbp of 5′-flanking region.

Together, these results confirm our previous data obtained in vitro in a cell-free transcription system (39) and ex vivo in transient CAT expression assays (6). From studies with these systems, it was suggested that the HNF1 binding site would be essential and sufficient to confer tissue specificity. This transcriptional factor has recently been implicated in vivo in the case of the α_1-antitrypsin gene, in which a mutation in the HNF1 binding site (referred to as LFB1) has been shown to drastically reduce transcription from the transgene in adult mouse liver (37).

Upstream sequences are involved in the modulation of L-PK gene expression in liver and small intestine. The comparison of expression from the various 5′-deleted transgenes has permitted some specific properties to be ascribed to distal sequences.

(i) The sequence between bp −183 and −392 seems to be involved in L-PK gene expression, positively in the liver and negatively in the small intestine. Ex vivo, the region from bp −183 to −283 (with effects similar to the region between bp −183 and −392) has also proved to behave as a positive or negative regulatory element according to cell context and transfection method; with electroporation, it is a repressor in hepatocytes in primary culture; with calcium phosphate coprecipitation, it has no significant effect in hepatocytes but is an activator in hepatoma HepG2 cells (2, 6, 41).

The only DNA-binding protein we were able to detect in this region is a protein termed L5BF (31), between bp −248 and −275, whose identity is still unknown. It is interesting that this L5BF is present in both the liver and small intestine but with different electrophoretic mobilities, as judged by gel retardation. When linked to a heterologous thymidine kinase promoter, the L5 box is able to inhibit transcription of a reporter gene in vitro in the presence of hepatic nuclear extract (31). Recently, such a dual DNA regulatory element has been described for the apolipoprotein AII gene (5). In this case, as for the L-PK gene, a 5′ internal deletion of the region from bp −230 to −614 decreased hepatic transcription while it increased intestinal transcription from constructs transfected into hepatic or intestinal cells, respectively.

(ii) Sequences located between bp −1170 and −2080 seem to have a negative influence in the liver and a much greater negative influence in the small intestine. This extinguishing effect was previously described for hepatoma cells but was not found in hepatocytes in primary culture (6). Since deletion of this negative regulatory region does not result in reexpression of the L-PK gene in tissues that do not synthesize L-PK, we can propose that the role of this element is to regulate transcription in tissues that are able to activate the tissue-specific proximal promoter, i.e., containing HNF1 and HNF4 transactivators (22, 33). In this sense, this negative region might cooperate with element L5 in modulating L-PK gene expression at different stages of differentiation of different tissues—liver, kidney, small intestine, and, per-

haps, pancreas (4, 23)—while the main mechanism leading to the absence of L-PK gene expression in other tissues could be the absence of HNF1 and HNF4.

Negative sequences have now been described for several genes in a number of different cell types in tissue culture (17, 18), but only a few examples in vivo have been described. They are thought to act on gene expression in a manner similar to the activating elements (19).

(iii) Upstream sequences between bp −2080 and −3200 behave, in contrast, as activating sequences. This is deduced from the very low expression detected from the minigene II and 400/−392 constructs. Although they have a very limited activating effect compared with that observed in vivo, these sequences were first shown to enhance transcription of the CAT gene in transfected hepatoma cells (6). Furthermore, in these cells, the smallest fragment of 400 bp (between bp −2500 and −2900) was able to reproduce the full activating effect of the largest fragment. This was not true in vivo, as we have found that this distal 400-bp fragment, corresponding exactly to an Alu-like sequence (7), is not able to reproduce the effect of the region from bp −2080 to −3200 in activating the bp −392 promoter fragment. Similar discrepancies between in vivo and ex vivo appreciation of the functional influence of *cis*-acting DNA elements have been reported in the literature for several other genes (10, 15). The distal activator in the sequences between bp 2080 and 3200 corresponds to a liver-specific DNase I-hypersensitive site (3) but seems to be very different in nature from the enhancers associated with the hypersensitive sites found in other liver-specific genes, e.g., the albumin and α-fetoprotein genes (11, 30). While deletion of these distal enhancers precludes tissue-specific transgene expression (15, 30), the results obtained with minigene II show that the distal activator of the L-PK gene is not necessary to tissue-specific expression and correct regulation by diet and hormones, but is required to confer a quantitatively correct level of expression of the transgene.

Finally, this distal element does not seem to be responsible for the LCR activity of the L-PK regulatory region, since the two lines harboring minigene II conserve a direct relationship between the number of integrated copies and the level of expression (3). In brief, this activator element activates the transcriptional complex built up on the proximal promoter but is not indispensable to its assembly during development and to opening of the chromatin structure.

Hormonal response elements are located in the proximal −183 bp of the L-PK promoter. While the positive regulation of gene expression by glucagon and cyclic AMP is beginning to be very well documented (14), nothing is known about the negative influence of these agents on gene expression. Similarly, the mechanism by which insulin and carbohydrates act on gene expression remains obscure (25). From this point of view, the L-PK gene is the model of choice, since its expression is modulated by these nutrients and hormones.

Our data demonstrate that the hormone- and diet-responsive elements are located within the very proximal L-PK promoter between bp −183 and +11.

Very recently, we have demonstrated, by electroporation of hepatocytes in primary cultures, that within this bp −183-proximal fragment, the L4 box (nt −168 to −144), a degenerated low-affinity binding site for MLTF, is the positive glucose and insulin response element and is also an element required for inhibition by cyclic AMP, both effects requiring close cooperation with box L3 (2). This L-PK glucose-insulin response element is significantly different from those described so far in the literature (1, 16, 24, 26, 29). These elements have usually been identified through transient- (1, 24, 29) or stable-transfection experiments in cultured cells (26), except for the insulin-dependent element of the α-amylase gene, initially discovered in transgenic mice (16).

In conclusion, analyzing the *cis*-acting DNA elements of the L-PK gene regulatory region in transgenic mice has allowed us to propose the model of a tissue-specific and hormone- and diet-responsive proximal promoter, whose exact level of expression is tissue-specifically modulated by the interplay of negative and positive upstream elements. These upstream modulator elements might be implicated in the control of L-PK gene expression in tissues that are able to activate the proximal promoter.

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