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Differential Regulation of the Rat Phosphoenolpyruvate Carboxykinase Gene Expression in Several Tissues of Transgenic Mice

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The selective expression of a unique copy gene in several mammalian tissues has been approached by studying the regulatory sequences needed to control expression of the rat phosphoenolpyruvate carboxykinase (PEPCK) gene in transgenic mice. A transgene containing the entire PEPCK gene, including 2.2 kb of the 5′-flanking region and 0.5 kb of the 3′-flanking region, exhibits tissue-specific expression in the liver, kidney, and adipose tissue, as well as the hormonal and developmental regulation inherent to endogenous gene expression. Deletions of the 5′-flanking region of the gene have shown the need for sequences downstream of position −540 of the PEPCK gene for expression in the liver and sequences downstream of position −362 for expression in the kidney. Additional sequences upstream of position −540 (up to −2200) are required for expression in adipose tissue. In addition, the region containing the glucocorticoid-responsive elements of the gene used by the kidney was identified. This same sequence was found to be needed specifically for developmental regulation of gene expression in the kidney and, together with upstream sequences, in the intestine. The apparently distinct sequence requirements in the various tissues indicate that the tissues use different mechanisms for expression of the same gene.

In multicellular organisms, each tissue exhibits a distinct phenotype, corresponding to its specialized function. This is a consequence of differential expression of tissue-specific genes. However, there are instances when different tissues share a specialized phenotype. One such example is gluconeogenesis. This pathway is selectively used by the liver and kidney cortex, as a result of the expression of three genes coding for the gluconeogenic enzymes in these two tissues (34). Since the liver arises from the endoderm and the kidney cortex arises from the intermediate mesoderm, and gluconeogenesis initiates long after these two tissues have traversed through independent tracks of differentiation, it is puzzling how the two tissues share the same gluconeogenic phenotype.

We have asked whether the same or different mechanisms govern the phenotype shared by these two disparate tissues by studying the mechanisms that regulate expression of the gene encoding the rat cytosolic phosphoenolpyruvate carboxykinase (EC 4.1.1.32) (PEPCK). PEPCK catalyzes the first and rate-limiting step of gluconeogenesis. The PEPCK gene is selectively expressed in the gluconeogenic liver and kidney cortex and in glyceroneogenic adipose tissue (4, 15, 34). PEPCK is encoded by a unique copy gene, whose transcription in all three PEPCK-expressing tissues is initiated from the same promoter (3, 25). Thus, it serves as an excellent example of a phenotype shared by three tissues.

In addition to tissue-specific expression, previous studies have shown that PEPCK gene transcription is regulated by multiple hormones, in accordance with the identification of distinct hormonal responsive elements in the 5′-flanking region of the gene (15, 20, 30). We have shown that glucocorticoids, which stimulate gene transcription in the liver and kidney (20), repress transcription of the gene in adipose tissue (25). Thus, hormonal control of PEPCK gene transcription is tissue specific. The PEPCK gene begins to be expressed in the kidney several days before birth and in the liver at birth (4, 15, 21, 34). Therefore, the developmental onset of this expression is also tissue specific. Together, these features constitute a hierarchy of mechanisms that differentially regulate PEPCK gene expression in the three tissues.

The first indication that different mechanisms might regulate PEPCK gene expression in the three tissues emerged from experiments in cultured cell lines. Using transient expression assays of chimeric PEPCK constructs in the PEPCK-expressing cell lines of hepatocytes, adipocytes (6), and kidney cells (33), we have shown that each cell line needs a different minimal sequence of the PEPCK promoter for its specific expression.

Transgenic mice provide a suitable system to analyze the organization of the regulatory regions needed for the complex regulatory features that govern PEPCK gene expression. McGrane et al. (19) have shown that a chimeric gene containing up to 2,000 bp of the 5′-flanking region of the rat PEPCK gene fused to the bovine growth hormone gene was expressed at a high level in the liver and poorly in the kidney. Since growth hormone on its own exhibits multiple metabolic effects, its unrestrained production in transgenic mice has various deleterious consequences (2, 10, 27). Therefore, we have chosen an alternative approach using as the transgene the entire rat PEPCK gene, including considerable flanking regions, to enable the analysis of *cis*-regulatory regions involved in the multiple control features of gene expression.

Our experiments in transgenic mice have now defined the

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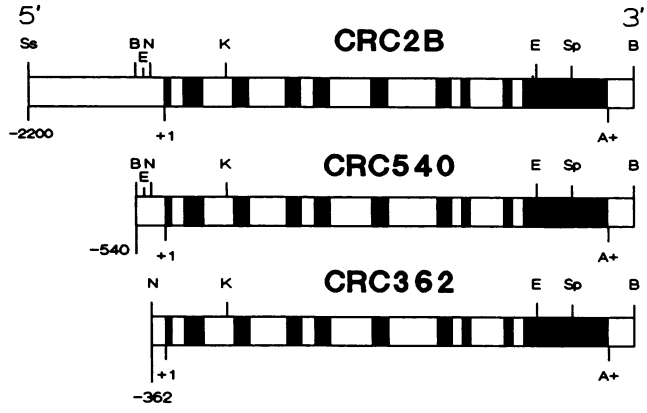

FIG. 1. Schematic representation of the chimeric gene constructs. The linearized *CRC2B*, *CRC540*, and *CRC362* gene constructs are represented as follows: black boxes indicate exons, stippled region indicates the chicken PEPCK cDNA-derived insert, and open boxes indicate introns and flanking regions. Abbreviations: E, *Eco*RI; Sp, *Sph*I; B, *Bam*HI; K, *Kpn*I; Ss, *Sst*I; N, *Nde*I; +1, transcription start site; A+, polyadenylation signal.

minimal size of 5'-flanking sequences of the PEPCK gene needed for expression of this gene in the kidney and adipose tissue and suggest the minimal sequence required by the liver. The distinct sequence requirements by each of the three PEPCK-expressing tissues support the conclusion that these tissues use different mechanisms for expression of this gene.

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MATERIALS AND METHODS

Gene constructs. The gene constructs used are schematically represented in Fig. 1. The *CRC2B* gene consists of the entire rat PEPCK gene and flanking regions and contains a small replacement fragment from the chicken PEPCK cDNA in the last exon as a marker. To achieve this substitution, we first cloned a 1.4-kb *Eco*RI-*Bam*HI fragment containing 0.9 kb of the 3' end of the rat PEPCK gene through 0.5 kb of its 3'-flanking region into the appropriate cloning sites of pUC13. Digestion of this resulting plasmid with *Eco*RI and *Sph*I deleted a 465-bp fragment in the last exon of the PEPCK gene, into which the 485-bp *Eco*RI-*Sph*I nonhomologous counterpart fragment from the chicken PEPCK cDNA (16) was inserted. Subsequently, a 5.4-kb *Eco*RI fragment containing the entire upstream portion of the rat gene was inserted into the *Eco*RI site and the correct orientation was selected, giving rise to a chimeric rat-chicken PEPCK gene construct, *RC$_3$18*. However, this construct contained only 460 bp of the 5'-flanking region of the rat PEPCK gene. To further enlarge the 5'-flanking region of the *RC$_3$18* plasmid, we subcloned a 5.5-kb *Bam*HI-*Kpn*I fragment containing the 3' portion of the chimeric gene and its flanking region into the appropriate sites of the vector plasmid pBS (Stratagene), giving rise to *CRC1B*. The *Kpn*I site resides within the second intron of the rat PEPCK gene at position +876 with respect to the transcription start site (3). Finally, a 3.0-kb *Sst*I-*Kpn*I fragment obtained from a genomic clone of the rat PEPCK gene (5), spanning positions +876 to about −2200 of the rat PEPCK gene, was inserted into the appropriate sites of CRC1B to generate the rat-chicken PEPCK gene construct *CRC2B* (Fig. 1).

The deletion mutant construct containing 540 bp of the 5'-flanking region of the *CRC2B* gene was generated by *Bam*HI digestion of the *CRC2B* construct, which gave rise to the appropriate 7-kb deletion mutant construct *CRC540*. The deletion mutant construct containing 362 bp of the 5'-flanking region of the *CRC2B* gene was achieved as follows. A 1,012-kb fragment spanning the *Nde*I site at position −362 to the *Hin*dIII site at position +640 was excised from the rat PEPCK gene. The *Nde*I site was filled in, and the fragment was subcloned into the *Hin*cII and *Hin*dIII sites of the pBS vector (Stratagene). The unique *Hin*dIII site of this plasmid served to insert a 5.5-kb *Hin*dIII fragment containing the 3' portion and flanking region of the *CRC2B* gene which, after selecting for the correct orientation, gave rise to the deletion construct *CRC362*.

Probes used for hybridization analysis. (i) PCK-10. PCK-10 is the rat PEPCK cDNA in pBR322 (37). The cDNA insert was excised from the vector by *Pst*I digestion, yielding two fragments. The 5' 1.6-kb DNA fragment was used to probe endogenous mouse and transgene DNAs, yielding different size fragments upon genomic DNA digestion with *Bam*HI or *Eco*RI. *Bam*HI generates a 9-kb mouse PEPCK gene fragment and 7 kb of the transgene. *Eco*RI generates 11 kb of mouse PEPCK gene fragment and 5.4 kb of the transgene. The 465-bp *Eco*RI-*Sph*I sequence from the 3' 1.0-kb fragment of the rat PEPCK cDNA was used to probe the endogenous PEPCK transcripts.

(ii) *Hin*d 1.2. *Hin*d 1.2 is the 1.2-kb *Hin*dIII fragment of the *CRC2B* gene used to probe the *CRC540* transgene.

(iii) c-485. c-485 is the 485-bp *Eco*RI-*Sph*I chicken PEPCK cDNA (9, 16) fragment. It was used to probe the transgene transcripts.

(iv) Mouse actin. Mouse actin is a 1.15-kb *Pst*I fragment from the mouse β-actin cDNA (24), and rat L7 is composed of two *Pst*I fragments (428 and 408 bp) from the rat ribosomal L7 cDNA (18). These cDNAs were used as control probes for RNA blots, where indicated.

Microinjection of DNA into mouse fertilized eggs and breeding of progenies. Transgenic mice were produced by using linearized *CRC2B*, *CRC540*, and *CRC362* genes from which the vector sequences were completely removed. This was accomplished by excising the genes from the pBS vector using the unique restriction enzymes on both sides: digestion of the *CRC2B* construct by *Sal*I and *Sst*I generated *CRC2B*, and digestion with *Bam*HI generated *CRC540*. The *CRC362* construct was digested by *Bam*HI. Fertilized eggs were flushed from the oviducts of (C57BL × BALB/c)F_1 females mated with (C57BL/6J × DBA)F_1 males. Manipulation of mice and eggs and the microinjection techniques were done as previously described (11, 32). Founder (F_0) transgenic mice were bred back to C57BL/6J control mice to produce positive F_1 generation progenies. The F_1 male and female littermates were inbred to give F_2 generation progenies, some of which were homozygous for the transgene used. Inbreeding the F_2 homozygote with F_2 heterozygote littermates resulted in the F_3 generation progenies, all of which were positive for the respective transgene. F_1 generations were maintained by outbreeding the positive F_1 generation of the transgenic mice with C57BL/6J control mice.

Transfection of the *CRC2B* gene into mouse L cells. Ten micrograms of *CRC2B* DNA was cotransfected with 5 μg of the selection marker gene herpes virus thymidine kinase (TK) to TK^- mouse L cells by using the DEAE-dextran method (13). Mass cultures of stable transfectants (about 100

colonies) were grown in medium containing hypoxanthine-aminopterine-thymidine.

Glucocorticoid treatment. Male F_2 progenies of transgenic mice were injected intraperitoneally with dexamethasone (1.25 mg/100 g of body weight) and sacrificed 5 h later.

Preparation and analysis of genomic DNA. DNA was extracted from 2-cm-long tails of 4- to 6-week-old mice and from fetal hind limbs by digesting the tissues in 1 ml of a solution containing 0.1 M EDTA, 10 mM NaCl, 10 mM Tris-HCl (pH 8.0), and 1% sodium dodecyl sulfate (SDS) containing 200 μg of proteinase K and incubated at 55°C with a shaker for 12 h. Digestion was terminated by phenol-chloroform extraction and treatment with chloroform, and the DNA was precipitated with a solution containing 2.5 M NH_4 acetate and 2.5 volumes of 100% isopropanol. After the DNA was spooled and washed with 70% ethanol, it was dissolved in 10 mM Tris-HCl (pH 8.0)–1 mM EDTA. DNA samples were digested with *Bam*HI or *Eco*RI, electrophoresed on 0.7% agarose gels, transferred to Nytran membranes, and hybridized at 67°C by the method of Schuler et al. (31) with a nick-translated (28) ^{32}P-labeled probe. The filters were washed as described previously (31) and exposed for autoradiography.

RNA extraction and analysis. Total RNA from cells and tissues was extracted with guanidinium thiocyanate and centrifuged through a CsCl cushion by the method of Chirgwin et al. (7). Slot blot and Northern (RNA) blot analysis of the RNA, using 50 μg of RNA per lane, were done by the method of Meyuhas et al. (23) with Nytran instead of nitrocellulose. After washes, the filters were exposed to X-ray films for autoradiography.

RESULTS

Characterization of the rat-chicken PEPCK gene construct (*CRC2B*). To study the multiple regulatory features of the PEPCK gene, we introduced into fertilized mouse eggs the entire rat PEPCK gene, including 2.2 kb of the 5′-flanking region and 0.5 kb of the 3′-flanking region. Because of the high degree of homology between the rat and mouse PEPCK transcripts (8), it was necessary to substitute a 465-bp fragment in the last exon of the gene with the corresponding nonhomologous 485-bp fragment of the chicken PEPCK gene, to serve as a probe for the expression of the chimeric construct. To assess the competence of this chimeric construct (*CRC2B* gene), it was stably transfected into mouse L cells. Northern blot hybridization analysis of total RNA from the transfected cells readily detected *CRC2B* transcripts when probed with the 485-bp chicken DNA fragment but not when probed with the counterpart rat sequence (Fig. 2B). This established the specificity of the chicken-derived probe to detect *CRC2B* transcripts.

Tissue specificity of the *CRC2B* transgene expression in transgenic mice. The *CRC2B* construct was microinjected into mouse fertilized eggs, producing two male founder lines (F_0; M7 and M8, carrying from two to four copies of the transgene per haploid genome). After the F_0 males were outbred to generate independent *CRC2B* lines, the expression of the transgene in different tissues was determined by Northern blot hybridization. The analysis revealed that the transgene was specifically expressed in the liver, kidney, and adipose tissue of both mouse lines, similar to the expression of the endogenous PEPCK gene (results of one of the two lines is shown in Fig. 2). Longer exposure of the autoradiographs revealed a low level of transcript expression in the intestine (estimated to be about 2 orders of magnitude lower

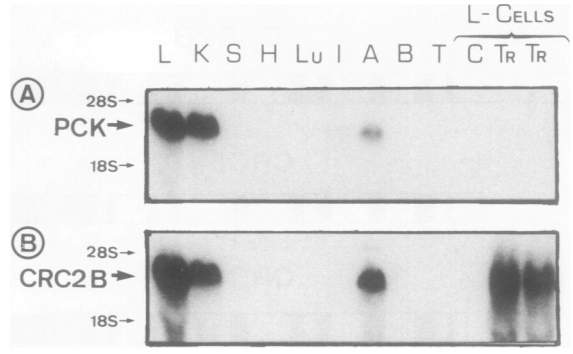

FIG. 2. Tissue-specific expression of the *CRC2B* transgene. Northern blot analysis of total RNA (50 μg/lane) from tissues of transgenic mouse line M8F1. The tissues analyzed were liver (L), kidney (K), spleen (S), heart (H), lung (Lu), intestine (I), adipose tissue (A), testis (T), and from *CRC2B*-transfected (Tr) or nontransfected (C) L cells. The same Northern blot was first probed with a fragment from the 3′ region of the rat PEPCK cDNA, which detected only the endogenous PEPCK transcripts (A), and subsequently probed with the 485-bp *Eco*RI-*Sph*I fragment that contains the chicken PEPCK cDNA (c-485) (B).

than in the liver, kidney, and adipose tissue). An even lower level of transcripts was detected in the lung (data not shown). No transcripts could be detected in the spleen, heart, brain, and testis even after longer exposures. The correct tissue-specific expression of the *CRC2B* gene in the two independent transgenic lines indicates that the sequences included in this construct contain sufficient information to accurately mimic the pattern of expression of the endogenous gene in the appropriate tissues.

The 5′-flanking sequences of the PEPCK gene required for tissue-specific expression. To define the minimal sequence of the 5′-flanking region of the PEPCK gene needed for tissue-specific expression, deletion mutations of the *CRC2B* gene were constructed.

The first deletion mutant construct introduced into the mouse fertilized eggs was a *CRC2B* derivative which contained the 5′-flanking region up to position −540 (*CRC540*). Four independent lines of transgenic mice containing the *CRC540* transgene (F8, M9, M12, and M16 carrying between 8 to 50 copies per haploid genome) were generated. Northern blot analysis of tissue RNA revealed that all four lines expressed the transgene in the kidney. Expression of the transgene in the liver, similar to that of the endogenous PEPCK gene, was evident in three lines, two of which are shown (Fig. 3). Minimal expression in the liver, noted only upon five-times-longer exposure of the autoradiogram, was found in the fourth (M12) line (results not shown). Assuming that the minimal expression in the liver in just one of four lines results from a position effect, it is apparent that sequences upstream of position −540 are not required for transgene expression in either the kidney or liver. These results corroborate those previously obtained in cultured cells (6, 33). Unexpectedly, however, we could not detect any *CRC540* transcripts in the adipose tissue of all four lines. These results, which differed strikingly from those previously observed by transient expression assays in adipocytes (6), clearly indicated a requirement of sequences upstream of position −540 for in vivo PEPCK gene expression in adipose tissue.

In the second *CRC2B* derivative mutant construct, *CRC362*, we deleted sequences upstream of position −362,

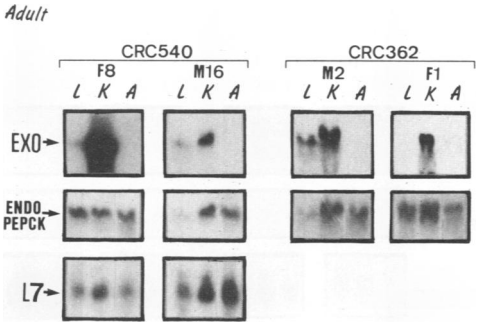

FIG. 3. Analysis of *CRC540* and *CRC362* transgene expression in PEPCK-expressing tissues. Representative results of Northern blot analysis of total RNA from the liver (L), kidney (K), and adipose tissue (A) from two independent lines of transgenic mice each containing either the *CRC540* transgene (F8 and M16) or the *CRC362* transgene (M2 and F1) are shown. Transgene expression was analyzed by Northern blot hybridization with c-485 (upper panels). Subsequently, the same blot was rehybridized with the counterpart rat PEPCK cDNA (middle panels) and again with the mouse L7 ribosomal protein (lower panels).

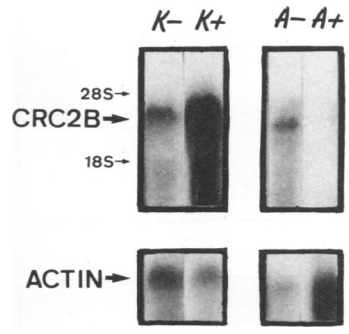

FIG. 4. Control of *CRC2B* transgene expression by glucocorticoids. F_2 male progenies of transgenic line M8F1, containing *CRC2B*, were injected intraperitoneally with dexamethasone (1.25 mg/100 g of body weight) and sacrificed 5 h later. Samples of total RNA from adipose tissue (30 μg per lane) before (A−) and after (A+) the glucocorticoid treatment and from the kidney (50 μg per lane) before (K−) and after (K+) treatment were analyzed by Northern blotting, probed with c-485 (upper panel). For a control, the same blot was probed with mouse β-actin cDNA (bottom panel). Each lane represents RNA from two or three animals.

relative to the transcription start site. This deletion was based on our previous results from transient expression assays in hepatocytes and adipocytes (6) and in kidney cells (33), which indicated that the 362-bp sequence of the PEPCK promoter retained full promoter activity in adipocytes and kidney cell lines but exhibited about 10% of the normal activity in hepatoma cells (6). Five transgenic lines containing the *CRC362* transgene were generated (F1, F7, F14, M2, and M9 carrying between 6 to 26 copies per haploid genome). Analysis of tissue RNA revealed that all five lines expressed the transgene in the kidney (results of two lines are presented in Fig. 3), thus corroborating previous findings from transient transfection experiments in a PEPCK-expressing kidney cell line (33). Expression of the *CRC362* transgene in the liver was either minimal or barely detectable in four of the five lines (represented by line F1 in Fig. 3). The normal liver expression, which was observed in just one line (M2, Fig. 3), may result from a position effect. Thus, compared with *CRC540* transgene expression in the liver (in three of four lines) and congruent with the experiments in hepatoma cells, the 180-bp sequence deleted from the *CRC362* transgene appears to be needed for normal liver-specific expression but not for that in the kidney. We have previously identified, in this 180-bp region, a protein-DNA-binding sequence (35) important for PEPCK promoter activity in hepatoma but not in kidney cell lines (33). Again, we failed to detect transgene expression in the adipose tissue in any of the five independent lines of transgenic mice containing the *CRC362* transgene.

Tissue-specific hormonal control of *CRC2B* transgene expression. To determine whether *CRC2B* transgene expression was regulated by glucocorticoids like the endogenous PEPCK gene, transgenic mice were treated for 5 h with the synthetic glucocorticoid dexamethasone. Northern blot analysis of the RNA isolated from treated animals revealed a significant decrease of *CRC2B* transcripts in adipose tissue and an increase in the kidney (Fig. 4) and liver (results not shown). Thus, the *CRC2B* gene contains the information for the typical reciprocal hormonal regulation of PEPCK gene expression.

Since both *CRC540* and *CRC362* deletion mutant constructs failed to be expressed in adipose tissue, only the positive response to glucocorticoids of these transgenes could be examined. Upon treatment of mice with glucocorticoids for 5 h, we noted a clear positive response of transgene expression in the kidney and liver in all four lines containing the *CRC540* transgene (results from kidney analysis of two representative lines are shown in Fig. 5). We conclude that the *CRC540* transgene includes a glucocorticoid-responsive element needed for positive hormonal control of gene expression. Similar experiments with three independent lines containing the *CRC362* transgene have failed to show any response in the kidney, which constitutively expressed the transgene (results from two lines are shown in Fig. 5). Clearly, the *CRC362* transgene lacks the glucocorticoid-responsive element. The results indicate, therefore, that a glucocorticoid-responsive element used by the kidney resides in the 180-bp sequence missing in the *CRC362* construct.

Developmental onset of *CRC2B* transgene expression. The onset of PEPCK gene expression during development is also tissue specific. In the rat, expression is first observed in the

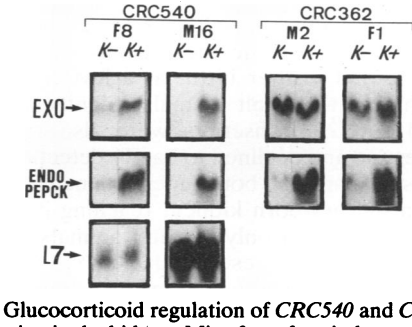

FIG. 5. Glucocorticoid regulation of *CRC540* and *CRC362* transgene expression in the kidney. Mice from four independent *CRC540*-containing lines and from three independent *CRC362*-containing lines were treated with dexamethasone as described in the legend to Fig. 4. Northern blot analyses of kidney RNA from two representative lines of each transgene are shown. The blots were first probed with c-485 (upper panels) and then rehybridized with the counterpart rat PEPCK cDNA (middle panels) and with the mouse L7 ribosomal protein (lower panels).

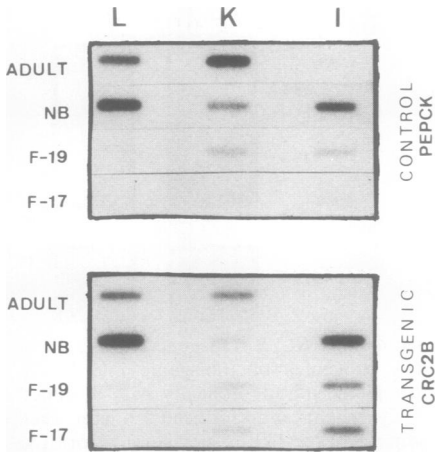

FIG. 6. Analysis of *CRC2B* gene expression during development. Total RNA from the liver (L), kidney (K), and intestine (I) of adult, newborn (NB), 19 day (F-19) and 17 day (F-17) fetuses of control and transgenic mice was analyzed by slot blot hybridization. Upper panel shows RNA from control mice, probed with 1.6 kb DNA fragment of PCK-10. Lower panel shows RNA from transgenic mice, probed with the c-485.

kidney 4 days before birth and in the liver at birth (4, 15, 21, 34). To determine whether the *CRC2B* transgene is similarly regulated, we studied its expression in different tissues of fetal and newborn transgenic mice. The analysis from pooled tissues was facilitated by utilizing F_3 generation offsprings, of which all fetal littermates contained the transgene. The presence of *CRC2B* and endogenous transcripts were initially determined by Northern blot analysis of total RNA from pools of various tissues of individual litters at days 16, 17, 18, and 19 of gestation and of 1-day-old newborns. The onset of expression of the *CRC2B* gene in the PEPCK-expressing tissues was very similar to that of the endogenous gene, with no ectopic expression of the transgene (results not shown). To determine the timing of the developmental onset of expression for the transgene and endogenous gene in the PEPCK-expressing tissues, slot blot hybridization analysis was performed with the same RNA preparations (Fig. 6). In this manner, we detected the onset of PEPCK and *CRC2B* expression in the kidney and intestine on day 17 of gestation. No PEPCK or *CRC2B* transcripts could be detected at this stage in the liver. A marginal expression of both genes in the liver was first detected on day 19 of gestation. The liver became the predominant PEPCK-expressing tissue 1 day after birth, at a level of expression exceeding that in the adult animals. Significant levels of PEPCK and *CRC2B* transcripts were also found in the newborn intestine and declined to barely detectable levels in adult animals. In contrast, both genes retained a low level of expression in the newborn kidney, reaching its full prominent level of expression only in adult animals. The results establish that the sequences included in the *CRC2B* gene contain the information for the proper timing of onset expression in the different tissues during development. In addition, this transgene faithfully exhibits the characteristic induced expression in the livers, transient expression in the intestines, and minimal expression in the kidneys of newborn animals.

Analysis of the 5′-flanking sequences involved in the developmental regulation of PEPCK gene expression upon birth. The sequence requirements for the features of expression

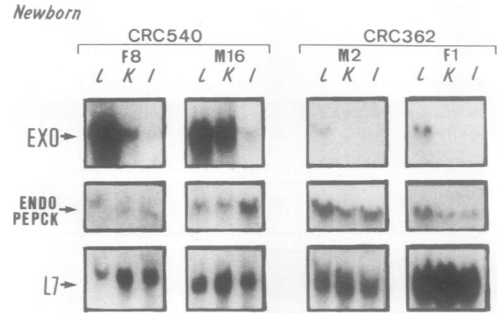

FIG. 7. Analysis of *CRC540* and *CRC362* transgene expression in newborn transgenic mice. Results from representative independent lines containing either the *CRC540* or *CRC362* transgene are shown. Northern blots were first probed with c-485 for transgene expression (upper panels) and then the same blots were rehybridized with the counterpart rat PEPCK cDNA (middle panels) and with mouse L7 ribosomal protein cDNA (lower panels).

inherent to the newborn animal were examined using the deletion mutant transgenes. Analysis of newborn mice containing the *CRC540* transgene demonstrated the typical induction of prominent transgene expression in the liver in all four lines, including the one line (M12) in which transgene expression in adult liver was minimal. In fact, transgene expression at this age considerably exceeded that of the endogenous gene in all four lines. Expression of the transgene in the intestine was also noted in all four lines but at a level lower than that of the endogenous PEPCK gene. It is therefore clear that the *CRC540* transgene contained the information for developmental induction of its expression in the liver and for partial expression in the intestine (results from two representative lines are shown in Fig. 7). In contrast, transgene expression in the newborn kidney varied considerably among the four transgenic lines between overexpression (M16) to a barely detectable level of expression (M9). These varied levels of expression disappeared in the adults, for which three of the four lines (M9, M12, and M16) exhibited a level similar to that of the endogenous PEPCK gene (compare the results of M16 in Fig. 3 and 7).

Analysis of newborn mice containing the *CRC362* transgene revealed a lower level of transgene expression in the liver compared with that of the endogenous PEPCK gene (Fig. 7). This lower expression, however, was consistent in the three independent lines examined, regardless of whether the transgene expression in the adult liver was normal (M2) or absent (F1). We failed to detect any transgene expression in the intestine in any of the three lines tested but could detect in all of these lines a minimal expression in the kidney (seen in the original autoradiograms) which, however, was lower than that of the endogenous PEPCK gene (results from two representative lines are shown in Fig. 7). Apparently, when compared with the *CRC540* transgene expression, the 180-bp sequence missing in the *CRC362* construct seems to be needed for the fully induced expression in the newborn liver, as well as for expression in the newborn intestine and kidney.

DISCUSSION

The 5′-flanking regions of the rat PEPCK gene which are required for this gene expression in several tissues have been analyzed in transgenic mice. Using the level of the endogenous PEPCK gene expression as an internal reference for

TABLE 1. Expression of transgene in transgenic mouse lines

Length (kb) of 5'-flanking sequence	No. of lines expressing the transgene/ total no. of lines examined[a]								
	Adult tissue			Glucocorticoid control			Newborn tissue		
	L	K	A	L	K	A	L	K	I
2.2	2/2	2/2	2/2	1/1	1/1	1/1	2/2	2/2	2/2
0.54	3/4	4/4	0/4	4/4	4/4	0/4	4/4	4/4	4/4[b]
0.36	1/5	5/5	0/5	ND[c]	0/3	ND	3/3[b]	3/3[b]	0/3

[a] Transgene expression comparable to endogenous PEPCK gene expression. Abbreviations: L, liver; K, kidney; A, adipose tissue; I, intestine.
[b] Expression considerably lower than endogenous gene expression.
[c] ND, not determined.

this analysis is especially appropriate in view of the fact that PEPCK gene expression in vivo readily changes in response to many stimuli (4, 15, 34).

The results (summarized in Table 1) enabled us to draw several conclusions regarding the 5'-flanking sequences required for gene expression in adult PEPCK-expressing tissues and for hormonal control of gene expression and its developmental regulation.

Sequences needed for tissue-specific PEPCK gene expression in adult mice. The fully sustained transgene expression in the adult kidney in all lines, regardless of whether the transgene harbored 2.2, 0.54, or 0.36 kb of the 5'-flanking region of the PEPCK gene, indicates that sequences downstream of position −362 are sufficient for expression in the kidney. This conclusion is emphasized by the high level of expression in the kidney exhibited by the transgenes used in the present study. The previously reported (19) low level of expression of transgenes containing the structural growth hormone as a reporter gene in the kidney might be a consequence of the known deleterious effect of unrestrained growth hormone production on the kidney (10).

A clear picture regarding the sequences required for expression in adipose tissue emerges. The loss of transgene expression in adipose tissue of all lines containing the *CRC2B* deletion mutant constructs (*CRC540* and *CRC362*) indicates that sequences upstream of position −540 are explicitly required for expression in this tissue. This requirement, however, differs strikingly from that obtained by transient expression analysis in which 362 bp of the PEPCK promoter appeared sufficient to direct adipocyte-specific transcription (6). The additional sequence requirement in transgenic mice might reflect developmental interactions that are needed for the activation of PEPCK gene expression in adipose tissue. Other instances have been reported when the sequence requirements differed between those sufficient for transient expression assays and those required in transgenic mice (1, 12, 14). One such instance is the adipocyte-specific aP2 gene for which a 168-bp segment of the promoter was sufficient for adipocyte-specific transcription in cell culture, whereas 5.4 kb of the 5'-flanking region was required for proper expression in the adipose tissue of transgenic mice (12).

The sequence requirements for gene expression in the liver, however, appear to be more complex, since expression was less consistent regarding the number of independent lines expressing the transgenes. Nevertheless, the normal expression in three of four lines containing the *CRC540* transgene compared with one of five lines containing the *CRC362* transgene argues that there is an important sequence required for liver-specific expression between positions −540 and −362. This notion agrees with results of McGrane et al. (19), using a chimeric transgene of the PEPCK promoter driving the bovine growth hormone gene. They reported that the percentage of transgenic lines producing blood growth hormone dropped from 60% with a transgene containing 460 bp of the PEPCK promoter to 18% of lines with a transgene containing 355 bp of the promoter. This decreasing percentage, which most likely reflects a decreasing production of blood growth hormone in the liver is consistent with that occurring in transgenic lines expressing *CRC540* (75%) and *CRC362* (20%) transgenes in the liver. This region contains an AF1 recognition site (35) whose deletion has been shown, using transient expression assays, to be important for PEPCK promoter activity only in hepatoma but not in kidney cells (33).

It appears that our analysis has defined the minimal requirements of 2.2 kb of the PEPCK gene 5'-flanking sequence for expression in adipose tissue and of 0.36 kb which is sufficient for expression in the kidney. The results also imply a minimal requirement of 0.54 kb of the 5'-flanking sequence for expression in the liver.

Control of PEPCK gene expression by glucocorticoids. Studies of hormonal regulation of *CRC2B* transgene expression enabled us to conclude that this transgene contains the elements needed for positive control of gene expression by glucocorticoids in the kidney and negative control in adipose tissue.

The positive response of *CRC540* transgene expression in the kidney to glucocorticoids in all four lines tested and its absence in the three examined *CRC362*-containing lines clearly indicate that the sequence between positions −540 and −362 contains a positive glucocorticoid-responsive element. Although liver expression of the *CRC540* transgene was likewise induced by glucocorticoids, the lack of basal liver expression of the *CRC362* transgene precludes a conclusion regarding the use of such an element by the liver. It is noteworthy, however, that transient expression experiments in hepatoma cells have documented the existence of a glucocorticoid-responsive element in this region (17, 30). Such an element may, therefore, be shared by both kidney and liver. It remains to be seen whether the sequence responsible for negative control of PEPCK gene transcription by glucocorticoids in adipose tissue resides in this same region.

5'-flanking regions involved in the developmental regulation of PEPCK gene expression. The induced PEPCK gene expression in the newborn liver, its transient expression in the intestine, and the low level of expression in the kidney constitute specific features of expression inherent to the newborn animal. These features are faithfully reproduced by the *CRC2B* transgene. The summary of the resulting levels of expression of the deletion mutant constructs (Table 1) clearly reveals that in the newborn, the sequence spanning positions −540 to −362 is required for the fully induced expression in the liver and for expression in the intestine and kidney. In the intestine, however, this sequence accounts only for a partial level of expression and additional upstream sequences are needed to attain the level of expression of the endogenous gene. In view of the repressed PEPCK gene expression in the intestines of adult animals, the sequence requirements exhibited by this tissue are confined to the newborn stage. Likewise, the requirement of this sequence by the kidney is also specific for the newborn stage, as it is not needed for the subsequent buildup of gene expression in the adult kidney. This sequence contains the glucocorticoid-responsive elements of the PEPCK gene used by the kidney,

as shown in the present study, and an AF1 recognition sequence (35). Previous results indicated that the AF1 site is important for PEPCK promoter activity in hepatoma but not in kidney cell lines (33). Therefore, the specific requirement of this region in the newborn kidney might be explained if glucocorticoids were involved in controlling PEPCK gene expression in the newborn kidney.

In the liver, unlike the intestine and kidney, this sequence is required for full expression of the PEPCK gene in both newborn and adult animals. It is evident, however (Table 1), that sequences downstream of position −362 are involved in the newborn liver expression, since the *CRC362* transgene retains a fraction of this induced expression. The consistent transgene expression in the three transgenic lines examined, including the single M2 line (one of five lines) which exhibits a normal transgene expression in the adult liver, emphasizes the exceptional transgene expression of this line in the adult liver. Therefore, while sequences downstream of position −362 are involved in the newborn liver expression of the PEPCK gene, these sequences are unable in themselves to sustain the expression in the adult liver. In contrast, these sequences are sufficient for the buildup of the prominent expression in the adult kidney. Thus, the role played by the sequences downstream of position −362 appears to be different for the liver and kidney.

This conclusion lends further support from footprinting analysis of the PEPCK promoter (26, 29, 35). Thus, the 362-bp region of the PEPCK promoter comprises several recognition sites for transcription factors. These include sequences containing the C/EBP motif which interact with nuclear proteins that are present in the liver but not in the kidney (29, 35, 36). Furthermore, two of these sites interact with nuclear proteins that are absent in the fetal liver and appear in the newborn liver together with the onset of PEPCK gene expression (35). It is also intriguing that according to McGrane et al. (19), a specific mutation in just one of these C/EBP-containing motifs was sufficient to reduce transgene expression in the liver but not in the kidney. Taken together, these data might implicate the C/EBP recognition sites in the developmental activation of the liver PEPCK gene. These sites, however, should function in conjunction with the upstream sequences spanning positions −540 to −362 to elicit full expression of the PEPCK gene in the liver in either the newborn or adult stage.

Unlike the situation in the liver, nuclear proteins from the kidney interact with only three sequences in the region downstream of position −362 of the PEPCK promoter (29, 36). These include the ubiquitous TFIID and NF1 recognition sequences and the recognition sequence of the HNF1 transcription factor which is specifically expressed in the liver and kidney (for a recent review, see reference 22). These three sites constitute an apparent simple sequence requirement which is capable of maintaining full expression of the PEPCK gene in the adult kidney.

Taken together, the results from the present study seem to imply that the various PEPCK-expressing tissues exhibit distinct sequence requirements for the expression of the same gene. These sequences could be composed of different modular components of the 5′-flanking region of the PEPCK gene. The glucocorticoid-responsive element, which seems to be shared by the liver and kidney, represent one such modular component.

If the most minimal sequence required by the adult kidney is viewed as a basic element, the additional sequences required by the liver, adipose tissues, and the newborn intestine might be viewed as modules added to satisfy the requirements needed by these tissues. It might be interesting to make a phylogenetic comparison of other animals to see whether the PEPCK gene consists of just the basic element or, alternatively, whether there are animals whose PEPCK gene is expressed only in the kidney. Indeed, in birds PEPCK gene expression is confined to the kidney, with no expression in the liver (15, 16). However, whether the chicken PEPCK gene contains just a basic element has yet to be determined.

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