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Tissue-Specific, Developmental, Hormonal, and Dietary Regulation of Rat Phosphoenolpyruvate Carboxykinase-Human Growth Hormone Fusion Genes in Transgenic Mice

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The cytosolic phosphoenolpyruvate carboxykinase (PEPCK) gene is expressed in multiple tissues and is regulated in a complex tissue-specific manner. To map the *cis*-acting DNA elements that direct this tissue-specific expression, we made transgenic mice containing truncated PEPCK-human growth hormone (hGH) fusion genes. The transgenes contained PEPCK promoter fragments with 5′ endpoints at −2088, −888, −600, −402, and −207 bp, while the 3′ endpoint was at +69 bp. Immunohistochemical analysis showed that the −2088 transgene was expressed in the correct cell types (hepatocytes, proximal tubular epithelium of the kidney, villar epithelium of the small intestine, epithelium of the colon, smooth muscle of the vagina and lungs, ductal epithelium of the sublingual gland, and white and brown adipocytes). Solution hybridization of hGH mRNA expressed from the transgenes indicated that white and brown fat-specific elements are located distally (−2088 to −888 bp) and that liver-, gut-, and kidney-specific elements are located proximally (−600 to +69 bp). However, elements outside of the region tested are necessary for the correct developmental pattern and level of PEPCK expression in kidney. Both the −2088 and −402 transgenes responded in a tissue-specific manner to dietary stimuli, and the −2088 transgene responded to glucocorticoid stimuli. Thus, different tissues utilize distinct cell-specific *cis*-acting elements to direct and regulate the PEPCK gene.

Cytosolic phosphoenolpyruvate carboxykinase (PEPCK; EC 4.1.1.32), an enzyme that catalyzes the conversion of oxaloacetic acid to phosphoenolpyruvate, performs a variety of important tissue-specific metabolic roles in mammals (21, 30, 58, 68, 70). For example, it is rate limiting for gluconeogenesis in hepatocytes and in proximal tubular epithelia of the kidney, is also ammoniagenic in the kidney, and is glyceroneogenic in white adipocytes. In addition to being present in liver, kidney, and white fat, PEPCK is found in similar amounts in brown fat, intestinal epithelium, and mammary gland, though its function has not been investigated in the latter three tissues (1, 22, 75). Lesser amounts of PEPCK have been reported in colon, heart, skeletal muscle, lung, ovary, certain smooth muscles, and sublingual gland, where its function is also not known (30, 75).

Regulation of PEPCK is complex, as it is expressed in multiple cell types and changes in response to multiple hormones, environmental factors, cell-cell interactions, and development (5, 7, 11, 15, 24, 43, 45, 54, 68, 69, 71, 72). Unlike many other metabolically important enzymes, PEPCK is not regulated by allosteric or phosphorylation-dephosphorylation mechanisms (68). Instead, it is regulated by changes in the transcription of its single-copy gene from a single promoter site (14, 24, 39, 42, 43, 46–48, 63, 68, 74), though regulation of PEPCK mRNA turnover also plays a role (33, 55). PEPCK gene transcription is regulated by a variety of effectors in a tissue-specific pattern. For example, in liver, cyclic AMP (cAMP), glucocorticoids, thyroid hormone, prolactin, and retinoic acid are inducers, and insulin and diacylglycerol are inhibitors (14, 24, 39, 42, 43, 63, 74). Glucocorticoids are also inducers in kidney, as are hydrogen ions (acidosis), while cAMP and insulin are weak effectors (47). In contrast, glucocorticoids inhibit transcription of the PEPCK gene in adipocytes (48). The PEPCK gene is also developmentally regulated (for a review, see reference 23) and has been well characterized in rat liver, where it is competent but dormant as much as 6 days prior to birth but is induced at parturition in response to changes in the insulin/glucagon ratio. Because of its unique and pleiotropic regulation, the PEPCK gene is a valuable model for studying the mechanisms of hormonal, tissue-specific, and developmental control of genes expressed in multiple tissues. Indeed, several laboratories have identified a number of upstream *cis*-acting DNA elements (and some of the cognate *trans*-acting factors) involved in regulating PEPCK expression in rat liver (6, 35, 43, 45, 49, 53, 57, 59, 65, 69).

While cultured cell lines are most often used to identify *cis*-acting elements in a gene, such studies are often hampered by the limited selection and state of differentiation of such cell lines. For example, transcription rates of numerous liver-specific genes are greatly diminished in cultured hepatoma cells and primary hepatocytes (16, 17). In addition, in some systems more regulatory sequence information is required for expression in cultured cells than in transgenic animals, and therefore fundamental differences exist between animals and differentiated cells in culture (67). Transgenic mice are at present the most rigorous system available for identifying and characterizing *cis*-acting DNA se-

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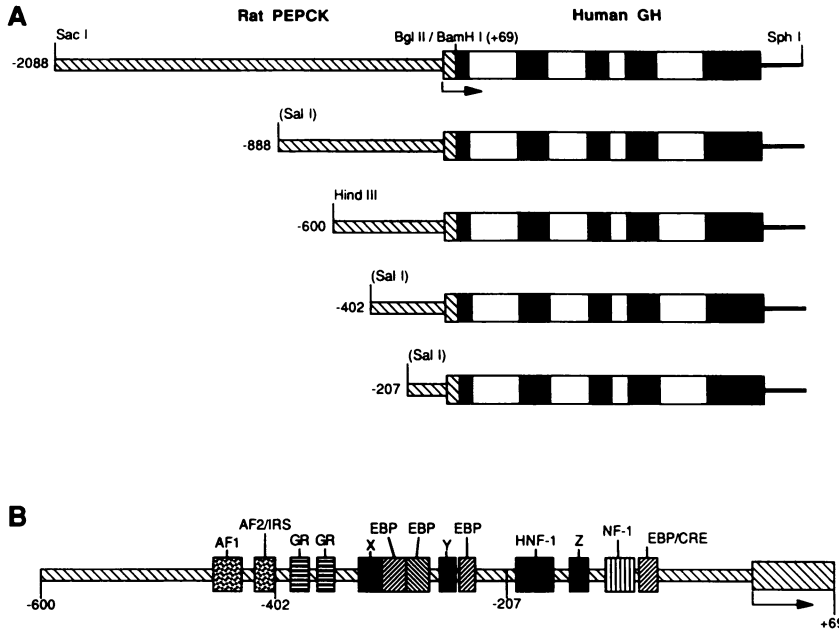


FIG. 1. PEPCK-hGH constructs tested in transgenic mice. (A) DNA derived from the PEPCK gene is indicated by hatched areas, and hGH sequences are indicated by solid boxes (exons), open boxes (introns), and a thin line (3′ flanking DNA). A *Bgl*II site at +69 bp in PEPCK was the 3′ terminus for all PEPCK constructs; this site was fused to a *Bam*HI site in the hGH gene. The start site and direction of transcription from the PEPCK promoter are indicated by an arrow. The positions of the 5′ deletion endpoints are shown at the left of each construct; these numbers are used in the text to refer to their respective transgenes. Restriction enzyme sites referred to in Materials and Methods are shown; those in parentheses were artificially created from linkers (see Materials and Methods for details). (B) PEPCK DNA from −600 to +69 bp is shown as in panel A. Transgene deletion endpoints and the locations of hepatic *cis*-acting elements and *trans*-acting factor binding sites (designated as boxes superimposed on the DNA) are shown: AF1 and AF2, accessory factors 1 and 2 (part of the glucocorticoid response unit) (35); EBP, CCAAT/enhancer-binding protein (C/EBP) (40, 53, 59, 69); GR, glucocorticoid receptor (35); IRS, insulin response sequence (49); HNF-1, hepatic nuclear factor 1 (37, 57, 59); NF-1, nuclear factor 1 (57, 59, 62); CRE, cAMP response element (57, 65); and X, Y, and Z, unknown factors (59). A retinoic acid response element also coincides with the 5′-most accessory factor (43).

quences. Transgene expression can be assessed in numerous cell types as well as examined during both embryonic and fetal development.

This study was undertaken to determine whether distinct cell types utilize common or unique *cis*-acting elements to regulate transcription of the PEPCK gene. Because of the rich diversity of tissues that express and uniquely regulate the PEPCK gene, we chose to use PEPCK-human growth hormone (hGH) fusion genes in transgenic mice to map the *cis*-acting elements controlling its tissue- or cell-specific expression. Indeed, transgenic mice bearing hGH reporter genes have been used successfully to map and characterize *cis*-acting elements that regulate genes expressed in single cell types, such as the genes encoding elastase, protamine, and albumin (9, 56, 67), and genes expressed in multiple cell types, such as those encoding metallothionein, transferrin, and fatty acid-binding protein (28, 32, 34), to name a few. We tested five PEPCK promoter fragments with 5′ deletion endpoints of −2088, −888, −600, −402, and −207 bp. To ascertain the fidelity of transgene expression, we also chose to quantitate both PEPCK mRNA and hGH mRNA molecules per cell by a solution hybridization assay. We were thus able to identify PEPCK DNA sequences that recapitulated wild-type levels of transgene expression in most tissues. Our results indicate that different cell types use distinct *cis*-acting elements to direct expression of the PEPCK gene.

MATERIALS AND METHODS

Construction of PEPCK-hGH fusion genes and production of transgenic mice. Five rat PEPCK-hGH fusion genes that contained various amounts of PEPCK 5′ flanking DNA were made (Fig. 1A). The PEPCK genomic DNAs were derived from subclones of λPC112 (4). A *Bgl*II site in the 5′ untranslated region of the rat PEPCK gene at +69 in the first exon served as a common 3′ endpoint for linking the PEPCK DNA fragments to a *Bam*HI site in the first exon of the hGH gene. The largest PEPCK-hGH fusion gene contained 2,088 bp of PEPCK 5′ flanking DNA and was constructed by restriction with *Sac*I, blunting of the 3′ overhang with T4 DNA polymerase, and addition of a *Sal*I linker. After digestion with *Bgl*II and *Sal*I, the resulting DNA fragment was ligated into the *Sal*I and *Bam*HI sites of pOGH (64). The constructs containing 888, 402, and 207 bp of 5′ flanking PEPCK DNA were derived from a series of PEPCK deletion mutants generated with Bal 31 exonuclease, ligated to a *Sal*I linker, and subcloned into pPLFCAT (57). The PEPCK DNA fragments in these plasmids were released by digestion with *Sal*I and *Bgl*II and then inserted into the *Sal*I and *Bam*HI sites of pOGH. The construct containing 600 bp of 5′ flanking PEPCK DNA was made by ligating a *Hin*dIII-*Bgl*II fragment of PEPCK DNA into the *Hin*dIII and *Bam*HI sites of pOGH.

DNA fragments for microinjection were separated from plasmid DNA by digestion with *Sph*I and an appropriate

TABLE 1. Glucocorticoid regulation of PEPCK and PEKCK-hGH expression in transgenic mice

Transgene	Group	mRNA molecules/cell (mean ± SEM)[a]							
		Liver		Kidney		Jejunum		White fat	
		hGH	PEPCK	hGH	PEPCK	hGH	PEPCK	hGH	PEPCK
−2088[b]	Control	5,672 ± 2,561	1,510 ± 109	30 ± 18	1,056 ± 410	36 ± 36	85 ± 37	340 ± 46	121 ± 14
	Dex[c]	16,731 ± 621***	2,684 ± 23**	159 ± 63	2,992 ± 460	262 ± 52*	413 ± 16**	276 ± 34	21 ± 12*
−402[d]	Control	1,684 ± 1,305	638 ± 36	16 ± 8	598 ± 208	ND	75 ± 74	ND	
	Dex	2,985 ± 1,047	5,981 ± 1,189**	16 ± 10	6,115 ± 808***	ND	245 ± 122	ND	

[a] hGH and PEPCK mRNA levels were measured by solution hybridization as described in Materials and Methods. All groups contain three or more animals except those for −2088 PEPCK mRNA determinations, which contain two. Sample means were compared for significance by Student's *t* test. Values significantly different from those for saline-treated transgenic littermates: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$. ND, undetectable hGH mRNA levels.
[b] Mice are 1-month-old third-generation progeny from line 50-3, which has four copies of the −2088 PEPCK-hGH transgene integrated per cell.
[c] Mice were given two intraperitoneal injections of physiological saline (control) or dexamethasone (Dex; 10 mg/kg) administered 18 and 6 h before sacrifice.
[d] Mice are 1- to 4-month-old second-generation progeny from line 99-3, which has eight copies of the −402 PEPCK-hGH transgene integrated per cell.

enzyme to release the 5′ ends, resolution by agarose gel electrophoresis, dissolution of the gel in sodium perchlorate, binding the DNA to glass filters (Whatman GF/F), and elution with 1 mM Tris-HCl (12). The DNA concentration was measured and diluted to 3 ng/μl for microinjection. About 2 pl of the DNA solution was microinjected into the pronuclei of fertilized (C57BL/6 × SJL)F_2 hybrid mouse eggs which were cultured to the two-cell stage. Two-cell embryos were transferred to day 1 pseudopregnant recipients as previously described (10). Transgenic animals were identified by dot blot analysis of tail nucleic acids, using a ^{32}P-labeled hGH cDNA probe (50).

Transgenic mouse lines. Several lines of transgenic mice were established during the course of this study. Two lines were particularly important for parts of this study (Fig. 3 to 7; Tables 1 and 2); they were derived from founder mice 50-3 and 99-3, which bore the −2088 (four copies per haploid genome) and the −402 (eight copies per haploid genome) PEPCK-hGH transgenes, respectively. These particular lines were used because they typify the overall expression patterns of their respective transgenes (shown in Fig. 4 and described in Results). Because of the impaired fertility resulting from hGH expression (3, 29), established lines were maintained through ovarian transplants (61).

Animal treatments. To determine the effects of glucocorticoids on −2088 and −402 transgene expression, mice of the 50-3 and 99-3 lines (described above) were treated with dexamethasone. Dexamethasone was administered intraperitoneally as a 1:1 mixture of dexamethasone acetate and dexamethasone phosphate (Merck, Sharp & Dohme, West Point, Pa.) at a final dose of 10 mg/kg of body weight. To assess the dietary responsiveness of the −2088 and −402 transgenes, mice from the 50-3 and 99-3 lines were fed isocaloric diets of either 10% protein–70% carbohydrate–8% fat (carbohydrate diet) or 65% protein–10% carbohydrate–10% fat (protein diet) (Purina Mills, Inc., Richmond, Ind.) or a normal maintenance diet of 51% protein–25% carbohydrate–4% fat (Teklad, Madison, Wis.).

Measurement of PEPCK mRNA and hGH mRNA abundance. Total nucleic acids were isolated by homogenizing 50 to 100 mg of tissue in 4 ml of 1% sodium dodecyl sulfate–1 mM EDTA–10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.5) (SEH) buffer with 100 μg of proteinase K per ml, incubating the extract at 45°C, and then isolating total nucleic acids by phenol-chloroform extraction and ethanol precipitation (20). The number of mRNA molecules per cell was determined by solution hybridization (20), using a ^{32}P-labeled 26-bp oligonucleotide probe complementary to nucleotides 29 to 54 of hGH mRNA and a single-stranded T7 polymerase RNA transcript of pPCPst (5) complementary to nucleotides 225 to 1460 of PEPCK mRNA (4). Standards for the hGH and PEPCK solution hybridization assays were single-stranded M13 DNAs with inserts that were, respectively, a 2.15-kb *Bam*HI-*Eco*RI hGH genomic DNA fragment and a 1.26-kb *Pst*I-*Pst*I PEPCK cDNA fragment from pPC201 (4). The number of mRNA molecules per cell was calculated relative to the level of DNA in each sample (38), assuming 6.4 pg of DNA per cell (20). The RNA/DNA ratios determined for each tissue were as follows: liver, 4.1; kidney, 1.0; fat, 1.6;

TABLE 2. Dietary regulation of PEPCK and PEPCK-hGH expression in transgenic mice

Transgene	Diet[b]	mRNA molecules/cell (mean ± SEM)[a]			
		Liver		Kidney	
		hGH	PEPCK	hGH	PEPCK
−2088[c]	Carbohydrate	2,403 ± 670	534 ± 109	52 ± 20	1,542 ± 530
	Protein	10,111 ± 949*	1,161 ± 136*	583 ± 132*	4,372 ± 670*
−402[d]	Carbohydrate	467 ± 283	1,087 ± 453	61 ± 31	2,583 ± 633
	Protein	6,149 ± 2,602*	3,686 ± 835*	155 ± 53	8,346 ± 2,751*

[a] hGH and PEPCK mRNA levels were measured by solution hybridization as described in Materials and Methods. All groups contain four animals except for the group containing −2088 mice fed a high-protein diet, which contains three. Sample means were compared for significance by Student's *t* test. *, Value significantly different ($P < 0.05$) from those obtained in carbohydrate-fed transgenic animals.
[b] Mice from either the 50-3 or 99-3 line were fed ad libitum diets high in either carbohydrate (70% carbohydrate, 10% protein) or protein (65% protein, 10% carbohydrate) for 1 week before sacrifice.
[c] Mice are 1- to 5-month old third-generation progeny from line 50-3, described in Table 1, footnote *b*.
[d] Mice are 2- to 5-month old third-generation progeny from line 99-3 (402 PEPCK-hGH transgene), described in Table 1, footnote *d*.

ileum, 1.3; jejunum and colon, 1.1; heart, 1.3; lung, 0.53; gonad, 0.9; skeletal muscle, 2.1; brain, 1.0; spleen, 0.55; and salivary gland, 1.7. Northern (RNA) blot hybridization was carried out as described elsewhere (27). Briefly, total cellular RNA was extracted from tissues by the guanidinium thiocyanate-cesium chloride procedure (13), separated on a glyoxal-agarose gel, and blotted onto a nylon membrane. hGH mRNA was detected with a random-primed 660-bp *Sma*I fragment from pMThGH#111 (52), and PEPCK mRNA was detected with a random-primed 1.2-kb *Pst*I fragment from pPCPst (5). All washes were carried out in 0.1× SSC (1× is 0.15 M NaCl plus 0.015 M sodium citrate)–0.5% sodium dodecyl sulfate at 65°C.

Immunocytochemistry. All tissues were fixed for 12 to 24 h in Carnoy's reagent with the exception of brown fat and gonadal white fat, which were fixed in Bouin's fixative. Specimens were embedded in paraffin, cut into 5-μm sections, and placed on poly-L-lysine-coated slides. Slides with cut sections were then heated at 58°C for 30 min, and sections were deparaffinized in xylene and rehydrated by using standard procedures. Indirect immunocytochemistry was performed by using the reagents and suggested protocols of Zymed SABC kits (Zymed Laboratory, Inc., San Francisco, Calif.). The primary antibody, rabbit anti-hGH, was obtained from Dako Corp. (Carpinteria, Calif.), and sections were incubated in diluted primary antibody overnight at 4°C. Sections of liver, kidney, ileum, jejunum, and colon were incubated at a final dilution of 1/1,000. Sections of other tissues were incubated dilutions of 1/250 (lung), 1/100 (white fat), and 1/50 (brown fat and sublingual gland).

The secondary antibody was biotinylated goat anti-rabbit immunoglobulin G (Vector Laboratories, Inc., Burlingame, Calif.). Diaminobenzidine-nickel substrate (Zymed) was used as the substrate chromagen. Sections were counterstained in Mayer's hematoxylin. Negative controls included the use of both the primary and secondary antibodies on tissues isolated from nontransgenic animals.

RESULTS

PEPCK is expressed in multiple tissues and is not affected by hGH. Five PEPCK-hGH fusion genes which contained variable portions of PEPCK 5′ flanking DNA linked to the hGH gene were constructed (Fig. 1A). These PEPCK-hGH fusion genes contained PEPCK DNA fragments with 5′ endpoints of −2088, −888, −600, −402, and −207 bp. In all five constructs, the PEPCK DNA was fused to the hGH gene at the *Bgl*II site (+69) of PEPCK. For reference, Fig. 1B shows the three proximal deletion endpoints superimposed on a map of known *cis*-acting elements that have been identified by transfection and DNA-binding assays. The PEPCK-hGH fusion genes were separated from plasmid DNA and used to produce 9 to 16 founder animals per construct. A total of 49 transgenic founder mice were generated; 37 of these expressed the transgenes in one or more tissues. Of the mice identified as transgenic, 75, 73, 73, 54, and 0% of the −2088, −888, −600, −402, and −207 constructs, respectively, grew to approximately twice the size of their nontransgenic littermates and displayed a phenotype typical of transgenic mice expressing hGH (51, 52). The number of transgenes integrated into the DNA of each founder mouse varied from 1 to 500 copies per cell; however, no correlation was observed between copy number and expression of hGH mRNA (unpublished data).

As a measure of PEPCK gene expression in various tissues, PEPCK mRNA was quantitated in a solution hybridization assay (Fig. 2). PEPCK mRNA was always detectable in liver, kidney, white fat, brown fat, ileum, jejunum, and colon but was detectable in the hearts of only three of five mice and in skeletal muscle of only two of five mice. PEPCK mRNA was not detectable in lung, gonads, spleen, or brain.

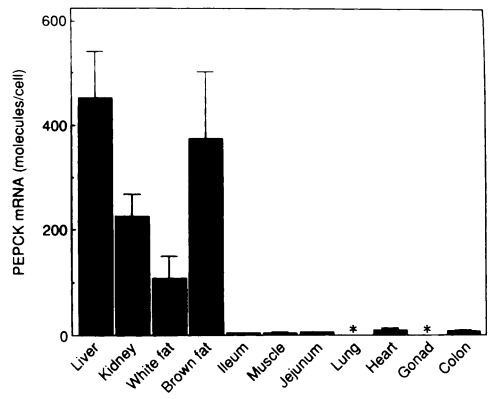


FIG. 2. Tissue survey of PEPCK mRNA levels in nontransgenic mice. PEPCK mRNA was measured in five mice (two males and three females) by solution hybridization (see Materials and Methods for details). The data are presented as means ± standard errors. Asterisks indicate that PEPCK mRNA was undetectable. Not shown are data for spleen and brain, which contained neither PEPCK nor hGH mRNA. Skeletal muscle was isolated from the hind limb, white fat was from ovarian or epididymal fat pads, and brown fat was from the suprascapular regions.

To determine whether expression of hGH had an effect on PEPCK gene expression (which would render hGH inappropriate as a reporter gene), PEPCK mRNA levels were measured in liver, kidney, white fat, and brown fat in five transgenic mice. The PEPCK mRNA levels (615 ± 119, 435 ± 106, 176 ± 39, and 780 ± 147 molecules per cell in liver, kidney, white fat, and brown fat, respectively) in these transgenic mice were not significantly different from those in control mice. These five transgenic mice had high serum hGH levels (data not shown), exhibited enhanced growth, and had hepatic hGH mRNA levels in excess of 4,000 molecules per cell. Thus, elevated serum hGH did not affect expression of the PEPCK gene, and therefore the hGH gene appears to be an appropriate reporter.

Expression of the −2088 PEPCK-hGH transgene is similar to PEPCK expression in most but not all tissues. Expression of the construct bearing the longest piece of PEPCK DNA (−2088) was examined first by Northern blot analysis of RNA isolated from 15 tissues. Figure 3 shows a representative blot using tissues from a mouse of the 50-3 lineage bearing the −2088 PEPCK-hGH transgene. The blot was probed sequentially for PEPCK and hGH mRNAs in order to compare transgene expression with that of the endogenous PEPCK gene. PEPCK mRNA was detected in liver, kidney, ovarian white fat, suprascapular brown fat, sublingual gland, skeletal muscle, jejunum, colon, and ileum but not in spleen, brain, stomach, lung, heart, or pancreas of this mouse. hGH mRNA was detected in all of the tissues that expressed PEPCK mRNA and additionally in stomach, lung, and heart. This result indicated that the essential tissue-specific control elements are present in the −2088 to +69 bp region, as there was good qualitative correspondence between tissues that express PEPCK and those that express the transgene (except for stomach, lung, and heart). The quantitative discrepancies may be caused by a combination

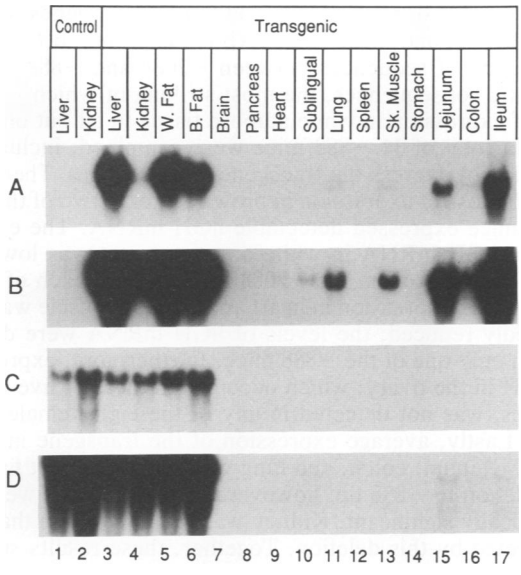


FIG. 3. Northern blot analysis of PEPCK and hGH expression in a −2088 transgenic mouse. Total RNA was extracted from the indicated tissues of a mouse of the 50-3 lineage (bearing the −2088 transgene shown in Fig. 1A) described in Materials and Methods. Six micrograms of each RNA was resolved by electrophoresis on a 1.5% agarose–glyoxal gel, transferred to a nylon membrane, and hybridized with a ^{32}P-labeled hGH cDNA probe. After exposure to XAR-5 film at −70°C with intensifying screens for 2.5 h (A) and 16 h (B), the membrane was stripped and rehybridized with a ^{32}P-labeled PEPCK cDNA probe and exposed to film for 3 h (C) and 40 h (D).

of factors. First, hGH mRNA is approximately an order of magnitude more stable than PEPCK mRNA (4, 36). Second, the site of transgene integration, which is random, can have profound effects on the level of its expression as a result of endogenous regulatory elements near the integration site (52). Third, PEPCK is localized in minor cell types in some tissues such as lung and skeletal muscle (75); hence, PEPCK mRNA levels measured in whole tissue extracts of lung and skeletal muscle may be at or below the detection limit, whereas hGH mRNA is not. Finally, additional *cis*-acting elements involved in control of the PEPCK gene might lie outside of the −2088 to +69 bp region tested.

To control for the effects of transgene integration site, expression of the −2088 PEPCK-hGH transgene was quantified in 10 transgenic mice in a solution hybridization assay. As shown in Fig. 4, a total of 10 founders (or their offspring) gave an overall pattern of expression from the −2088 transgene that was qualitatively similar to that shown in the Northern analysis (Fig. 3). In addition, low levels of hGH mRNA were detected in heart and skeletal muscle of several mice. Because no two mice are likely to have the same integration site, the average level of transgene expression determined from multiple mice likely reflects the *cis*-acting elements present within the transgene.

We also determined whether expression of the −2088 transgene was quantitatively correct in the tissues surveyed. If all of the *cis*-acting elements that control PEPCK expression are included in the −2088 transgene, then the expected hGH/PEPCK mRNA ratio in a given tissue should be directly proportional to the ratio of their half-lives. The reported half-lives suggest that hGH mRNA is approximately an order of magnitude more stable than PEPCK mRNA (4, 36), and we made the initial assumption that this ratio would hold constant for all transgenic mouse tissues that express these two mRNA species. Transgene expression in liver, white fat, ileum, jejunum, skeletal muscle, and possibly heart and colon (Fig. 4) were close to the predicted values of 4,450 ± 890, 1,070 ± 420, 30 ± 10, 50 ± 10, 30 ± 20, 90 ± 40, and 70 ± 30 molecules per cell, respectively. In contrast, expression in kidney (153 ± 100) and brown fat (300 ± 160) was significantly lower than expected (2,250 ± 430 and 3,740 ± 1,290, respectively), and expression in lung (50 ± 20) and gonad (30 ± 30) was higher than expected (0 for both).

Cellular localization of hGH. Immunohistochemistry was used to analyze the precise cellular distribution of transgene expression within tissues surveyed for hGH mRNA. Animals used for these studies were second- and third-generation progeny of the 50-3 line (−2088 transgene). hGH immunoreactivity was evident in liver, kidney, white adipose tissue, brown adipose tissue, ileum, jejunum, colon, lung, vagina, and sublingual gland (Fig. 5 and 6). hGH-specific staining was unambiguous in these 10 tissues, as there was no staining when nonimmune serum was used as a control (data not shown). In liver (Fig. 5A and B), hGH staining was restricted to hepatocytes in the same pattern as that for PEPCK (2, 75): a decreasing gradient of hGH immunoreactivity from the periportal to the perivenous parenchymal cells. A gradient of expression was also found in the renal cortex, where hGH was localized to the proximal tubule epithelia of the kidney cortex, with decreasing reactivity in the medulla (Fig. 5C and D). This staining pattern is restricted to the same cell type and has the same gradient as does renal PEPCK (75). Staining of hGH in the ileum, jejunum, and colon was localized to the epithelial cells lining the villi; staining was more intense in the ileum than in the jejunum and colon (Fig. 5E to H; Fig. 6A and B). There was a gradient of expression that was highest toward the tips of the villi and decreased toward their bases. This same pattern occurs with immunohistochemical staining for PEPCK (75). However, the strict epithelial localization of hGH was in contrast to the staining of PEPCK, which is localized to both epithelial cells and smooth muscle cells along the entire digestive tract (75). Both white and brown adipose tissue and ductal epithelial cells in sublingual gland had identical staining patterns with anti-PEPCK (75) and anti-hGH (Fig. 6E, F, and H). These observations suggest that the region of PEPCK DNA between −2088 and +69 bp contain *cis*-acting elements that direct cell-specific expression of PEPCK in hepatocytes, proximal tubular epithelia of the renal cortex, small intestinal epithelium, colonic epithelium, and white and brown adipocytes.

Finally, low levels of immunoreactive hGH were localized to the vascular smooth muscle of the lung and the muscular wall of the vagina in the −2088 transgenic mice (Fig. 6C, D, and G). This finding suggests the presence of cell-specific *cis*-acting elements in these tissues, as PEPCK immunoreactivity is also found in these same cell types (75). We were unable to confirm the cellular localization of hGH (in excess of background) in adrenal gland, bladder, brain, heart, stomach, pancreas, skeletal muscle, and parotid gland, tissues which exhibit hGH mRNA or PEPCK immunoreactivity (75).

Low-resolution mapping of tissue-specific *cis*-acting elements by deletion analysis. The tissue-specific expression of four truncated PEPCK-hGH constructs was examined in transgenic mice to more precisely map the locations of sequences directing multitissue expression of the PEPCK

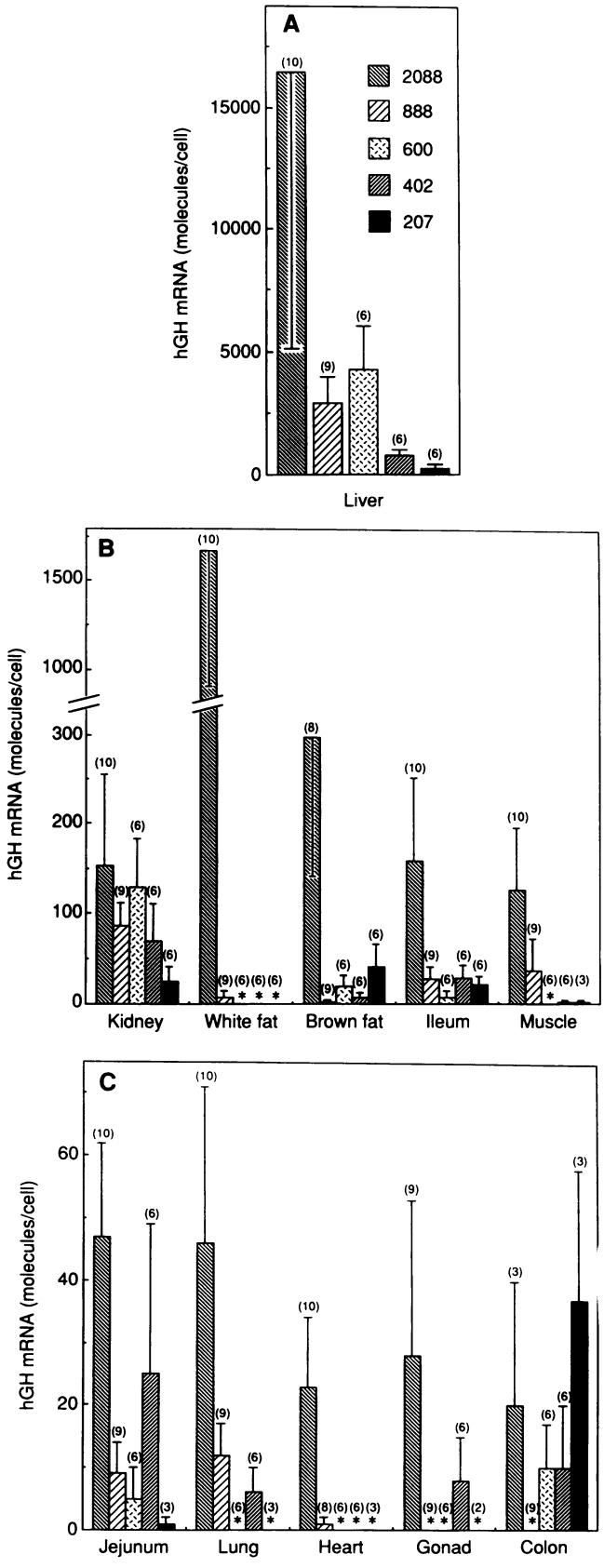


FIG. 4. Tissue survey of hGH mRNA levels in PEPCK-hGH transgenic mice. hGH mRNA was measured by solution hybridization as described in Materials and Methods. Tissues are from individual founder or offspring mice (lines were established with some −2088 and −402 constructs). The data are presented as means ± standard errors. The number of founder transgenic animals that contributed to each group is shown in parentheses; transgenic animals which had no detectable hGH mRNA were not included in the calculations. The total number of transgenic animals in each group was 12 with the −2088 transgene, 11 with the −888 transgene, 11 with the −600 transgene, 6 with the −402 transgene, and 9 with the −207 transgene. Asterisks indicate that hGH mRNA was undetectable. Not shown are data for spleen and brain, as they contained neither PEPCK nor hGH mRNA. Tissues are grouped in panels A to C according to their hGH mRNA content; note the different ordinate scales in the three panels. Skeletal muscle was isolated from the hind limb, white fat was from ovarian or epididymal fat pads, and brown fat was from the suprascapular regions.

gene. Expression of these additional transgenes (−888, −600, −402, and −207 [Fig. 1A]) is illustrated in Fig. 4.

Deletion of sequences between −2088 and −888 bp had dramatic consequences, the most striking of which was the loss of expression in white adipose tissue in all but one of 9 mice (a total of 11 −888 mice were examined, including 2 that did not express the transgene in any tissue). There was a similar loss of expression in brown fat: only two of the nine −888 mice expressed detectable hGH mRNA. The expression of hGH mRNA in white and brown fat was low (less than 5% of that from the −2088 transgene) in each of these three mice. Expression in heart and skeletal muscle was also markedly reduced; the levels of hGH mRNA were detectable in only one of the −888 mice. Furthermore, expression of hGH in the ovary, which occurred in three of five −2088 females, was not detected in any of the eight female −888 mice. Lastly, average expression of the transgene in liver, ileum, jejunum, colon, and lung was reduced about fivefold by deletion to −888 bp; however, these decreases were not statistically significant. Kidney was the only organ that was unaffected by this deletion. Together, these results suggest that the DNA between −2088 and −888 bp contains elements essential for expression in white and brown fat, heart, skeletal muscle, and ovary. This region may also contain an element(s) that augments expression in liver, ileum, jejunum, colon, and lung.

The truncation of PEPCK sequences to −600 resulted in relatively minor changes (compared with the −888 mice) in the tissue distribution of hGH mRNA (Fig. 4). While expression of hGH was maintained in liver and kidney, the low levels of expression that remained in skeletal muscle, lung, and heart of the −888 mice were completely abolished in the −600 mice. Perhaps the putative regulatory elements for these tissues are several in number and span the −888 deletion endpoint. There were slight increases in −600 transgene expression (compared with the −888 transgene) in brown fat and colon. This finding suggests that the −888 to −600 bp region might contain a weak inhibitory element that functions in brown fat and colon.

Deletion of PEPCK 5′ DNA to −402 bp had no significant effect (compared with the −600 transgene) on expression in tissues other than liver, in which a four- to fivefold decrease in the mean level of hGH mRNA per cell occurred. This finding suggests that the −600 to −402 bp region of PEPCK DNA contains *cis*-acting elements that are important for quantitative expression of PEPCK in liver; they are not, however, essential for liver-specific expression. *cis*-acting elements to which hepatic accessory factors 1 and 2 bind are located in this region (Fig. 1B).

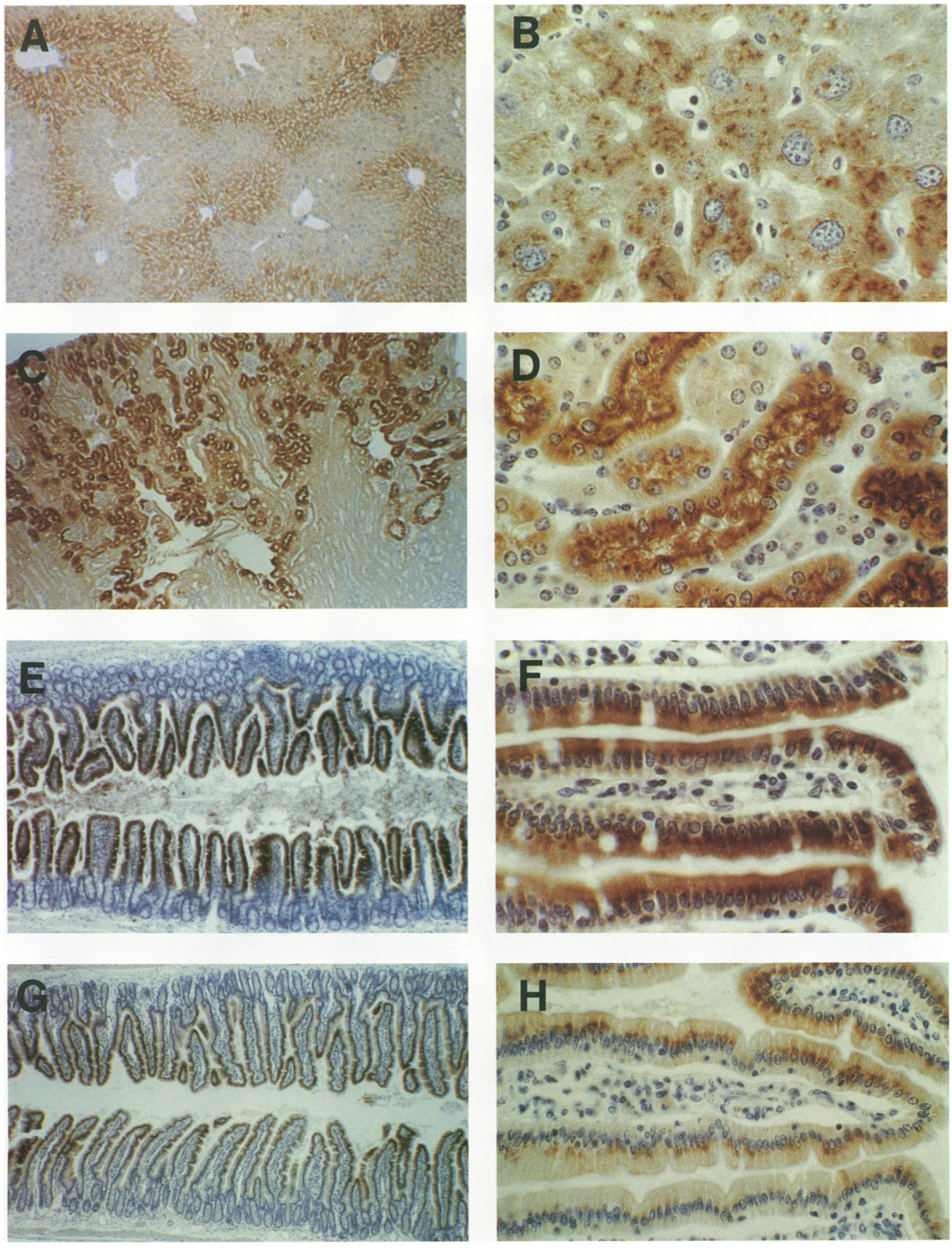
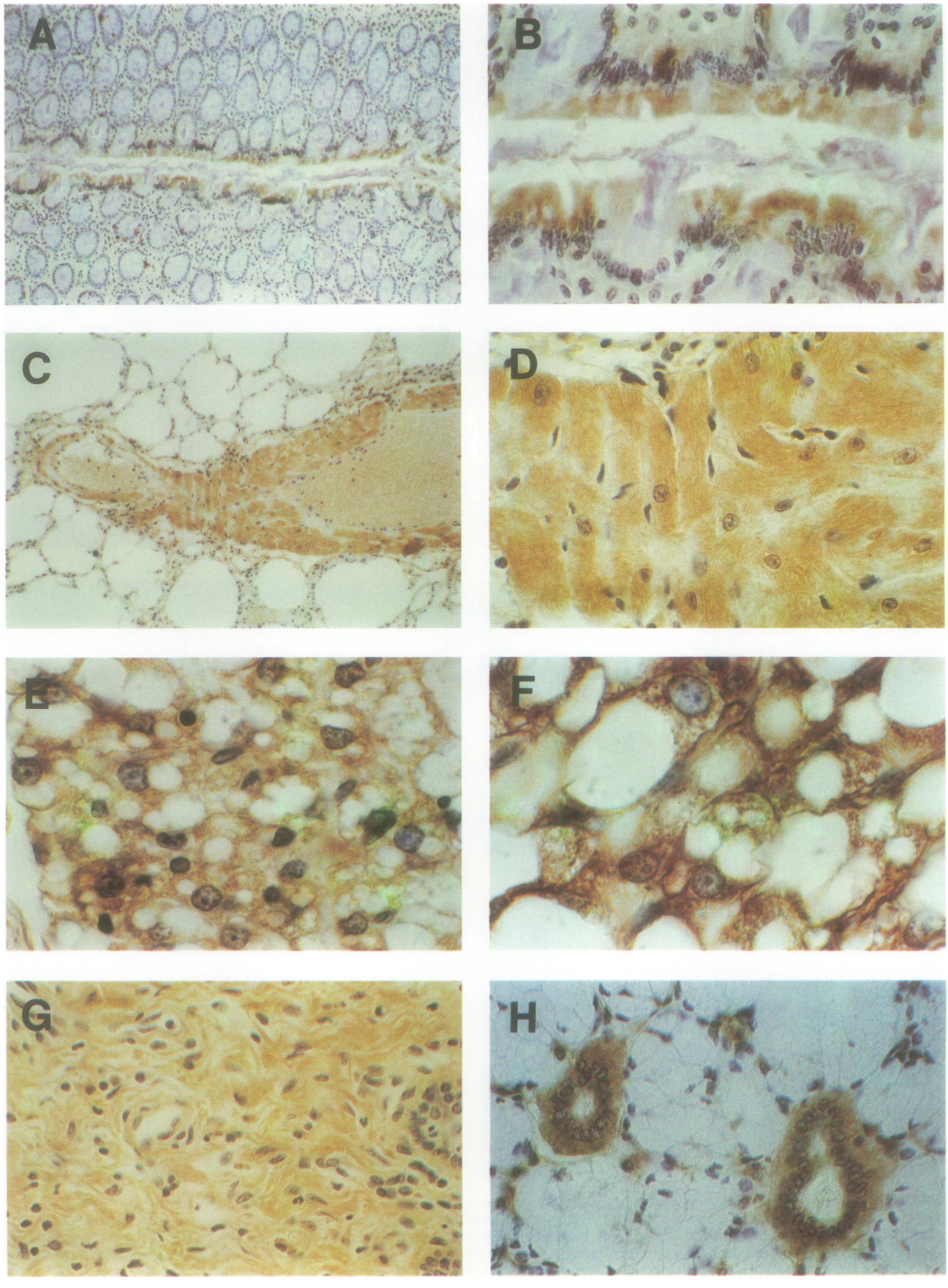


FIG. 5. Immunocytochemical localization of hGH in liver, kidney, ileum, and jejunum. Organs from a 4-month-old −2088 mouse were fixed, embedded, sectioned, and incubated with a rabbit anti-hGH primary antibody and a peroxidase-conjugated goat anti-rabbit secondary antibody; hGH staining is brown, and hematoxylin counterstaining is blue. (A) Liver (magnification, ×37); (B) periportal region of liver (×324); (C) kidney cortex and medulla without counterstain (×37); (D) kidney proximal tubule (×360); (E) distal ileum (×43); (F) ileal villi with lumen to the right (×288); (G) proximal jejunum (×43); (H) jejunal villi with lumen to the right (×288).


A
B
C
D
E
F
G
H

Truncation of PEPCK to −207 bp had minimal effects compared with the −402 construct; only two tissues, liver and kidney, exhibited significant changes in transgene expression. The content of hGH mRNA decreased fourfold in liver and threefold in kidney. This decrease is most evident when viewed in terms of the number of mice expressing hGH mRNA: while all of six and four of six −402 mice had detectable levels of hGH mRNA in liver and kidney, respectively, only three of nine −207 mice had detectable hGH mRNA in either liver or kidney.

Correct developmental expression of the −2088 transgene occurs in liver but not kidney. Developmental expression of the PEPCK gene in liver is induced at parturition in response to changes in the insulin/glucagon ratio (23); however, it can be hormonally induced 6 days prior to birth. It seems likely that the correct ontogenic expression of PEPCK requires the interaction of both differentiation-specific *trans*-acting factors with hormone responsive *cis*-acting elements. Thus, an additional test for the presence of essential tissue-specific *cis*-acting elements in a transgene is to determine whether the pattern of developmental activation is correct. We assayed PEPCK and hGH mRNAs in fetal and neonatal mice of the 50-3 line to determine whether the −2088 PEPCK-hGH transgene directed the correct pattern of developmental expression in liver and kidney (Fig. 7).

As expected, the levels of hepatic PEPCK mRNA were low during the fetal period, increased at parturition, and remained constant thereafter. Expression of the transgene in liver followed a very similar developmental profile. There was an abrupt induction of hGH mRNA at birth, with averages of 350 and 1,100 molecules per cell at days 1 and 7, respectively. The somewhat delayed induction and higher levels of hGH mRNA (compared with PEPCK mRNA) are likely explained by the greater stability of hGH mRNA. Half-lives of greater than 1 day for hGH mRNA and much less than 1 day for PEPCK mRNA can be inferred from the kinetics of induction in Fig. 7A. The similarity between PEPCK gene activation and transgene activation during liver development suggests that the region between −2088 and +69 bp contains elements essential for liver-specific expression (as well as responsiveness to insulin and glucagon).

In contrast to the liver, the developmental pattern of expression from the −2088 transgene in kidney did not correspond with expression of the PEPCK gene. As shown in Fig. 7B, the hGH mRNA level was approximately 24 molecules per cell 4 days prior to parturition and increased to a peak of approximately 210 molecules per cell at day −1. There was a subsequent decline in hGH mRNA so that adult levels of approximately 40 molecules per cell were attained by day 7. In contrast, PEPCK mRNA remained extremely low during the fetal and early neonatal period and showed a marked induction between days 1 and 7. The abnormal level of transgene expression in kidney versus liver in several mice (Fig. 4A and B) suggests that this pattern is not unique to the 50-3 lineage and that additional control elements outside of the −2088 to +69 bp region are required to achieve both the correct magnitude and correct developmental pattern of PEPCK expression in kidney.

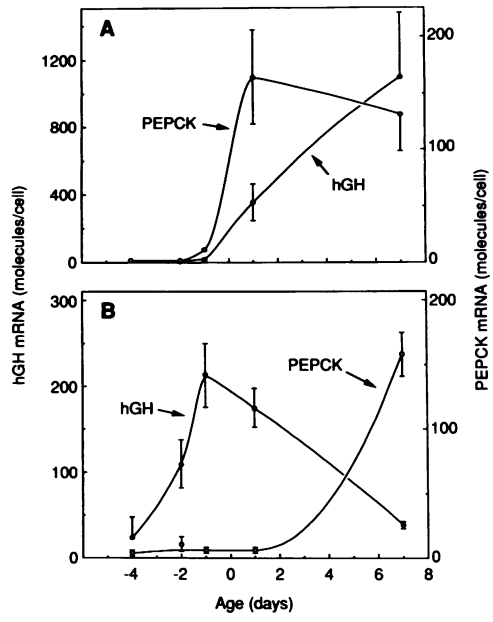


FIG. 7. Developmental analysis of PEPCK and hGH expression in liver and kidney of −2088 transgenic mice. Transgenic mice of the 50-3 lineage (bearing the −2088 transgene shown in Fig. 1A) described in Materials and Methods were analyzed for hepatic (A) and renal (B) PEPCK mRNA and hGH mRNA levels by solution hybridization. Values are means ± standard errors, with a minimum of four organs analyzed for each time point. Day −4 kidney determinations were made on two groups of three kidneys per group. Day 0 represents the day of parturition.

Regulation of transgene expression by glucocorticoids. Transcription of the PEPCK gene responds to glucocorticoid hormones in a tissue-specific pattern; it is induced in liver and kidney and repressed in adipose tissue (48). The elements necessary for response to glucocorticoids in liver are thought to span a region of ~110 bp between −455 and −350 bp (35) (Fig. 1B). The −2088 transgene thus contains an intact glucocorticoid response unit and should be induced by dexamethasone, whereas the −402 transgene should be unresponsive. We tested the response of the −2088 and −402 transgenes to dexamethasone in mice of the 50-3 and 99-3 lines, respectively (Table 1). Following dexamethasone treatment of the −2088 mice, PEPCK mRNA levels in the liver increased twofold and hGH mRNA showed a similar threefold increase. In kidney, PEPCK mRNA increased threefold, while hGH mRNA increased fivefold. The induction of PEPCK expression in jejunum was of greater magnitude than that in liver, with a fivefold increase in PEPCK mRNA from 85 to 413 molecules per cell. At the corresponding time, hGH mRNA increased sevenfold. In adipose tissue, dexamethasone caused a fivefold decrease in PEPCK mRNA, while hGH mRNA did not exhibit any significant change (Table 1). Dexamethasone treatment of the −402 mice did not result in any changes in hepatic, renal, or

FIG. 6. Immunocytochemical localization of hGH in colon, lung, brown fat, white fat, vagina, and sublingual gland. Additional organs were prepared from the mouse used for Fig. 3 and 5; experimental details are described in the legend to Fig. 5. (A and B) Distal colon (magnifications, ×87.5 and ×315, respectively); (C) aveoli and vasculature of lung (×74); (D) higher magnification of vascular smooth muscle of lung (×280); (E) suprascapular brown fat (×385); (F) gonadal fat pad (×420); (G) vaginal smooth muscle (×210); (H) sublingual gland (×263).

adipose tissue hGH mRNA concentrations. The effects of dexamethasone on transgene expression in jejunum could not be assessed, as hGH mRNA levels were below the sensitivity of the assay. These results suggest that sequences required for glucocorticoid responsiveness in liver, kidney, and small intestine are located between −2088 and −402 bp. In contrast, either the inhibitory element(s) required for dexamethasone response in adipose tissue is absent from the −2088 and −402 constructs or the regulation of the fusion genes in this tissue is affected by the site of transgene integration.

Regulation of transgene expression by diet. Expression of the PEPCK gene in liver is reciprocally regulated by plasma glucagon and insulin levels (23). While both the −2088 and −402 transgenes contain a cAMP response element, the −402 transgene lacks an insulin response sequence (Fig. 1B), and so differences in the dietary responsiveness of the two constructs were expected. To test this prediction, we compared the effects of feeding animals either a high-carbohydrate diet or a high-protein diet on liver and kidney PEPCK and hGH mRNAs (Table 2). In the −2088 mice, liver PEPCK mRNA levels were approximately twofold higher in mice fed a high-protein diet than in mice fed the high-carbohydrate diet, and in the same animals hGH mRNA levels increased approximately fourfold. In kidney, PEPCK mRNA and hGH mRNA were induced about 3- and 11-fold, respectively, by feeding the mice a diet high in protein. The effect of these two diets on PEPCK mRNA in liver and kidney of the −402 mice was similar to that in the −2088 mice. However, the response of the transgene in liver was far more dramatic, as there was a >13-fold (467 to 6,149 molecules per cell) increase in protein-fed versus carbohydrate-fed mice. These results suggest that diet-responsive elements (probably cAMP and/or insulin response elements) are located between −402 and +69 bp and that sequences between −402 and −2088 bp probably modulate the effects of the more promoter-proximal elements. Indeed, O'Brien et al. (49) have shown that there is an additional, previously unmapped insulin responsive sequence in the promoter-proximal region between −402 and +69 bp.

DISCUSSION

Study of the PEPCK gene provides a unique model of genes expressed in multiple tissues and regulated by numerous hormones, environmental factors, and cell-cell interactions (5, 7, 11, 15, 24, 43, 45, 54, 68, 69, 71, 72). For this study, we sought to determine whether multiple tissues, especially liver, kidney, and fat, utilize common or unique tissue-specific *cis*-acting elements to direct and regulate the PEPCK gene. Since the only available cell lines that express PEPCK were hepatoma cells and adipoblasts, we used transgenic mice to assess expression of PEPCK fusion genes in diverse cell types and during development. A total of 49 transgenic founder mice from a series of five PEPCK-hGH fusion genes were made; 37 of these mice expressed the PEPCK/hGH transgene in one or more tissues. Our results indicate that deletion of various upstream regions of PEPCK DNA had differential effects on transgene expression in liver, kidney, and fat as well as in various other tissues. Although this finding does not rule out the possibility that different cell types share some common *cis*-acting elements, it does strongly suggest that different cell types must utilize different *cis*-acting elements to direct and regulate the PEPCK gene.

Control of PEPCK expression in liver. Our results indicate that the region between −2088 and +69 bp of the PEPCK gene is both necessary and sufficient to direct hepatocyte-specific, developmental, hormonal, and dietary regulation of the PEPCK gene. To more precisely localize the *cis*-acting elements that direct liver-specific expression, we analyzed mice that contained various deletions of PEPCK 5′ DNA. Two reports by McGrane et al. (44, 45), who produced transgenic mice with five different PEPCK 5′ regions fused to the bovine growth hormone gene, indicate that there are two liver elements, one between −460 and −355 bp and the other between −355 and −174 bp. Our results suggest that there are at least three elements or domains in this proximal region, one between −600 and −402 bp, the second between −402 and −207 bp, and the third between −207 and +69 bp. Although the −207 to +69 bp region directed only a very low level of expression (only two of six mice had detectable hGH mRNA, and neither mouse exhibited enhanced growth), it clearly exhibited functional ability in liver, and thus we propose that it contains basal, liver-specific promoter elements. Expression from the −207 transgene was not surprising, since several groups (53, 57, 59, 69) have described elements in this region that support basal promoter activity in cultured hepatoma cells.

Several *trans*-acting factors (C/EBP, HNF-1, NF-1, CREB, and glucocorticoid receptor; Fig. 1B) have been shown to interact with *cis*-acting elements upstream of the PEPCK promoter in liver. Both CREB and NF-1 are present in a variety of tissues and thus probably support transcription in numerous tissues (57, 59, 69). However, since both CREB and NF-1 are families of transcription factors, various members of each family could have functional differences and tissue-specific distributions (25, 62). Thus, neither should be ruled out as contributors to tissue-specific expression. C/EBP binds a total of four sites upstream of the PEPCK promoter (53), has an expression pattern similar to that of PEPCK (liver, kidney, fat, and intestine but not spleen, brain, or testis), and appears in liver just before birth when PEPCK is activated (8, 69). Thus, C/EBP could participate in driving PEPCK expression in hepatocytes as well as intestine, fat, kidney, and lung. HNF-1 expression is restricted to liver, kidney, and intestine (37). In liver, it binds to the upstream regions of several liver-specific genes such as the fibrinogen and α1-antitrypsin genes (19). The observation that a −174 to +69 bp PEPCK-bovine growth hormone chimera is completely inactive in transgenic mice (44) may be particularly meaningful when compared with the data for our transgene. Specifically, the only obvious difference between our −207 transgene and the −174 transgene is an HNF-1 binding site (Fig. 1B). Thus, HNF-1 could act alone or in combination with another, more proximal factor(s) such as CREB, NF-1, C/EBP, or the factor that binds at site X (indicated in Fig. 1B) to impart basal liver-specific expression. Indeed, X could be a binding site for HNF-3, another hepatocyte-enriched *trans*-acting factor, as 11 of 14 bases from −156 to −143 are identical to bases in the sequence of a known HNF-3 binding site (GATTATTGACT TAG) (73).

Our observations along with published reports clearly show that additional elements upstream of position −207 are involved in expression of PEPCK in hepatocytes. These upstream elements are also candidates for control of liver-specific expression, though they may function only in hormonal regulation and nonspecific enhancement. The region between −402 and −207 bp includes two glucocorticoid receptor binding sites, three C/EBP binding sites, and at least two binding sites for unidentified transcription factors.

The third domain (from −600 to −402 bp) contains the two glucocorticoid response unit accessory elements, a retinoic acid response element, and the insulin response sequence indicated in Fig. 1B (35, 43, 49). We do not know whether the elements in this third domain function as liver-specific elements, though it is interesting to note that the accessory factor region (accessory factors 1 and 2) contains three HNF-4/LF-A1 binding site homologies [(G/T)GC(A/T)A(A/G)G(G/T)(T/C)CA(T/C)] spaced so that their orientation would be on the same face of the double helix (69). HNF-4/LF-A1 is a liver-specific transcription factor associated with several liver-specific genes, including the α1-antitrypsin, apolipoprotein CIII, and haptoglobin genes (31, 41, 66). Finally, a fourth domain that stimulates PEPCK transcription in liver may reside between −888 and −2088 bp. McGrane et al. (44, 45) presented evidence supporting this possibility, as they found stimulatory elements between −2088 and −460 bp.

Control of PEPCK expression in kidney. Expression of hGH mRNA in the kidney was affected by two regions of the PEPCK promoter: −402 to −207 bp and −207 to +69 bp. However, the time of developmental activation and the overall level of transgene expression in adults were incorrect with all of the transgenes used (including the −2088 construct). McGrane et al. (45) also found that a −2088 transgene was underexpressed in adult mice. These results suggest that at least three domains direct PEPCK expression in kidney. The first element is between −207 and +69 bp (which exhibited basal promoter activity as in the liver), the second is between −402 and −207 bp (which stimulated expression), and the third lies outside of the −2088 to +69 bp region. The missing element(s) could be involved in regulating either a transcriptional or a posttranscriptional event. An example of a posttranscriptional control sequence would be the *cis*-acting element in the 3′ nontranslated region of PEPCK mRNA which was recently reported to control the mRNA's turnover rate (55). In either case, it will be necessary to design additional transgenes containing more extensive PEPCK DNA sequences and other reporter genes to test such possibilities.

Despite the missing elements, the cellular localization of hGH corresponds exactly with that of PEPCK as determined by immunohistochemistry; both proteins were in the proximal convoluted tubular epithelial cells of the renal cortex. This observation, taken together with the fact that there were no differences in expression of the −2088 and −402 transgenes, indicates that the promoter proximal elements are cell type specific and that any missing elements serve only to enhance and modulate renal PEPCK expression. The probable location of the renal cell-specific element(s) is not immediately obvious, since very little is known about renal control elements and their putative binding sites on the PEPCK gene. HNF-1 and low levels of C/EBP are expressed in kidney (8, 37); hence, these factors may be involved. However, it is not known whether HNF-1 and C/EBP are expressed in the same cells that express renal PEPCK. HNF-4 is also expressed in kidney (18), although, as mentioned above, its putative binding sites are localized upstream of −402 bp and thus it seems to be an unlikely participant in kidney-specific PEPCK expression.

Control of PEPCK expression in white and brown adipose tissues. Transgene expression in white fat was detectable only in mice bearing the −2088 construct, suggesting that a white fat-specific *cis*-acting element(s) is located between −2088 and −888 bp. Expression in brown fat was also highly stimulated by this same −2088 to −888 bp region. There were, however, two effects unique to brown fat (compared with white fat): (i) the region between −888 and −600 was slightly inhibitory, and (ii) low levels of expression occurred with the remaining constructs, including the −207 transgene. Thus, the two types of fat differ in that a white fat element is exclusively localized distally, between −888 and −2088 bp, while more proximal elements support basal promoter activity in brown fat.

Our localization of fat-specific elements differs with results of two previous reports. Benvenisty et al. (6), using transient expression assays in cultured 3T3-F442A adipoblasts, found that the region between −362 and −98 bp stimulated reporter expression and proposed that this was due to a fat-specific *cis*-acting element. Such a discrepancy could be due to differential requirements for cell-specific expression in transfected cells versus transgenic mice (26, 60, 67). In the second report, McGrane et al. (45), using transgenic mice as an assay system, detected bovine growth hormone mRNA in white fat of animals bearing 2,088 bp of upstream PEPCK DNA and in one mouse bearing 460 bp of upstream DNA; they detected no expression in any −355 mice. This finding led them to propose that a fat element was localized between −460 and −355 bp. However, we propose the existence of a distal element between −2088 and −888 bp because of the striking expression pattern that we observed: hGH mRNA (a predicted average of 1,670 molecules per cell) was in white fat of 7 of 9 −2088 mice, only 1 (59 molecules per cell) of 9 −888 mice, and none of the remaining 18 transgenic mice.

Control of PEPCK expression in other tissues. Although the levels of transgene expression in heart, skeletal muscle, and ovary are very low in comparison with levels in liver, kidney, and fat, they are detectable and reproducible among animals. This finding suggests that, as in white fat, elements between −888 bp and −2088 bp are necessary for expression in these three tissues and organs.

The patterns of expression of the transgenes in the ileum indicate that basal promoter elements are localized between −207 and +69 bp and that additional stimulatory elements are present between −888 and −2088 bp. However, the low levels and high variance preclude making conclusions regarding control of expression in jejunum, colon, and lung. In general, the patterns of expression and regulation of PEPCK gene expression in gut are similar to those in liver. This similarity may stem from their similar embryonic origins from endoderm. If this is correct, then the control mechanisms are likely similar if not identical.

Tissue-specific regulation of PEPCK expression by glucocorticoids and diet. In view of the foregoing evidence for distinct tissue-specific control elements, we reasoned that there may also be tissue-specific responses to diet and glucocorticoids. Therefore, we tested the effects of dexamethasone and diet on the −2088 and the −402 transgenes. In agreement with Imai et al. (35), who showed that the glucocorticoid response unit consists of the two accessory factor elements plus the two glucocorticoid receptor binding sites (Fig. 1B), the −2088 transgene was induced by dexamethasone in the liver, whereas the −402 transgene did not respond. A similar response pattern occurred in kidney, suggesting that the same stimulatory glucocorticoid response unit functions in both organs. In contrast, dexamethasone treatment in fat did not elicit a response by either transgene, whereas PEPCK mRNA levels decreased. The simplest explanation for this observation is that adipocytes utilize a unique fat-specific *cis*-acting element for this inhibitory response.

The −2088 and −402 transgenes displayed interesting

differences between liver and kidney in their responses to feeding with high-protein versus high-carbohydrate diets. Specifically, liver hGH mRNA was increased 4-fold in the −2088 mice and 13-fold in the −402 mice fed a high-protein diet. In kidney, there was an 11-fold increase in the −2088 mice and no significant change in the −402 mice. These differences might be positional effects, though it seems likely to be due to tissue-specific differences in the *cis*-acting elements involved in dietary response.

Summary. Previous studies indicated that most *cis*-acting elements necessary for expression and regulation of the PEPCK gene are located within a pleiotropic control domain of approximately 500 bp of upstream DNA. We have expanded the functions attributable to this domain by showing that tissue-specific basal promoter elements for liver, kidney, brown fat, and intestine are between −207 and +69 bp, and two additional domains at −600 to −402 and −402 to −207 bp augment expression in liver. However, we have also shown that elements between −2088 and −888 bp are essential for tissue-specific expression in white and brown adipose tissues and ovary, and these elements may also enhance expression in liver. Moreover, we demonstrated that the tissue-specific *cis*-acting elements between −2088 and +69 bp direct temporally correct developmental activation in liver, and these elements are, in fact, cell type specific in hepatocytes, proximal tubular epithelia of the renal cortex, and intestinal epithelium (with normal expression gradients within each tissue). Finally, in kidney we were unable to obtain temporally correct developmental activation and normal levels of adult transgene expression, and in fat we did not observe the inhibitory glucocorticoid response. Hence, elements outside of the −2088 to +69 bp region of PEPCK DNA are required for normal function in kidney and white fat. We have thus shown that different cell types utilize a striking assortment of *cis*-acting elements to direct and regulate the PEPCK gene. This newfound diversity further enhances the utility of the PEPCK gene as a model for studying the mechanisms by which genes that are expressed in multiple cell types are differentially regulated.

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