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Separate cis-regulatory elements confer expression of phospho*enol*pyruvate carboxykinase (GTP) gene in different cell lines

(cell specificity/gene expression/hepatoma cells/adipocytes/DNA-mediated gene transfer)

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ABSTRACT The gene encoding cytosolic phospho*enol*pyruvate carboxykinase (GTP) [PEPCK; GTP:oxaloacetate carboxy-lyase (transphosphorylating), EC 4.1.1.32], a key enzyme in gluconeogenesis and glyceroneogenesis, is expressed in tissues that arise from different embryonal origins: the gluconeogenic liver arises from endoderm, whereas the gluconeogenic kidney cortex and glyceroneogenic adipose tissue arise from the mesoderm. To identify the cis-regulatory elements conferring the differential gene expression, PEPCK chimeric genes were transfected into two rat hepatoma cell lines (H4IIEC3 and HTC-M1.1) and mouse adipocytes (3T3F442A), which express the endogenous gene, and into myoblasts and preadipocytes, which do not express it. The results demonstrate that 597 base pairs of the 5′ flanking region of the PEPCK gene are sufficient to confer cell-specific gene expression in the PEPCK-expressing hepatoma cells and adipocytes. However, different elements within this 597-base-pair region enhance the gene expression in the hepatoma cells (endoderm) and adipocytes (mesoderm). In the hepatocytes, expression is conferred by two elements—one 5′ of position −362 and the other 3′ of position −98 with respect to the transcription start site. The region in between these two elements (from −362 to −98), which seems to inhibit the gene expression in the hepatocytes, confers enhanced expression in the adipocytes. Moreover, the distal positive regulatory element of the hepatocytes seems to be orientation and PEPCK promoter dependent. In contrast, the positive regulatory element of the adipocytes seems to act as a more typical enhancer. These results suggest that separate cis-regulatory elements confer cell-specific expression of the PEPCK gene.

Major progress in unraveling mechanisms underlying tissue differentiation has been made by studying the control of expression of genes that are exclusively expressed in a single cell type (1). Yet, it is intriguing to study differentiation of another class of genes where a single copy gene is specifically expressed in several different tissues, rather than in just one tissue. Such instances should reconcile how different tissues express the same gene, from a single promoter, in their independent course of differentiation. Up until now, various instances have been studied in *Drosophila* (2–11), but only a few have involved mammalian genes (12–14).

The rat phospho*enol*pyruvate carboxykinase (GTP) [PEPCK; GTP:oxaloacetate carboxy-lyase (transphosphorylating), EC 4.1.1.32] gene, which codes for a key enzyme in gluconeogenesis, provides an excellent model to explore specific gene expression in several tissues. This single copy gene is specifically expressed in three tissues that arise from different embryonal origin: liver (endoderm) and kidney cortex and adipose tissue (mesoderm) (for reviews, see refs. 15–17). We have recently shown that in all three tissues the gene is transcribed from the same promoter (18). Moreover, the onset of the gene transcription in the liver occurs late in development, later than the early embryonic differentiation of this tissue (19). Previous studies from our laboratory (20, 21) have shown close correlation between the methylation pattern and the tissue-specific and developmental regulation of the gene expression, indicating that the gene is methylated in nonexpressing tissues and undermethylated in PEPCK-expressing tissues. However, in all three PEPCK-expressing tissues, the gene is similarly undermethylated (ref. 20; H.N. and L.R., unpublished results). In the present study, we have demonstrated that 597 base pairs (bp) of the PEPCK gene 5′ flanking region are sufficient to confer specific gene expression in PEPCK-expressing hepatoma cells (endoderm) and adipocytes (mesoderm). However, distinct elements within this 597-bp region enhance the gene expression in these two cell types.

MATERIALS AND METHODS

Plasmids Used in Transient Expression Assays. pSV2cat is a plasmid harboring the structural gene encoding the bacterial chloramphenicol acetyltransferase (CAT) fused to the simian virus 40 (SV40) early promoter–enhancer region in pBR322 (22). The 597-pck-CAT chimeric gene contains a PEPCK gene sequence spanning from position +69 (*Bgl* II site) to position −597 (*Hin*dIII site), relative to its transcription start site. The 666-bp *Hin*dIII–*Bgl* II fragment was inserted into the *Hin*dIII and *Sma* I sites in the multicloning sites of p106-CAT (23) in front of the structural CAT gene. The 4600-pck-CAT gene was constructed by insertion of the 4-kilobase *Hin*dIII fragment, residing between positions −4600 and −597 of the PEPCK gene, into the *Hin*dIII site of 597-pck-CAT. A series of progressive deletion mutants were prepared by using appropriate restriction sites within the PEPCK gene sequence in 597-pck-CAT and the unique 5′ *Hin*dIII site. 597-362-pck-CAT and 362-597-pck-CAT were constructed by inserting a 236-bp *Hin*dIII–*Nde* I fragment (from position −597 to −362), in both orientations, into position −208 of the PEPCK promoter (see Fig. 1). The PEPCK-tk-CAT constructs were prepared by inserting 5′ flanking sequences of the PEPCK gene in front of the thymidine kinase (tk) promoter, driving CAT expression, in the plasmid pTE1 [obtained from M. Walker (24)].

Cell Lines and Transfection Conditions. The cell lines used were rat hepatoma H4IIEC3 cells (25) derived from Reuber H35 hepatoma (26), rat hepatoma M1.19-HTC cells (27) derived from Morris hepatoma (28), the L-8 rat myoblast cell line (29), and the 3T3-F442A mouse preadipocyte cell line (30). Conversion to adipocytes was induced, at cell confluence, by the addition of 0.5 mM 3-isobutyl-1-methylxanthine

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Abbreviations: PEPCK, phospho*enol*pyruvate carboxykinase; CAT, chloramphenicol acetyltransferase; SV40, simian virus 40; tk, thymidine kinase.

(Sigma) in a medium containing 10% fetal calf serum for 3 days. Subsequently, the cells were maintained in the presence of insulin at 20 milliunits/ml for 9 to 14 days until 50–80% conversion had been observed.

Twenty micrograms of supercoiled plasmids, except where indicated otherwise, was introduced to the cells as follows: H4IIEC3 hepatoma cells according to Ott *et al.* (31), M1.19-HTC hepatoma cells according to Miesfeld *et al.* (27), and myoblasts and 3T3-F442A preadipocytes in the presence of DEAE-dextran (32, 33) by using 30 μg of DNA for the preadipocytes. 3T3-F442A adipocytes were transfected by using electroporation according to Chu *et al.* (34). We have found that 200 volts and 100 μg of supercoiled plasmid with 10^7 cells per ml were optimal for the DNA transfer. CAT activity was determined with 100–150 μg of 12,000 × *g* supernatant protein according to Gorman *et al.* (22), with the modifications described by Ott *et al.* (31).

DNA Isolation and Southern Blot Hybridization. Cellular DNA was extracted according to Hewish and Burgoyne (35). Southern blot hybridization analysis (36) was performed with Nytran membranes (Schleicher & Schuell). Prehybridization and hybridization were carried out under the conditions recommended by the supplier by using DNA probes labeled with ^{32}P by nick-translation (37).

RESULTS

Cell-Specific Expression of the PEPCK Gene Is Conferred by 597 bp of the 5′ Flanking Region of the Gene. To identify the sequences that confer cell-specific expression of the rat cytosolic PEPCK gene, a 666-bp fragment of the PEPCK gene was linked to the bacterial CAT structural gene (see 597-pck-CAT in Fig. 1). This construct has been introduced into several cell lines that vary according to the expression of the endogenous PEPCK gene. These included two rat hepatoma cell lines that highly (H4IIEC3, ref. 24) and poorly (M1.19 HTC) express the PEPCK gene (38) and rat-derived L-8 myoblasts that do not express the gene. In parallel, the cells have been transfected with pSV2cat as a control for a promoter–enhancer whose function is not restricted to specific cell types. Determination of CAT activity revealed a differential pattern of expression of 597-pck-CAT in these cells, which is in accord with that of the endogenous PEPCK gene. This pattern clearly differed from the CAT activities driven by the SV40 promoter–enhancer, which were high and comparable in all three cell lines (Fig. 2 *A* and *C*). In each case, cellular DNA was extracted and analyzed by Southern blot hybridization, using the CAT sequence as probe, to monitor the amount of foreign DNA introduced into the cells. As shown in Fig. 2*B*, similar amounts of foreign DNA have been detected in PEPCK-expressing hepatoma cells (H4IIEC3) or non-expressing myoblasts (L-8) transfected with either 597-pck-CAT or SV2cat plasmids.

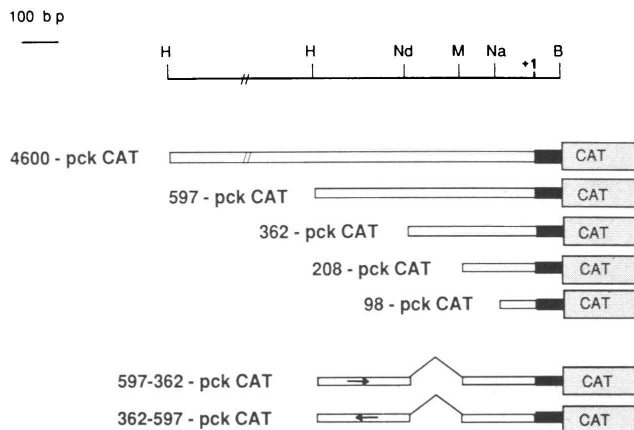

FIG. 1. Schematic illustration of PEPCK–CAT chimeric genes. (*Upper*) The 5′ end and flanking region of the PEPCK gene is presented with some of its restriction enzyme sites. H, *Hin*dIII; Nd, *Nde* I; M, *Mst* II; Na, *Nae* I; B, *Bgl* II. The transcription start site is indicated by +1. (*Lower*) PEPCK–CAT chimeric genes contain various 5′ flanking sequences and 69 bp of the end of the PEPCK gene inserted in front of the prokaryotic CAT gene. □, PEPCK gene 5′ flanking region; ■, 69 bp of the 5′ end of PEPCK gene; ▨, CAT structural gene. The size of the 5′ flanking sequences of the PEPCK gene in each construct is specified by the number of bp and the specific restriction site of its 5′ end: 4600-pck-CAT, 4.6 kb (up to the distal *Hin*dIII site); 597-pck-CAT, 597 bp (up to the proximal *Hin*dIII site); 362-pck-CAT, 362 bp (up to the *Nde* I site); 208-pck-CAT, 208 bp (up to the *Mst* II site); 98-pck-CAT, 98 bp (up to the *Nae* I site). 597-362-pck-CAT and 362-597-pck-CAT involve insertion of the sequence between positions −597 (*Hin*dIII site) and −362 (*Nde* I site) into position −208 (*Mst* II site) of the PEPCK–CAT chimeric gene in both orientations.

In addition to the liver (endoderm origin), the PEPCK gene is expressed in adipose tissue (mesoderm origin). Thus, mouse 3T3-F442A preadipocytes and adipocytes were transfected with either pSV2cat or 597-pck-CAT, and CAT activity was measured. pSV2cat was equally expressed in the two types of cells, whereas 597-pck-CAT was expressed only in the PEPCK-expressing adipocytes (Fig. 2*C*). Clearly, the 597 bp upstream of the transcription start site of the PEPCK gene are involved in controlling PEPCK cell-specific expression. Moreover, the CAT activities found by us correlated with the reported endogenous PEPCK activities in these various cell lines (15, 25, 38). For comparison, the published levels of PEPCK activities are given in a semiquantitative form (+, +/−, and − in Fig. 2*C*). To determine whether this 597-bp region is sufficient for directing the high level of expression in hepatoma cells, a 4000-bp fragment spanning positions −4600 to −597 of the PEPCK gene was inserted into the 597-pck-CAT construct (see 4600-pck-CAT in Fig. 1). Since 4600-pck-CAT does not yield an increased CAT activity (Fig. 3 *A* and *C*), we conclude that the 597 bp preceding the transcription start site of the PEPCK gene are sufficient to confer its cell-specific expression.

Separate cis Sequences of the PEPCK Gene Confer Its Cell-Specific Expression in Cells That Arise from Different Embryonal Origins. To better define the sequence(s) that confers cell-specific expression in the two different cell types that express the PEPCK gene (hepatoma cells and adipocytes), a series of deletions in the 597-bp region have been prepared (Fig. 1). As shown (Fig. 3 *A* and *C*), deletion of the distal region down to position −362, with respect to the transcription start site (362-pck-CAT), reduced by 10-fold the enhanced CAT activity in the H4IIEC3 hepatoma cells. Further deletion to position −208 (208-pck-CAT) had no additional effect (Fig. 3 *A* and *C*). However, deletion to position −98 (98-pck-CAT) revealed a partial enhanced activity (≈25% of that obtained with 597-pck-CAT) (Fig. 3*C*). These results suggest that the region 5′ to position −362 accounts for the enhanced gene expression in the hepatoma cells. To verify this possibility, the sequence between positions −362 and −597 was linked in both orientations to position −208 of 208-pck-CAT (597-362-pck-CAT in the correct and 362-597-pck-CAT in the reverse orientation, see Fig. 1) and introduced into H4IIEC3 hepatoma cells. Indeed, the results showed that insertion of this sequence restored about 50% of the enhanced activity, but only when placed in the correct orientation (Fig. 4).

In adipocytes, unlike the hepatoma cells, deletion of the distal region downstream to position −362 (362-pck-CAT) did not affect the activity as compared to that obtained with 597-pck-CAT. Deletion to position −208 (208-pck-CAT) reduced the activity by about 60%, and deletion to position −98 (98-pck-CAT) further reduced the activity to 15% of that of 597-pck-CAT (Fig. 3 *B* and *C*). It should be noted however that all the deletion mutants were negligibly expressed in L-8

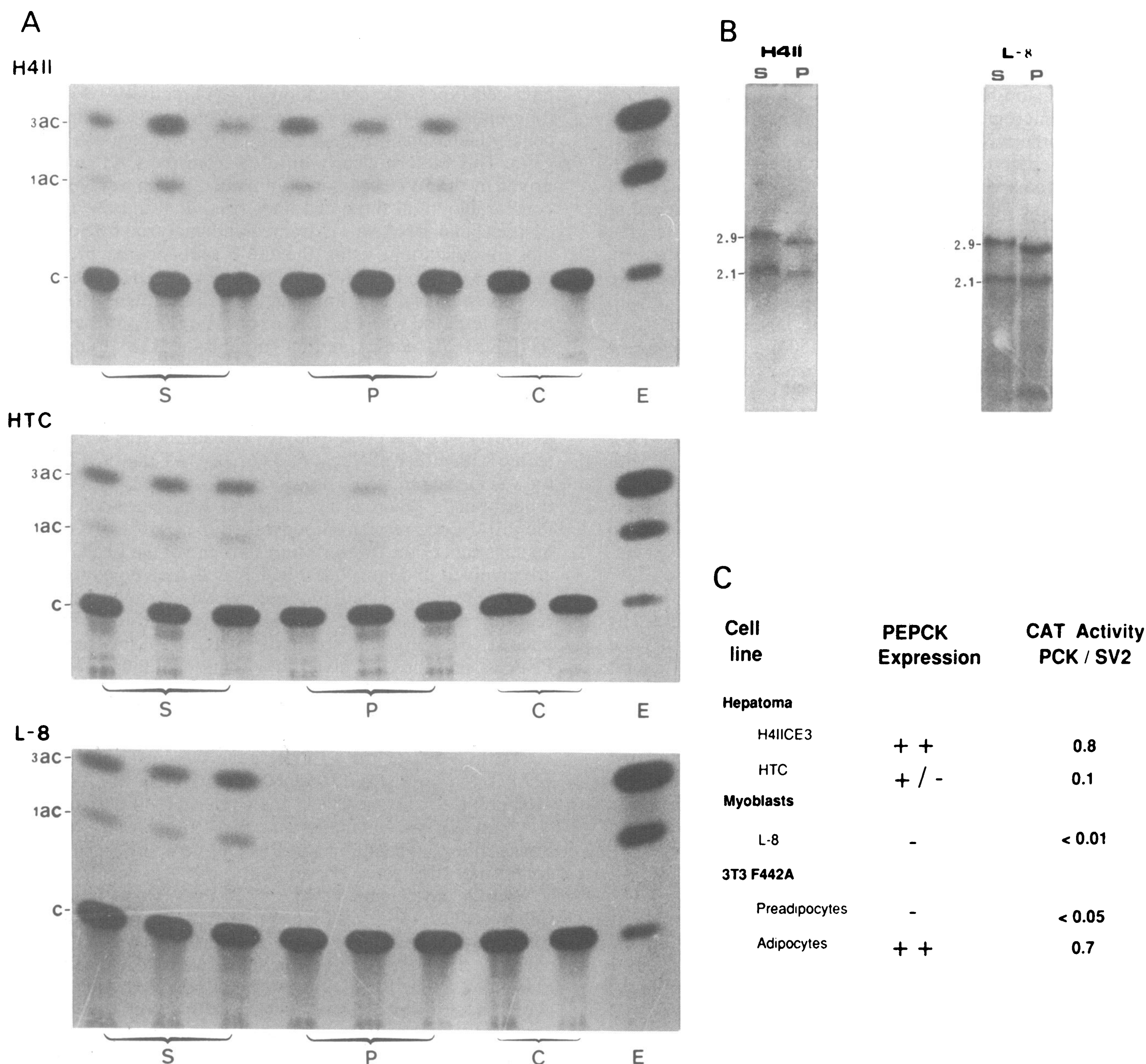

FIG. 2. Transient expression of the PEPCK-CAT chimeric gene in cultured cells. (*A*) H4IIEC3 hepatoma cells (H4II), M1.19-HTC hepatoma cells (HTC), and L-8 myoblasts (L-8) were transfected with pSV2cat (S) or 597-pck-CAT (P) on were mock transfected (C). CAT activity was determined in cell extracts 2 days after transfection, and the products were separated by thin-layer chromatography. c, free chloramphenicol; 1ac and 3ac, 1- and 3-acetylchloramphenicol, respectively; E, activity of purified CAT enzyme. Three independent transfection experiments are shown. (*B*) Detection of plasmid sequences in the transfected cells. Twenty micrograms of cellular DNA were digested with *Eco*RI and analyzed by Southern blot hybridization using 597-pck-CAT DNA as probe. Analysis of the digested DNA from L-8 myoblasts (L-8) and H4IIEC3 hepatoma cells (H4II), transfected with either pSV2cat (S) or 597-pck-CAT (P), is shown. The expected fragments of 2.9 and 2.1 kilobases of pSV2cat and of 2.7, 2.1, and 0.6 kilobases of 597-pck-CAT are revealed. (*C*) Densitometry of the 3-acetylated chloramphenicol spots was used to determine the mean CAT activities of 597-pck-CAT relative to pSV2cat in the various transfected cells. PEPCK activities, taken from published data (15, 25, 38), are given in semi-quantitative figures (+, +/−, and −) for comparison.

myoblasts and in non-PEPCK-expressing preadipocytes (results not shown).

These results clearly show that different sequences confer enhanced expression of the gene in the hepatoma cells and adipocytes. Thus, in the hepatoma cells, this sequence lies 5′ of position −362. Whereas in the adipocytes, the enhanced expression is conferred by a region located between positions −362 and −98.

Further analyses of the nature of the various regulatory elements were performed by inserting different elements in front of a heterologous promoter in tk-CAT chimeric gene (pTE1) (24). Using these constructs in transfection experiments, we have observed that the sequences containing the various regulatory elements (between positions −597 or −362 and −208 with respect to the transcription start site) were inactive in the hepatoma cells when linked to the tk promoter. In contrast, the adipocyte-specific sequence, residing between positions −362 and −208, increased CAT expression by 3- to 4-fold in adipocytes when linked to the heterologous promoter. This magnitude of the enhancement was comparable to that of the reduction in CAT activity (by about 2.5-fold) observed upon deleting this sequence (Fig. 3 *B* and *C*).

DISCUSSION

Separate Elements Confer Specific Expression of the PEPCK Gene in Different Cell Lines. Results in this report show that

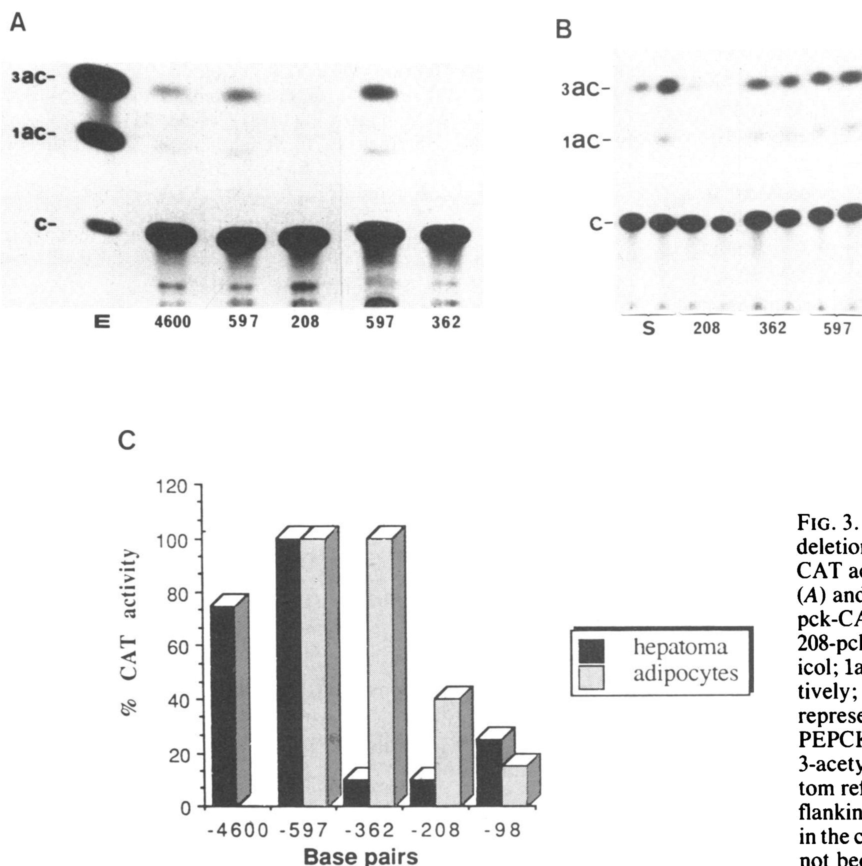

FIG. 3. Transient expression of a series of PEPCK–CAT deletion mutant genes in hepatoma cells and in adipocytes. CAT activity was determined in H4IIEC3 hepatoma cells (*A*) and 3T3-F442A adipocytes (*B*) transfected with 4600-pck-CAT (4600), 597-pck-CAT (597), 362-pck-CAT (362), 208-pck-CAT (208), or pSV2cat (S). c, free chloramphenicol; 1ac and 3ac, 1- and 3-acetylchloramphenicol, respectively; E, activity of purified CAT enzyme. (*C*) Schematic representation of the mean activity of CAT of the various PEPCK-CAT genes determined by the densitometry of the 3-acetylchloramphenicol spots. The numbers at the bottom refer to the position of the 5′ end of the PEPCK gene flanking region with respect to the transcription start site in the chimeric genes. Activity of 4600-pck-CAT (4600) has not been determined in adipocytes.

the 597 bp flanking the end of the PEPCK gene are sufficient to confer expression of the gene in the PEPCK-expressing hepatoma cells and adipocytes and exclude its expression from non-PEPCK-expressing cells (myoblasts and preadipocytes). It is noteworthy that Chin and Fournier (39) have shown extinction of a chimeric gene expression, driven by this region of the PEPCK promoter, in a somatic cell hybrid between a derivative of H4IIEC3 and mouse fibroblasts. Thus, this region contains the information for both the exclusive PEPCK gene expression and its enhancement in PEPCK-expressing cells.

Detailed analysis of the 597-bp region has provided clear evidence on separate elements, within this region, that confer enhanced PEPCK gene expression in the hepatoma cells (endoderm) and adipocytes (mesoderm). Thus, in the liver two regions—a distal (residing between positions −597 and −362) and a proximal (downstream of position −98) region—

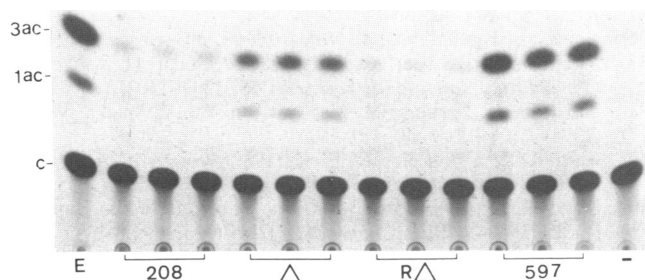

FIG. 4. Activity of the distal hepatocyte-specific positive element. H4IIEC3 hepatoma cells were transfected with 208-pck-CAT (208), 597-362-pck-CAT (△), 362-597-pck-CAT (R△), or 597-pck-CAT (597) (for details on the constructs see Fig. 1) or were mock-transfected (-). CAT activity was determined, and the products were separated on thin-layer chromatography. c, free chloramphenicol; 1ac and 3ac, 1- and 3-acetylchloramphenicol; E, activity of purified enzyme. Each experiment included triplicate transfections.

contain elements for enhanced cell-specific expression of the gene, whereas the region in between these elements contains the domain that enhances the gene expression in the adipocytes. This domain appears to be composed of two adipocyte-specific elements, the proximal of which (residing between positions −208 and −98) may harbor a liver-specific silencer whose removal reveals the activity of the proximal liver-specific element. [The existence of a silencer in this region has been previously suggested by Hanson and colleagues (40).] Since the PEPCK gene is transcribed from a single promoter in the various tissues (18), the finding that separate elements are used to confer transcription of this gene in the liver-derived hepatoma cells (endoderm) and adipocytes (mesoderm) provides a basis for the differentiation of this gene expression in the two embryonal remote tissues. Studies of a number of genes in *Drosophila* have shown that they comprise essentially two classes. The first involves genes that are expressed in tissues from related embryonal origins, where either separate or overlapped elements are found (6, 7, 11). The second class includes genes expressed in tissues from remote embryonal origins, where distinct elements for each tissue have been identified, such as in the YP1 and YP2 genes (fat body and ovary) (2, 3) and the white gene (testis sheath and malpighian tubes) (4, 5). Similarly, in the viral SV40 enhancers, separate elements were suggested to be active in different cells (41). Only a few instances have likewise been studied in mammals, where mammalian single copy genes are specifically transcribed from a single promoter in more than one tissue (12–14). Thus, the α-fetoprotein gene is expressed in three endoderm tissues: the yolk sac, liver, and gut. Likewise, the apolipoprotein A1 gene is expressed in the liver and intestine. Both these genes are expressed in tissues from related embryonal origins and thus belong to the first class described above. Indeed, for both genes more than one regulatory element has been described, out of which one or some are shared by the dif-

ferent tissues (13, 14). The regulation of the PEPCK gene is similar to those instances of the *Drosophila* genes or the SV40 enhancers, where separate elements confer gene expression in different tissues. Analysis of additional mammalian genes, like that of the PEPCK gene, will support the generality of this phenomenon.

Nature of the Regulatory Elements. The separate sequences, harboring the regulatory elements for PEPCK gene expression, in hepatoma cells and adipocytes also differ in nature. The distal liver-specific element enhances CAT expression only when linked to the homologous promoter in the correct orientation. Thus, this element is not a typical enhancer. Nevertheless, regulatory elements with such characteristics are not unprecedented. For instance, the obligatory requirement for a homologous promoter has been documented for regulatory elements in a number of genes (14, 42). The dependency of the PEPCK distal element on the correct orientation, even when linked to its homologous promoter, has also been documented for the α1-antitrypsin gene (43). Since the homologous PEPCK promoter (from position -98 to $+69$) also harbors a tissue-specific element, it is possible that correct spatial arrangement of the two elements is required for maximal expression of the gene in the liver. Unlike the liver element, the distal element of the adipocytes appears to be a more typical enhancer as it is capable of cooperating with a heterologous promoter. The results demonstrate qualitative differences in the nature of the regulatory elements used by the two tissues.

Taken together, the separate regulatory elements of the PEPCK gene used by the hepatoma cells (endoderm) and adipocytes (mesoderm) and the difference in nature of these distinct elements raise the intriguing issue of the way by which such architecture of the PEPCK promoter evolved. It is attractive to assume that sequences bearing adipocyte-specific elements have been merged with liver-specific elements thus creating the PEPCK promoter. Even more attractive is the possibility that the adipocyte-specific sequences have been inserted into the liver-specific PEPCK promoter thus producing the two separate liver elements. This possibility may be supported by the result that, unlike the liver, the adipocyte-specific regulatory element can cooperate with a heterologous promoter.

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