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Transcriptional Regulation of the Phosphoenolpyruvate Carboxykinase Gene by Cooperation Between Hepatic Nuclear Factors

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To study the transcriptional regulation of the liver gluconeogenic phenotype, the underdifferentiated mouse Hepa-1c1c7 (Hepa) hepatoma cell line was used. These cells mimicked the fetal liver by appreciably expressing the α-fetoprotein and albumin genes but not the phosphoenolpyruvate carboxykinase (PEPCK) gene. Unlike the fetal liver, however, Hepa cells failed to express the early-expressed factors hepatocyte nuclear factor 1α (HNF-1α) and HNF-4 and the late-expressed factor C/EBPα, thereby providing a suitable system for examining possible cooperation between these factors in the transcriptional regulation of the PEPCK gene. Transient transfection assays of a chimeric PEPCK-chloramphenicol acetyltransferase construct showed a residual PEPCK promoter activity in the Hepa cell line, which was slightly stimulated by cotransfection with a single transcription factor from either the C/EBP family or HNF-1α but not at all affected by cotransfection of HNF-4. In contrast, cotransfection of the PEPCK construct with members from the C/EBP family plus HNF-1α resulted in a synergistic stimulation of the PEPCK promoter activity. This synergistic effect depended on the presence in the PEPCK promoter region of the HNF-1 recognition sequence and on the presence of two C/EBP recognition sequences. The results demonstrate a requirement for coexistence and cooperation between early and late liver-enriched transcription factors in the transcriptional regulation of the PEPCK gene. In addition, the results suggest redundancy between members of the C/EBP family of transcription factors in the regulation of PEPCK gene expression.

The liver is specialized to perform a large variety of metabolic functions in accordance with its central role in mammalian metabolism. This is due to the selective expression in the liver of a subset of genes which code for key proteins that participate in these functions. In recent years, it became apparent that the genes in this subset share some specific, nonubiquitous, *cis*-regulatory elements which, by binding cognate liver-enriched transcription factors (for reviews, see references 18, 37, and 75), direct their liver-specific expression. To this date, four families of liver-enriched transcription factors have been characterized (for reviews, see references 18, 37, and 75). Analyses of a number of genes have shown that each contains a combination of some or all of the liver-specific *cis*-regulatory elements. It appears that the combination of *cis*-regulatory elements, rather than a single *cis*-regulatory element, is required for liver-specific gene expression (18, 37, 75). It follows that the corresponding cognate transcription activators of the combinatory sites cooperate to regulate the liver-specific transcription of a given gene and that this process requires that the factors in this subset coexist in the cells that express the gene. Direct evidence for cooperation between factors has been shown in a few instances (43, 44, 72).

The onsets of expression of various liver-specific genes do not occur simultaneously. Rather, liver development is characterized by a sequential course of appearance of various gene expression, providing a stepwise acquisition of the mature liver phenotype. For example, the genes encoding α-fetoprotein (AFP) and albumin are expressed at the very early stage of liver development (66, 50), thus serving as typical markers of the fetal liver. In contrast, the gene encoding the cytosolic form of phosphoenolpyruvate carboxykinase (PEPCK), which is the key gluconeogenic enzyme, starts to be expressed only at birth (3, 28, 67). Expression of this gene, therefore, serves as a marker of the acquisition of the liver gluconeogenic phenotype. The sequential onset of specific gene expression during development may reflect a differential requirement of liver-enriched transcription factors, some of which might themselves be developmentally regulated. This notion is supported by studies by us and by others showing developmentally regulated interactions of liver nuclear proteins with *cis*-regulatory elements of liver-specific genes (29, 33, 71).

Studies in our laboratory have focused on the mechanisms underlying the tissue- and cell-specific regulation of transcription of the PEPCK gene in the rat. We have previously shown that the PEPCK gene starts to be expressed at birth by the initiation of its transcription (5). The transcriptional regulation of the PEPCK gene therefore provides a suitable model for study of the transcription machinery associated with the gluconeogenic phenotype. Using transgenic mice, we and others have shown that 540 bp of the PEPCK promoter are sufficient to direct its expression in the liver (19, 42, 61). This region is also sufficient for the hormonal control of PEPCK gene expression by glucocorticoids and for the developmental induction of its expression in the newborn liver (19). Transient transfection experiments, using a recombinant PEPCK promoter fused to the reporter chloramphenicol acetyltransferase (CAT) gene, have established that 600 bp of the PEPCK promoter are sufficient to direct cell-specific expression in the PEPCK-expressing H4IIEC3 (H4) rat hepatoma cells (4). The

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promoter activity in these cells exceeded by more than 2 orders of magnitude its activity in PEPCK-nonexpressing cells such as myoblasts and preadipocytes (4).

Footprinting analyses of the liver-specific PEPCK promoter have characterized ubiquitous and tissue-specific protein-binding sequences as well as sequences that mediate the multiple hormonal control of PEPCK gene expression (8, 30, 31, 40, 41, 45, 47, 48, 51, 54, 60, 71). The tissue-specific sequences contain recognition sites for three families (C/EBP, hepatocyte nuclear factor 1 [HNF-1], and HNF-4) of liver-enriched transcription factors (25, 47, 48, 54, 71). Promoter mutants harboring internal deletions or block mutations of specific sites have implicated the involvement of such sites in the cell-specific activity of PEPCK. The region encompassing positions −487 to −417, upstream of the transcription start site, decreased promoter activity fourfold in PEPCK-expressing hepatoma cells (62). This region includes a consensus AF1 sequence (71) which constitutes an HNF-4 recognition site (25, 64). The AF1 site and an adjacent AF2 site, which contains an insulin-responsive element, are two liver-specific accessory components of the glucocorticoid-responsive unit of the PEPCK promoter (31). A similar decrease in promoter activity has been obtained by a 107-bp internal deletion encompassing positions −98 to −205 (62). This sequence contains an HNF-1 recognition sequence, an NF1 recognition sequence, and a cryptic cyclic AMP (cAMP)-responsive element (CRE) sequence (CRE2) (54). Block mutations of each of the protein-binding sites within the PEPCK promoter have indicated that none of the sites by itself had a marked effect on the promoter activity in PEPCK-expressing hepatoma cells. Rather, the combination of two such blocked sites appeared to result in a decreased activity (40). The PEPCK promoter thus shows a combinatorial requirement of sites typically observed in other liver-specific genes.

In the present work, we have used PEPCK-nonexpressing hepatoma cells, which mimic early stages of fetal liver, to examine whether distinct liver-enriched transcription factors cooperate in the regulation of PEPCK gene expression and whether the gluconeogenic phenotype is associated with a subset of liver-enriched transcription factors different from those sufficient for fetal liver gene expression. Transient transfection assays in this hepatoma cell line enabled us to show synergistic transactivation of the PEPCK promoter by two distinct liver-enriched transcription factors. These factors, which are not essential for expression of fetal liver marker genes, are present in the differentiated gluconeogenic cells.

MATERIALS AND METHODS

Cell culture and selection conditions. Cell lines, including rat hepatoma H4 cells (49), the derived subpopulation of H12G$^-$ cells, and mouse hepatoma Hepa-1c1c7 (Hepa) cells (26), were grown in 1:1 Dulbecco modified Eagle medium-F12 medium (Biological Industries, Kibbutz Beth Haemek, Israel) supplemented with 10% fetal calf serum and amino acids from minimal essential medium-amino acids solution and minimal essential medium-nonessential amino acids solution (Biological Industries).

H12G$^-$ cells were obtained by selection of H4 cells in glucose-free medium, using a 1:1 glucose-free mix of Dulbecco modified Eagle medium-F12 medium (Biological Industries) supplemented with 10% dialyzed fetal calf serum, amino acids, and 10 mM sodium pyruvate. The selected H12G$^-$ cells were all capable of multiplying in the selection medium and, as shown in Results, exhibited an enrichment of some transcripts that belong to the gluconeogenic phenotype.

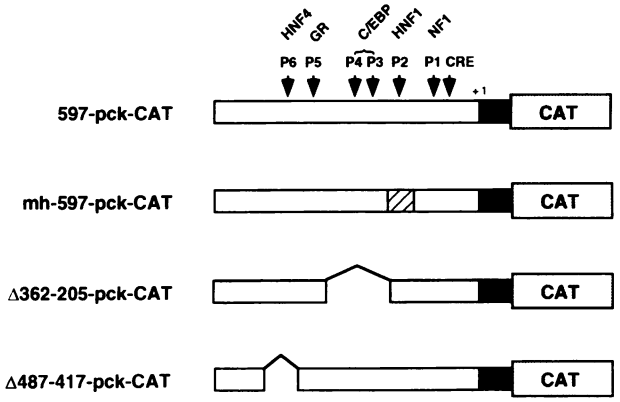

FIG. 1. Schematic description of the chimeric PEPCK constructs and the transcription factor recognition sites within the PEPCK promoter region. The wild-type construct, 597-pck-CAT (4), comprises 597 bp of the PEPCK promoter region (open box) and 69 bp of the 5′ end of the PEPCK gene (black box), fused to the CAT gene (hatched box). The letters at the top indicate regulatory elements: CRE at positions −78 to −94; NF1 recognition site (P1) at positions −94 to −119; HNF-1 recognition site (P2) at positions −162 to −203; C/EBP recognition sites (P3 and P4) at positions −231 to −250 and −274 to −293 (48, 71); glucocorticoid receptor (GR)-binding elements (P5) (31); and AF1 (HNF4) site (P6) at positions −433 to −457 of the PEPCK promoter region (71). The derived mutant plasmids include mh-597-pck-CAT, in which a 50-bp fragment of the P2 site has been replaced by a nonrelevant sequence; Δ362-205-pck-CAT, in which the sequence containing P3 and P4 has been deleted (4); and Δ487-417-pck-CAT, in which the AF1 site (P6) has been deleted (62).

Plasmids used in transfection. pSV2-cat is a plasmid harboring the structural gene encoding the bacterial CAT gene fused to the simian virus 40 (SV40) early promoter-enhancer region in pBR322 (23). Plasmid 597-pck-CAT, containing the 597 bp of the PEPCK promoter region fused to the CAT gene, has been previously described (4). The derived plasmid Δ362-205-pck-CAT contains an internal deletion in the PEPCK promoter, spanning positions −362 to −205 of the transcription start site, which harbors two C/EBP recognition sequences (48, 71). Plasmid Δ487-417-pck-CAT also derives from 597-pck-CAT and contains an internal deletion between positions −487 and −417 of the transcription start site (62) which harbors an AF1 site, the HNF-4 recognition sequence (22, 65). Plasmid mh-597-pck-CAT was constructed by first replacing a T for C at position −155 of plasmid 597-pck-CAT, using the method of Kunkel et al. (36), to create a *Hin*dIII site (plasmid h-597-pck-CAT). Subsequently, the sequence spanning positions −155 to −205 of plasmid h-597-pck-CAT was replaced by 50 bp of a polylinker sequence from the commercial plasmid pBluescript (Stratagene) to generate the mutant plasmid mh-597-pck-CAT (see Fig. 1 for a schematic presentation of plasmid 597-pck-CAT and its derivatives). The PEPCK-CAT-containing plasmid, in which the CRE site in the promoter was mutated, and its wild-type counterpart plasmid were previously described (40) and kindly provided by R. W. Hanson. Expression vectors encoding the liver-enriched transcription factors used in this work included HNF-1α (69), C/EBPα (21), C/EBPβ (55), and HNF-4 (64).

Transfection conditions and CAT assays. Hepa and H12G$^-$ cells were transfected by calcium phosphate precipitation (13). Hepa cells were transfected with 1 μg, and H12G$^-$ cells were transfected with 10 μg of each supercoiled plasmid and additional carrier pBluescript DNA (Stratagene), to make a

total of 20 μg. Where indicated, an optimal amount of 1 μg of each expression vector was added to the transfection mix. The precipitates were left on the cells for 5 h. After removal of the precipitates, the cells were rinsed and shocked for 2 min with 20% glycerol. The transfected cells were harvested after 2 days, and CAT activity was determined as previously described (4) except that the enzymatic assay mixture was incubated for 90 min. The amounts of cell extracts used for the assay were chosen to ensure CAT activities within the linear range. The percentage of acetylated ^{14}C-labeled chloramphenicol was determined in some experiments by scintillation fluid spectroscopy of acetylated and nonacetylated spots identified by autoradiography of the thin-layer chromatography plates and in most experiments by using a Fuji PhosphorImager to quantify the spots on the thin-layer chromatography plates. The transfection efficiency was determined in some of the experiments by the amount of the plasmid DNA in the transfected cells as described by Hirt (27). Alternatively, transfection efficiency was monitored by including as an internal standard 0.1 μg of plasmid pS16-GH (39), containing the human growth hormone (hGH) gene driven by the ribosomal protein S16 promoter, in the transfection mix. hGH levels secreted into the medium were determined by radioimmunoassay (39), using a commercial kit (St. Nichols, San Diego, Calif.) according to the supplier's instructions.

Northern (RNA) blot analysis. Northern blot analysis was done as previously described (19), using 10 μg of poly(A)$^+$ RNA except where indicated otherwise. Briefly, total RNA from tissues and cell lines was extracted with guanidine thiocyanate and centrifuged through a CsCl cushion by the method of Chirgwin et al. (14). Poly(A)$^+$ RNA was isolated by affinity chromatography on oligo(dT)-cellulose (2).

Molecular probes. The PEPCK cDNA probe was a 1.6-kb *Pst*I fragment (74). The fructose-1,6-bisphosphatase (FBPase) cDNA probe was a 0.55-kb *Hin*cII fragment (20). The rat albumin cDNA probe was a 1.2-kb *Hin*dIII fragment (35). The mouse albumin genomic DNA probe was a 6.4-kb *Eco*RI fragment (32). The AFP cDNA probes were 0.5-kb *Hin*dIII-*Bam*HI and 375-bp *Bam*HI-*Hin*dIII fragments (68). The HNF-1α cDNA probe was a 0.45-kb *Bam*HI-*Hin*dIII fragment excised from its expression vector (69). The vHNF1 (HNF-1β) cDNA was an 800-bp *Pvu*II fragment (52). The C/EBPα cDNA was a 2.1-kb *Bam*HI-*Hin*dIII fragment excised from its expression vector (21). The HNF-3β cDNA was a 2.2-kb *Eco*RI fragment (46); the HNF-4 cDNA was a 0.5-kb *Xba*I-*Xho*I fragment (63).

Nuclear extracts, gel shift assays, and Western blot (immunoblot) analysis. Nuclear extract from rat liver was prepared as previously described (71) by the method of Gorski et al. (24). Nuclear extracts from cell lines were prepared as described by Lassar et al. (38). Footprinting analysis was conducted as previously described (71). Gel shift assays were done by using as the DNA probe a 50-bp *Stu*I-*Hin*dIII fragment spanning positions −205 to −155, excised from plasmid h-597-pck-CAT (described above), which contained the HNF-1 recognition sequence. The protein-DNA interaction mixture, in a final volume of 25 μl, included 10 μg of nuclear proteins, 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer (pH 7.6), 5% glycerol, 50 mM NaCl, 1.5 mM $MgCl_2$, 3 μg of poly(dI-dC), and 10 fmol of ^{32}P-labeled DNA probe. After 30 min at room temperature, 5 μl of 0.05% bromophenol blue-xylene cyanol was added, and the mixture was loaded onto a 4% acrylamide gel containing 50 mM Tris base, 50 mM boric acid, and 1 mM EDTA and electrophoresed at 250 V for 3 to 4 h at 4°C.

Western blot analysis was performed as described by Towbin et al. (70). Immunoreaction with a polyclonal antibody to C/EBPα, kindly provided by S. L. McKnight, and antigen detection were performed by using a commercial kit from Amersham, with an anti-immunoglobulin G second antibody conjugated with alkaline phosphatase.

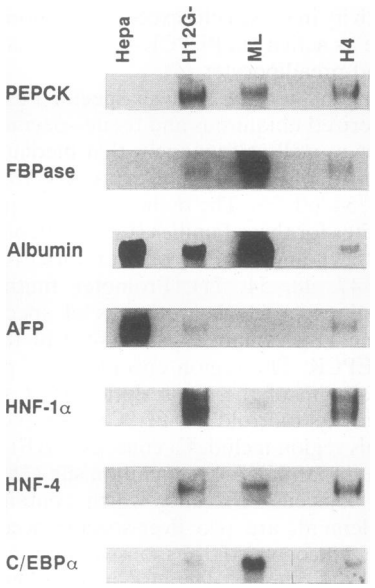

FIG. 2. Characterization of the liver phenotype of the Hepa cell line. Northern blots of 10 μg of poly(A)$^+$ RNA from Hepa, H12G$^-$, mouse liver (ML), and H4 cells were probed successively with PEPCK, FBPase, mouse albumin (Albumin), AFP, HNF-1α, HNF-4, and C/EBPα cDNA probes as described in Materials and Methods. Each blot was exposed to autoradiography for various time periods to enable quantitative comparisons, one of which is shown.

RESULTS

Phenotype of the Hepa hepatoma cell line. We have chosen for our studies the mouse Hepa hepatoma cell line, a spontaneous derivative of the Hepa-1c1 cell line (26) that expresses the liver-specific markers AFP and albumin and whose phenotype is considered to mimic the fetal liver (15). Further characterization of the cells has been made by determining the levels of transcripts coding for the gluconeogenic enzymes PEPCK and FBPase and for the liver-enriched transcription factors HNF-1α, C/EBPα, and HNF-4, whose recognition sequences exist in the promoter region of the rat PEPCK gene (25, 48, 54, 71) (Fig. 2). The transcript levels were compared with those of the mouse liver and the gluconeogenic hepatoma cell line H4. A more rigorous characterization of the gluconeogenic phenotype was done by growing H4 cells in glucose-free medium. A subpopulation of cells, termed H12G$^-$, that were capable of growing in this medium was derived. The phenotype of H12G$^-$ cells differed quantitatively from that of the parental H4 cells in the higher levels of transcripts encoding PEPCK, FBPase, albumin, and the liver-enriched transcription factor HNF-1α. The levels of the other transcripts, encoding AFP and the liver-enriched transcription factors HNF-4 and C/EBPα, remained the same.

In accordance with a previous characterization (15), Hepa cells abundantly expressed the AFP gene and appreciably expressed the albumin gene. The level of albumin gene expression exceeded by 10-fold that detected in H4 cells and was only 5-fold lower than that of mouse liver when either the mouse

albumin probe (Fig. 2) or the rat albumin probe (73) was used. The high level of expression of the fetal liver-like Hepa cell line was threefold higher than the level of albumin gene expression in the relatively differentiated H12G$^-$ line. In contrast, even very prolonged exposures of the autoradiographs could not detect in Hepa cells transcripts encoding PEPCK and FBPase nor those encoding the liver-enriched transcription factors HNF-1α and HNF-4. Transcripts encoding C/EBPα were barely detectable in Hepa cells. Only after prolonged exposure of the autoradiographs could we estimate their levels to be about six- to sevenfold lower than those found in either the H4 or H12G$^-$ cells. The failure to express PEPCK, FBPase, HNF-1α, and HNF-4, although these transcription factors are already expressed in the mouse fetal liver on gestation day 14 (7, 12), implied that the Hepa cells mimic an earlier stage of fetal liver development.

PEPCK promoter activity in the underdifferentiated Hepa cell line. To determine PEPCK promoter activity, transient transfection experiments of Hepa and H12G$^-$ cells were conducted with a plasmid harboring either the PEPCK promoter or the cell-nonspecific SV40 early promoter-enhancer driving the CAT structural gene as a reporter (597-pck-CAT [4] or pSV2-cat [23], respectively) (Fig. 3A). The percent conversions of chloramphenicol to its acetylated forms, driven by the SV40 early promoter-enhancer, were 80 ± 2.57 (n = 8) and 5.8 ± 2.3 (n = 6) with Hepa and H12G$^-$ cells, respectively. The values were the averages of results of several independent experiments, indicated in parentheses, in which the amount of cell extract used was chosen to ensure CAT activity within the linear range. These activities reflected a difference in the transfection efficiencies of the two cell lines that was evident from the content of the transfected DNA determined by the method of Hirt (27) as described previously (73). The activity of the transfected PEPCK promoter was therefore normalized according to that of the SV40 early promoter-enhancer, showing that Hepa cells support only a residual activity of the PEPCK promoter, 2.6% ± 0.35% versus 36.8% ± 5.07% in H12G$^-$ cells, in correlation with the lower degree of liver differentiation of these cells.

The residual promoter activity might result from a lack of liver-enriched transcription factors or from an inhibition of transcription driven by this promoter. We therefore used mutants of the PEPCK promoter (Fig. 1) in which sequences containing recognition sequences for each of the three liver-enriched transcription factors were either deleted (the HNF-4 recognition sequence [P6] in plasmid Δ487-417-pck-CAT and C/EBP recognition sequences [P3 and P4] in plasmid Δ362-205-pck-CAT) or replaced by a nonrelevant sequence (the HNF-1 recognition sequence [P2] in plasmid mh-597-pck-CAT). Analysis of these mutants (Fig. 3B) revealed either no effect (deletion of the HNF-4 recognition sequence), a slight increase (deletion of the two C/EBP recognition sequences), or a slight decrease (mutated HNF-1 recognition sequence) of PEPCK promoter activity. Therefore, despite these subtle changes, it appeared that the residual PEPCK promoter activity in Hepa cells resulted from absence of some required transcription factors.

To further ascertain this conclusion, footprinting analysis of the PEPCK promoter region, using nuclear extracts from Hepa cells and from the adult liver, was conducted. The analysis showed that Hepa-derived nuclear proteins failed to bind the C/EBP recognition sequences P3 and P4 but did bind the HNF-1 recognition sequence P2, albeit with a pattern slightly different from that obtained with the liver nuclear proteins (Fig. 4A). Gel shift analysis (Fig. 4B) using the HNF-1 recognition sequence as a probe revealed a shift obtained with

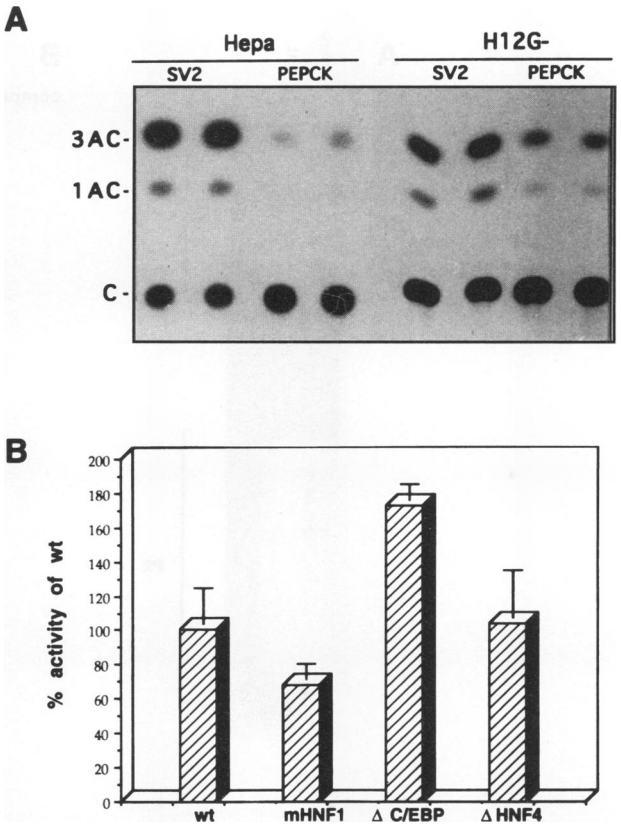

FIG. 3. PEPCK promoter activity in hepatoma cell lines. (A) Transient expression of pSV2-cat and 597-pck-CAT chimeric genes in the PEPCK-nonexpressing Hepa cell line and in the differentiated PEPCK-expressing H12G$^-$ cells. CAT activity was determined in cell extracts 2 days after transfection, and the products were separated by thin-layer chromatography. C, free chloramphenicol; 1AC and 3AC, 1- and 3-acetylated forms of chloramphenicol, respectively. (B) PEPCK promoter activities of 597-pck-CAT and derived mutants in Hepa cells. The histogram represents CAT activities driven by the wild-type construct (wt) and by three derived mutants: mh-597-pck-CAT (mHNF1), in which the HNF-1 recognition sequence has been replaced by a nonrelevant polylinker; Δ362-205-pck-CAT (ΔC/EBP), which contains an internal deletion of two C/EBP recognition sequences; and Δ487-417-pck-CAT (ΔHNF4), which contains an internal deletion of the HNF-4 recognition site. CAT activities were quantified by using a Fuji PhosphorImager, and the averages from 8 to 14 independent transfection experiments are expressed as percentages of the wild-type PEPCK promoter activity, with the standard errors indicated by the vertical bars.

Hepa-derived nuclear proteins that differed slightly in its mobility from that obtained with nuclear proteins from H4 or H12G$^-$ cells. In addition, the four-times-longer exposure of the autoradiographs needed to clearly visualize the shifted band implied that its concentration in Hepa nuclei was at least fourfold lower than that of the counterpart binding protein in the gluconeogenic cells. This binding factor is likely HNF-1β (vHNF1), whose steady-state level of transcripts detected in Hepa cells was about a quarter of that found in H4 cells (Fig. 4C). Even if this binding protein acted as a transcription activator of the PEPCK promoter, as suggested by the slightly reduced activity of the PEPCK promoter containing a mutated HNF-1 sequence, its transactivation appeared too low to play a significant role.

To ascertain the absence of C/EBPα in Hepa cells, whose

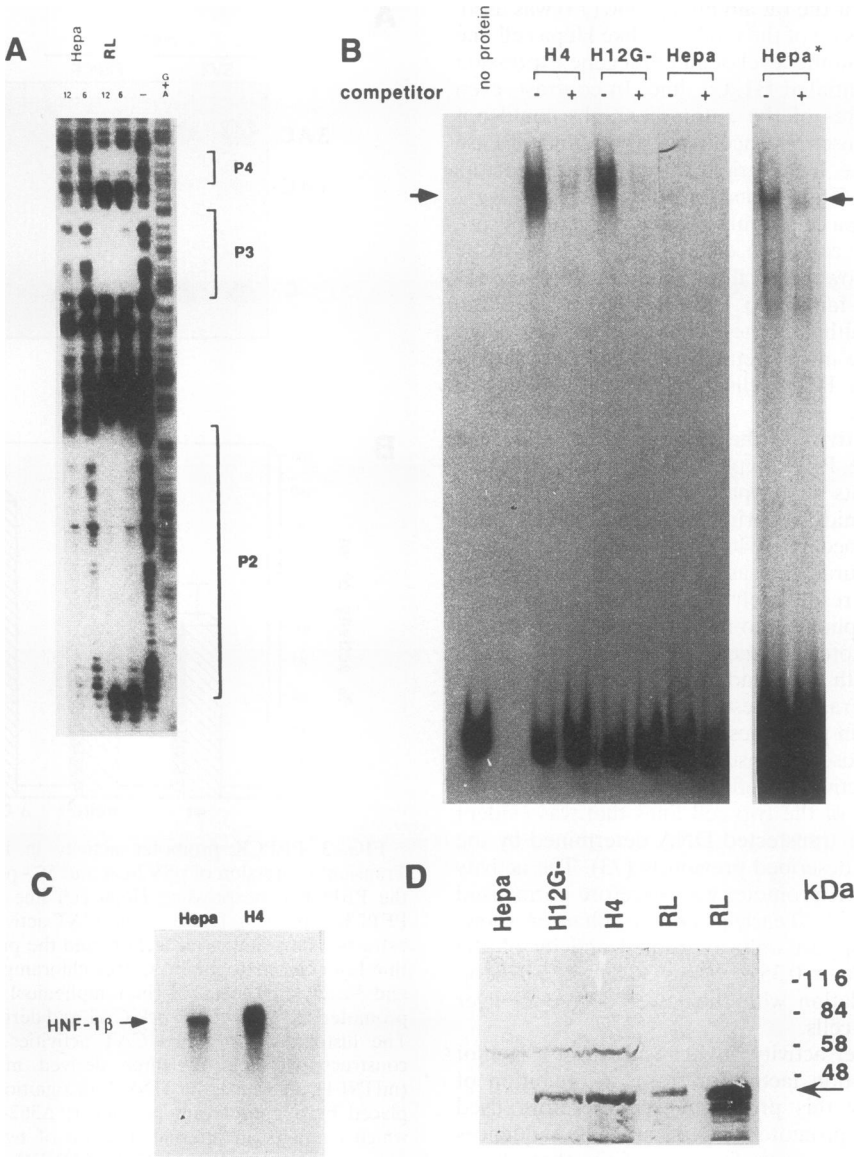

FIG. 4. Protein-DNA interactions and Western blot analysis. (A) Footprinting analysis of the PEPCK promoter region including the HNF-1 (P2) and two C/EBP (P3 and P4) recognition sequences, using 6 and 12 μg of nuclear proteins from rat liver (RL) and Hepa cells, as indicated at the top. (B) Binding of nuclear proteins to the 50-bp DNA fragment from the PEPCK promoter containing the HNF-1 recognition sequence. Gel shift assays of the ^{32}P-labeled DNA probe (10 fmol per assay) and nuclear proteins (10 μg per assay) were performed in the absence (−) or presence (+) of a 50-fold excess of the unlabeled competitor fragment. The asterisk denotes four-times-longer exposure of the autoradiograph. (C) Northern blot analysis of 4 μg of poly(A)$^+$ from Hepa and H4 cells, using the vHNF1 (HNF-1β) cDNA as a probe. (D) Western blot analysis of C/EBPα in nuclear extracts from hepatoma cell lines and rat liver. Eighty micrograms of nuclear proteins from the Hepa, H12G$^-$, and H4 cell lines and 16 and 3.2 μg of rat liver (RL) nuclear proteins were separated by gel electrophoresis, along with size markers, and analyzed by Western blotting using a C/EBPα antiserum. Detection of the bound antibody was done with a commercial kit containing a second antibody conjugated to alkaline phosphatase. The sizes of the detected C/EBPα proteins corresponded to 43 and 30, as determined by comparison with the coelectrophoresed size markers.

transcripts were detectable, albeit at a minimal level, we performed Western blot analysis (Fig. 4D). C/EBPα was detected in the gluconeogenic cells but not in the Hepa cells, corroborating the lower level of its transcripts. The absence of the liver-enriched transcription factors in Hepa cells rather than the presence of some inhibitory factors seems to account for the low PEPCK promoter activity in these cells.

Transactivation of the PEPCK promoter in Hepa cells. In the next set of experiments, we transiently supplemented the Hepa cells with expression vectors encoding the liver-enriched transcription factors, via transient cotransfection experiments with plasmid 597-pck-CAT (Fig. 5). Each factor by itself either did not affect (HNF-4) or slightly stimulated (HNF-1α and C/EBPα) PEPCK promoter activity. However, the combined cotransfection of HNF-1α and C/EBPα expression vectors resulted in a synergistic sevenfold stimulation of PEPCK promoter activity (Fig. 5A). The synergistic effect was also observed when the C/EBPα vector was replaced by the

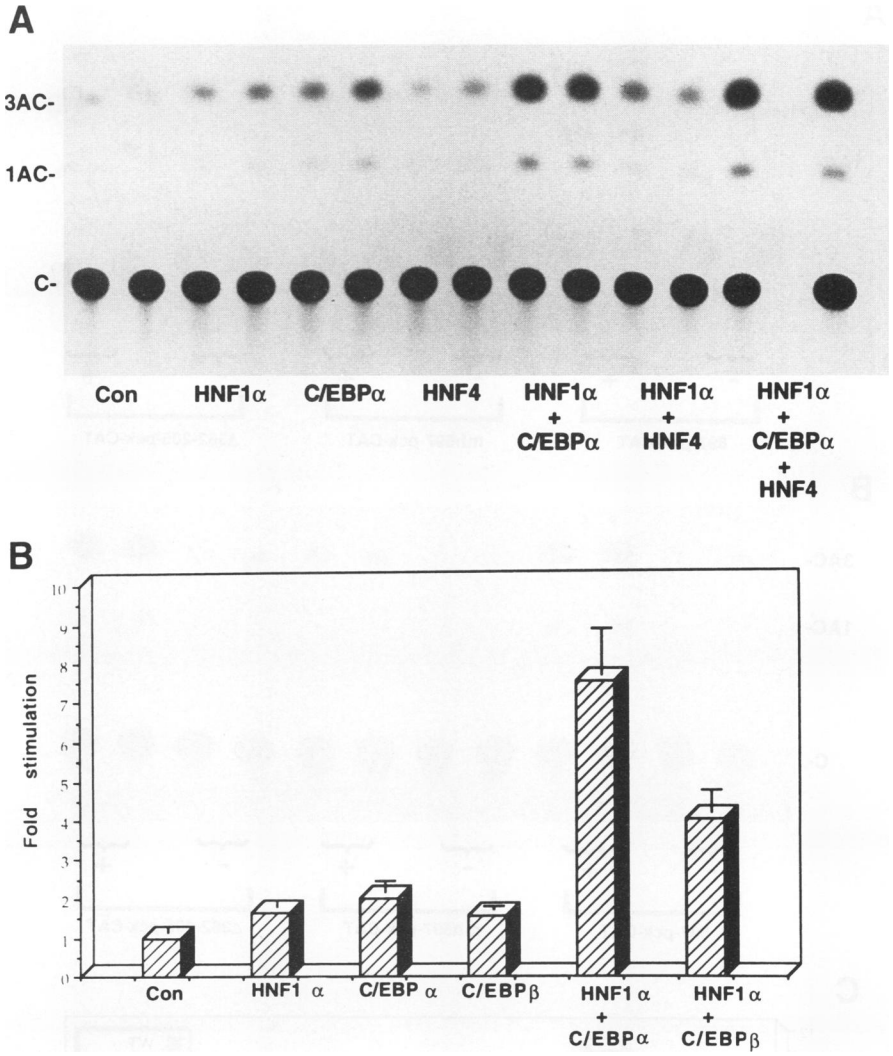

FIG. 5. Transactivation of the PEPCK promoter in Hepa cells by liver-enriched transcription factors. (A) Hepa cells were transiently transfected with 597-pck-CAT alone (Con) or cotransfected either with the HNF-1α, C/EBPα, or HNF-4 expression vector or with various combinations of these transcription factors, as indicated. The transfection mix included plasmid pS16-GH, whose hGH product served as an internal standard. Labels on the left are as defined for Fig. 3A. (B) Synergistic transactivation of the PEPCK promoter by HNF-1α together with either C/EBPα or C/EBPβ. CAT activities were quantified with a PhosphorImager as described for Fig. 3B and normalized according to the amounts of hGH secreted into the medium. The average fold effects of the various transcription factors from 6 to 12 independent experiments are shown by histograms. The standard errors are indicated by the vertical bars.

C/EBPβ expression vector, although the overall stimulatory effect was smaller than that obtained with C/EBPα (Fig. 5B). In contrast, cotransfection with the HNF-4 expression vector with either HNF-1α or C/EBPα or both did not further stimulate PEPCK promoter activity (Fig. 5A).

To assess the *cis*-regulatory elements that are involved in the synergistic effect, we used the mutated PEPCK promoter constructs. As shown (Fig. 6), mutation in the HNF-1 recognition sequence abolished the synergistic effect, and deletion of the C/EBP recognition sequences reduced the synergistic effect with either C/EBPα (Fig. 6A and C) or C/EBPβ (Fig. 6B and C). Previous experiments with HepG2 cells have shown the involvement of the CRE site in the transactivation of the PEPCK promoter by either C/EBPα or C/EBPβ (40, 47, 48). However, using the mutated CRE-containing PEPCK-CAT construct (40), we observed no effect of this mutation on the synergistic stimulation by HNF-1α with either C/EBPα or C/EBPβ (73).

Finally, the HNF-4 recognition sequence did not affect promoter activity even in the presence of all three transcription factors (73). Since this sequence plays an important role in PEPCK gene expression in the gluconeogenic H4 cells or in transgenic mice (19, 62), it appears that supplementing Hepa cells with HNF-1α and members of the C/EBP family only partially reconstitutes the transcription capacity driven by the PEPCK promoter.

DISCUSSION

In the course of liver development, HNF-4 and HNF-1α appear in the fetal liver (7, 12), while C/EBPα appears only in terminally differentiated hepatocytes (6, 21) and C/EBPβ protein is markedly induced at birth (17), when PEPCK gene transcription initiates (5). We have previously shown that interactions of nuclear protein factors with the C/EBP recognition sequences in the PEPCK promoter are absent in the

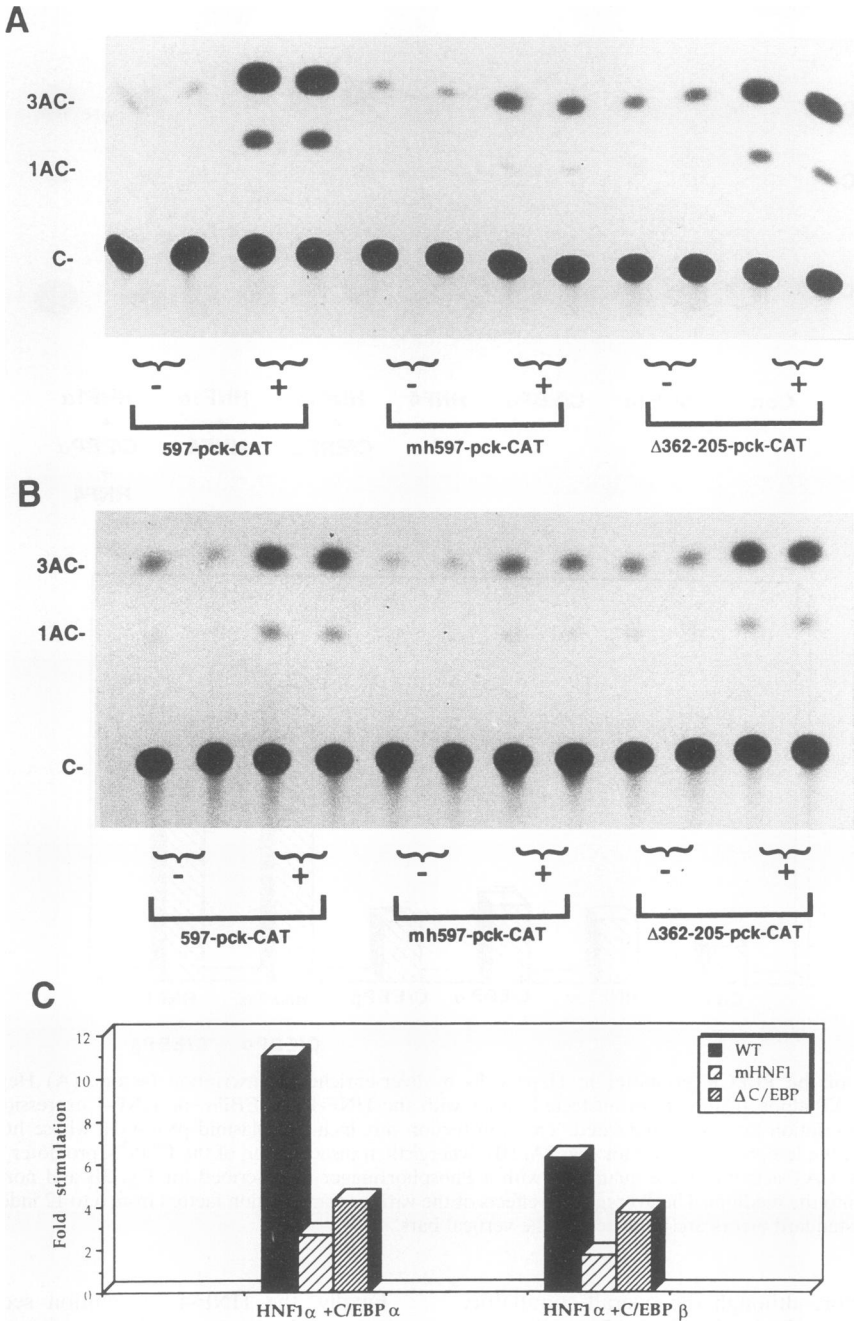

FIG. 6. Effects of mutations in the PEPCK promoter region on synergistic transactivation. Hepa cells were transfected either with wild-type 597-pck-CAT (WT) or with mutant mh-597-pck-CAT (mHNF1) or Δ362-205-pck-CAT (ΔC/EBP) alone (−) or cotransfected (+) with the HNF-1α expression vector together with either C/EBPα (A) or C/EBPβ (B). The transfection mix included plasmid pS16-GH, used as an internal standard. CAT activities were quantified with a Fuji PhosphorImager and normalized to the amounts of hGH as described for Fig. 5. Labels at the left are as defined for Fig. 3A. (C) Fold stimulation of each construct by the cotransfected transcription factors.

fetal liver and first appear at birth (71). Thus, together with the already existent HNF-4 and HNF-1α, C/EBPα and C/EBPβ characterize the newborn liver and conceivably also complete the requirements for the initiation of PEPCK gene expression. Indeed, we and others have previously documented that C/EBPα binds to several recognition sites in the PEPCK promoter region and that C/EBPα transactivates the promoter in the PEPCK-expressing cell lines H4 and HepG2 (48, 71). In fact, such transactivation has more recently been extended also to C/EBPβ/LAP (47) and DBP (53) members of the C/EBP family. Unlike the fetal liver (7, 12) or the PEPCK-expressing hepatoma cells (Fig. 2), which express the liver-enriched transcription factors HNF-1α and HNF-4, Hepa cells do not. The absence of these transcription factors in Hepa cells has enabled us to assess their role and possible cooperation in transcriptional regulation of the PEPCK gene. We now show that in the absence of HNF-1α in Hepa cells, C/EBPα or C/EBPβ only marginally affects PEPCK promoter activity.

Only a supplement of HNF-1α with either of these factors results in a marked transactivation of the PEPCK promoter. This finding clearly indicates that C/EBPα and C/EBPβ cooperate at least with HNF-1α in transcriptional regulation of the PEPCK gene. Regardless of whether additional cooperation with other transcription factors might participate in the activation of PEPCK gene expression, it is clear that this cooperation occurs between liver-enriched transcription factors that belong to two distinct families. Its physiological significance derives support from the coexistence of these factors in H12G$^-$ cells, which exhibit the newborn gluconeogenic liver phenotype, and suggests that this developmentally more advanced liver phenotype is associated with a new profile of transcription factors.

The cooperation of either C/EBPα or C/EBPβ with HNF-1α in control of the PEPCK promoter appears redundant. Both C/EBPα and C/EBPβ are capable of binding the several C/EBP recognition sites in the promoter region (47, 48, 71), and our present results have shown that the synergistic effect by either factor is dependent on the same C/EBP recognition sequences. It is attractive to discuss such a redundancy in view of the appearance of both C/EBP factors at birth, when PEPCK gene expression initiates. The appearance of PEPCK at birth, which initiates gluconeogenesis in all mammals, is critical for independent life (reference 3 and references therein). If the synergistic effect that we have observed plays a role in this critical event, the redundancy of the participating factors can be conceived in light of the redundancy of other factors that play critical roles in development. Thus, it has been shown that either MyoD or Myf-5 is crucial to the initiation of fetal muscle development. While the disruption of either of these genes has no significant consequence on myogenesis (10, 56), a null mutation of both factors together results in a severe impediment of muscle development (57). By analogy, it would be intriguing to find out whether the disruption of either C/EBPα or C/EBPβ or both is needed to obliterate the onset of PEPCK gene expression at birth and, as a consequence, results in newborn hypoglycemia incompatible with life.

Factors other than the liver-enriched factors have been implicated in the regulation of PEPCK gene expression during development and differentiation. The PEPCK gene belongs to a subset of genes whose onset of expression in the perinatal period is hormonally induced by glucocorticoids and glucagon (via cAMP) (3). Genetic approaches have defined two loci that repress expression of this subset of genes. First, mice homozygote for the albino lethal deletion locus on chromosome 7 die shortly after birth from hypoglycemia, conceivably due to the failure of the newborn liver to express the genes encoding for the gluconeogenic enzymes (22). It is thought that the deletion affects the hormonal regulation that induces the onset of expression of these genes, although the signal transduction pathways of glucocorticoids and cAMP themselves are not impaired (16, 58). Second, experiments using somatic cell hybrids have revealed the existence of a tissue-specific extinguisher (Tse-1) that has been recently cloned and identified as the regulatory subunit RIα of protein kinase A (9, 34). The Tse-1-mediated repression of chimeric PEPCK constructs in cultured cells can be relieved by cAMP (59, 65). The interrelationship between the liver-enriched transcription factors, such as HNF-1 and C/EBP, and the hormonal regulation of PEPCK chimeric constructs in Hepa cells remains to be examined.

Finally, since Hepa cells express both the AFP and albumin genes yet fail to express the liver-enriched transcription factor HNF-1α or HNF-4, these two factors are not required for expression of either AFP or albumin genes in these cells. In situ hybridization studies with mice have previously detected expression of the albumin gene in prehepatic cells prior to liver organization (11), when HNF-1α is not yet expressed (12). This early expression correlates, however, with the expression of HNF-1β (vHNF1) (12) and HNF-3β (1). Likewise, expression of the albumin gene in the absence of HNF-1α in Hepa cells also parallels the expression of HNF-1β (Fig. 4C) and HNF-3β (73) in these cells. These factors, however, are clearly not sufficient for PEPCK gene expression. In contrast, the more advanced gluconeogenic phenotype of H4 and H12G$^-$ cells correlates with the expression in these cells of at least the three liver-enriched transcription factors HNF-4, HNF-1α, and C/EBPα. It is conceivable, therefore, that the coexistence of both the early and late factors in the gluconeogenic cells favors cooperation capable of stimulating PEPCK gene expression.

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