CREB Binding Protein Coordinates the Function of Multiple Transcription Factors Including Nuclear Factor I to Regulate Phosphoenolpyruvate Carboxykinase (GTP) Gene Transcription*

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Nuclear factor I (NFI) binds to a region of the phosphoenolpyruvate carboxykinase (GTP) (PEPCK) gene promoter adjacent to the cAMP regulatory element (CRE) and inhibits the induction of transcription from the gene promoter caused by the catalytic subunit of protein kinase A. In vivo footprinting studies demonstrated that both the CRE and the NFI-binding site are occupied by transcription factors, regardless of the presence of factors that stimulate (dibutylryl cAMP or dexamethasone) or inhibit (insulin) transcription from the PEPCK gene promoter. The NFI effects on transcription from the PEPCK gene promoter were observed even in the absence of the NFI binding site, suggesting the possibility of other weaker binding sites on the promoter or an interaction of NFI with a transcriptional co-activator. A mammalian two-hybrid system was used to demonstrate direct interaction between the transactivation domain of NFI-C and the CRE-binding domain of the CREB-binding protein (CBP). Overexpression of a gene fragment encoding the CREB binding domain of CBP stimulates transcription from the PEPCK gene promoter. The inhibitory effect of NFI on transcription of the PEPCK gene induced by the catalytic subunit of protein kinase A appears to be the result of an interaction between NFI and the CREB-binding protein in which NFI competes with CREB for binding to the CRE-binding site on CBP. In contrast, glucocorticoids and thyroid hormone use the steroid hormone receptor binding domain of CBP to stimulate transcription from the PEPCK gene promoter. NFI-A combines with dexamethasone or thyroid hormone in an additive manner to stimulate PEPCK gene transcription. We conclude that CBP coordinates the action of the multiple factors known to control transcription of the PEPCK gene.

The regulation of phosphoenolpyruvate carboxykinase (GTP) (EC 4.1.1.32) (PEPCK) gene transcription is coordinated by the action of a number of transcription factors that bind to specific cis-elements on the promoter and mediate the effect of various hormones and other regulatory molecules (1, 2). Most of the cis-acting elements on the PEPCK gene promoter are located within the first 550 base pairs immediately 5’ to the transcriptional start site (3–6). The cAMP response element (CRE) is critically important for mediating both basal and cAMP-induced transcription (7, 8) and is located between –90 and –83 (9, 10) on the PEPCK gene promoter. A glucocorticoid regulatory unit, which contains an insulin response element and two glucocorticoid response elements, is also located from –415 to –440 in the promoter (5, 11). In this study we examine how nuclear factor I (NFI) transduces its signal to the initiation complex and how it interacts with other transcription factors, either competitively or cooperatively.

In general transcription factor activation domains fall into four main groups based on recurring motifs (12). NFI has a proline-rich transactivation domain and a DNA binding domain that is highly conserved among the various NFI isoforms but shows no homology to other well characterized domains (13). Bandopadhyay et al. (13) showed that four of five cysteine residues located in the N-terminal DNA binding domain of NFI are crucial for DNA binding. Crawford et al. (14) grouped the four isoforms of NFI proteins into two categories based on their ability to alter the level of basal transcription from the PEPCK gene promoter. NFI-A and -B stimulate basal transcription, whereas NFI-C and -X have mild inhibitory effects. All four isoforms of NFI markedly inhibited the induction of transcription from the PEPCK gene promoter caused by cAMP or by transfection of the gene for the catalytic subunit of protein kinase A (PKA-c) (14). Chaudhry et al. (15) used domain-swap experiments to show that these different transactivation capabilities of the NFI isoforms resided wholly in their C-terminal transactivation domains.

Many transcription factors are capable of interacting cooperatively. The NFI cis-element on the PEPCK promoter is located immediately adjacent to the CRE. Altering the relative positions of the NFI site and CRE on the PEPCK gene promoter did not affect the ability of NFI to abrogate CRE-mediated PKA-c induction of transcription in hepatoma cells (14). Thus, the proximity of P1 and CRE sites in the PEPCK gene promoter was not critical for the NFI effect. The ability of NFI to block CRE-mediated stimuli such as that achieved by the co-transfection of PKA-c, apparently without direct interaction between the proteins, suggested that another signal transduction pathway must exist.

Recently we have studied the effects of the various isoforms

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of NFI on cAMP induction of transcription of the PEPCK gene (14). The NFI site in the PEPCK gene promoter contains the consensus NFI binding sequence TTGGG(N2)GCCAA (16–18). There are four genes encoding NFI proteins, all of which are expressed in the liver (19). Mutating the NFI-binding site (P1) of the PEPCK gene promoter results in a marked increase in the level of basal expression of a linked structural gene (bGH) in the livers of transgenic mice and the premature expression of bGH mRNA in the liver in vivo, as compared with control mice carrying a transgene with an intact PEPCK promoter (20). We speculate that NFI serves the important function of maintaining a low basal level of transcription of the PEPCK gene in the liver until stimulation by hormones such as glucagon, glucocorticoids, and thyroid hormone occurs. In the current study we confirm the function of NFI in the control of PEPCK gene transcription, and we describe the key role of CBP in coordinating not only the effects of NFI but other transcription factors involved in determining the level of hepatic PEPCK gene transcription.

**EXPERIMENTAL PROCEDURES**

**Materials**

1H(Chloramphenicol (30–60 Ci/mmol), [32P]ATP (3,000 Ci/mmol), and [3H]dCTP (3000Ci/mmol) were purchased from NEN Life Science Products. Br2cAMP and butyryl-coA were bought from Sigma. HepG2 cells were originally purchased from ATCC (Rockville, MD). The Checkmate® mammalian two-hybrid assay kit was purchased from the Promega Corp. (Madison, WI). The expression vector for catalytic subunit of protein kinase A was a gift from Dr. Masa-Aki Muramatsu. Dulbecco’s modified Eagle’s medium/Ham’s F-12 cell culture medium and fetal calf serum were from Life Technologies Inc. All other reagents used in this study were of the highest quality obtainable. All protein-DNA binding vectors for the NFI proteins were the same as those used in an earlier study (19) and express murine proteins homologous to chicken NFL-A1,1, NFL-B2, NFL-C2, and NFL-X2 (39). The expression vectors for the full-length CBP and the fragments of CBP were generous gifts from Dr. Richard Goodman, Vollum Institute, Portland, OR.

**Methods**

*In vivo UV Photo-footprinting.* In vivo photo-footprinting was carried out essentially as described by Behrens and Greulich (21). Minimal deviation H4 hepatoma cells were grown to 70% confluence in 100-mm plates in a 1:1 mixture of Dulbecco’s modified Eagle’s medium and Ham’s F-12 media supplemented with 10% fetal calf serum. Before hormone treatment, cells were grown for 20 h in serum-free medium. Ham’s F-12 media supplemented with 10% fetal calf serum. Before

*Preparation of Cell Extracts for the Determination of CAT Activity—* Cells were harvested 48 h after transfection. Plates were drained and cells were rinsed in 1× PBS buffer. Ice-cold 1× PBS (1 ml) was added to each plate, and cells were scraped free of the surface and transferred to a 1.5 ml microcentrifuge tube kept on ice. Samples were centrifuged for 20 s at 10,000 rpm; the supernatant was removed and the pellet resuspended in 250 μl Tris-CI, at pH 7.8. Samples were subjected to three freeze-thaw vortex cycles using ethanol/dry ice and a 37 °C water bath, and then centrifuged for 10 min at 10,000 rpm. The supernatant was transferred to a new tube and placed in a 58 °C water bath for 10 min to denature inhibitors of CAT (25, 26). The samples were centrifuged again for 10 min at 10,000 rpm, and the supernatant was used to assay CAT activity using [3H] chloramphenicol, butyryl-CoA, and the xylene phase extraction method (27). All transfection studies were performed in duplicate or triplicate. Differences between means of each transfection were considered significant using Students t-test (one-tailed) (28). Experiments were repeated at least twice with three values per experiment. Values are expressed as the mean ± S.E. of the mean for the number of determinations noted in the legends to the figures.

*Mammalian Two-hybrid Assay for Direct Protein-Protein Interaction—* Plasmids encoding the CREB and E1A binding domains of CBP were a generous gift from Dr. Richard Goodman. The plasmid encoding the C-terminal transactivation domain (amino acids 451–682 and 1678–2441, respectively) linked to the steroid binding domain and 5′-CGCCGC-3′ (amino acid residues 451–682 and 1678–2441, respectively) linked to the luciferase promoter. Two other regions of CBP encompassing the steroid binding domain (amino acids 1–299) and a more restricted EIA binding domain (amino acids 1678–1843) were also subcloned into the pBIND vector supplied as a part of the mammalian two-hybrid kit (Promega). The primer pairs used for these were 5′-GCTAGGATCCCTACTGGGCCGGAAGCTTTGCTGCG-3′/5′-GCTAGCTAGCCGCGCGCTTTGTGCTGCTACTGCGG-3′ for the steroid binding domain and 5′-GCTAGGATCCATTCTCTTCTTCTTCTTTTCTGGCCCGC-5′/5′-GCTAGCCGCGCGCGCAGCAAGGGCAGCCAGGCGC-3′ for the restricted EIA binding domain. The region of the pPHANFI-C plasmid encoding the C-terminal transactivation domain (amino acids 220–499) was amplified using these polymerase chain reaction primers, 5′-GCTAGGATCCCTTGTCTACAGGTTTGTTGTTGTC-3′, in which the added BamHI site is underlined and immediately precedes the codon for amino acid 220 of NFI-C. The downstream primer 5′-GCTAGGCCGCCGCGCGCGCGTGCTGCGG-3′ introduced NotI 

*into the plasmid* immediately preceded the added BamHI codon of NFI-C. The amplified fragment was subcloned into the NotI/BamHI-digested pACT vector supplied as part of the Checkmate® mammalian two-hybrid kit (Promega). The resultant plasmid was named pAC220, the initial “A” indicating its putative “Activator” role in the two-hybrid system. The luciferase reporter gene contained five tandem repeats of the Gal4-binding site. The plasmids pBCBP451, pBCBP1678, and pAC200 were
RESULTS

UV Photo-footprinting of the PEPCK Gene Promoter in Vivo—DNA structural changes and the binding of sequence-specific binding proteins to DNA may be detected by comparing the rate of UV photoproduct formation in naked DNA irradiated in vitro with the rate of UV photoproduct formation in DNA irradiated in vivo. In order to determine the physiological circumstances under which the NFI-binding sites, CRE and TATA box on the PEPCK gene promoter (see Fig. 1A for organization of the promoter) were occupied by transcription factors, we carried out in vivo irradiation. UV photo-footprinting of the endogenous PEPCK gene was carried out using H4 cells treated in a manner calculated to provide widely divergent transcriptional states (Fig. 1B). These cells were maintained for 3 h in a serum-free (basal conditions) medium or were treated with a combination of Bt2cAMP and dexamethasone to stimulate PEPCK gene transcription or with a combination of three hormones (Bt2cAMP, dexamethasone, and insulin). Insulin inhibits PEPCK gene transcription (29). Cells treated as above were subjected to UV irradiation for the time indicated in Fig. 1B in order to introduce covalent bonding between consecutive pyrimidine moieties in the genomic DNA. Proteins bound to the DNA can act in a manner that either magnifies the effect of UV irradiation or deflects and thereby decreases the rate at which inter-pyrimidine covalent bonds are formed. Taq DNA polymerases used in subsequent primer extension analyses have difficulty reading through the modified pyrimidines leading to pausing and banding patterns that correlate with the location of pyrimidines (see Ref. 21 for a detailed description of the method and its limitations). Proteins bound to the DNA increase or decrease the banding patterns and thereby provide a unique footprint. Several significant differences in UV photoproduct signal intensity are evident between H4 DNA irradiated in vitro under different PEPCK transcription states (Fig. 1B, lanes 1–3) and H4 genomic DNA irradiated in vitro (lanes 4 and 5). The banding patterns in the P1, CRE, and TATA box sites in the PEPCK gene promoter, under a variety of hormonal treatment conditions, are different from the patterns seen in the control cells (lanes 4 and 5). Four changes of footprint map to the TATA box and early RNA coding regions. These have been designated with plus symbols next to the vertical bar and are seen only in the presence of the Bt2cAMP and dexamethasone. These bands are consistent with initiation site occupancy by the TATA box binding factor and RNA polymerase, respectively. The rate of UV photoproduct formation at the CRE and NFI-binding site (bands e and f in lanes 1–3) is markedly and reproducibly different in vivo from the corresponding DNA sites irradiated in vitro (bands e and f in lanes 4 and 5). We interpret the differences in the rate of UV photoproduct formation within the NFI-binding site/CRE in vivo, relative to the naked DNA, to signify that these promoter elements are continuously occupied regardless of the transcriptional state of the PEPCK gene. Faber et al. (30) have reported a similar finding using hepatoma cells treated with Bt2cAMP. Since the NFI-binding site appears to be continuously occupied by proteins, modulation of transcription caused by NFI may reflect the binding of different NFI isoforms during different physiological stimuli.

A Co-activator Molecule Is Involved in the Effect of NFI on PEPCK Transcription—The extent to which the P1 site on the PEPCK promoter is necessary for the NFI effects was determined by transfecting a PEPCK-CAT reporter gene containing a block mutation in the P1 site (“P1-CAT”) into HepG2 cells in the presence and absence of the various NFI-A and NFI-C isoforms (Fig. 2). NFI-A induced transcription from the intact PEPCK promoter 6-fold but increased transcription from the PEPCK gene promoter with a mutation in the P1 site only 2.5-fold (Fig. 2A). Similar results were observed for NFI-C (Fig. 2B). NFI-C blocked PKA-c-induced transcription from the intact PEPCK gene promoter by 60%, whereas PKA induction of the chimeric PEPCK-CAT gene with a mutation in the P1 site was inhibited by only 25%. Thus, removal of the NFI-binding site clearly reduced the effectiveness of NFI in altering PEPCK gene. There are two possible explanations for the partial effects observed in the absence of the NFI site: (i) NFI may be promiscuous in its binding and have other weaker binding sites on the promoter, and (ii) NFI may be capable of direct interaction with components of the initiation complex. Mutant transcription factors that cannot dimerize or bind DNA have been shown to be capable of activating or repressing transcription (31, 32). One candidate for mediating these site-independent effects is a co-activator molecule such as the CBP/p300 family of proteins. Members of this family have multiple binding domains and
have been implicated as co-activators in the signal transduction of members of the steroid receptor superfamily as well as members of the leucine zipper group of transcription factor such as c-Fos and c-Jun (33). Although the transcriptional regulation of the PEPCK gene promoter has never been linked directly to CBP/p300, such an interaction is likely since PECK gene expression is induced by thyroid hormone (34), glucocorticoids (5), cAMP (9, 7, 8), and c-Jun (35), all of which are known to bind to CBP. In addition, the adenovirus early protein, E1A, which binds to CBP, markedly inhibits cAMP-induced transcription from the PEPCK gene promoter in hepatoma cells (36). However, there has been no report of a direct interaction of members of the NFI family of transcription factors with CBP.

Co-transfection of increasing amounts of an expression vector for CBP caused a linear, 7-fold increase in expression of the PEPCK-CAT gene (Fig. 3A). Further increasing the amount of transfected CBP plasmid caused a dramatic decrease in CAT activity. Another indication that CBP coordinates the transcription of a stimulus to the initiation complex on the PEPCK gene promoter is its susceptibility to the action of E1A, which binds at the E1A binding domain located near the C-terminal of CBP (37). E1A has only a marginal effect on the level of basal transcription from the PEPCK gene promoter but can markedly inhibit the induction of transcription by PKA-c (Fig. 3B). An E1A mutant (Δ2-36), defective in its ability to bind CBP by virtue of a mutation in its N-terminal region, did not block the expected stimulation of expression of the PEPCK-CAT gene by PKA-c (Fig. 3B, inset).

Protein-Protein Interaction Assays Show That the Transactivation Domain of NFI-C Can Interact with the CREB-binding Domain of CBP—Chaudhry et al. (15) used domain swap experiments to show that the transactivation domain of NFI isoforms resides wholly in the C-terminal regions. The transactivation domain of NFI-C in particular appears to be the most active in transfection studies using the promoters for both the PEPCK gene (14) and the mouse mammary tumor virus.2 For these reasons we used a mammalian two-hybrid assay to test whether the NFI-C transactivation domain could interact with different regions of CBP protein. We used plasmids encoding fusion proteins comprising the Gal4 DNA binding domain linked to four different CBP domains representing amino acids 1–299 (pBCBP299), 451–682 (pBCBP451), 1678–1843 (pBCBP1678), and 1678–2441 (pBCBP2441). In addition, the transactivation domain of NFI-C comprising amino acids 220–499 was subcloned into the pACT vector containing the VP16 transactivation domain (pAC220). The two plasmids were then used in standard two-hybrid assays, the results of which may be seen in Fig. 4.

Basal activity (1st bar) was that seen when the reporter gene was co-transfected with the pACT and pBIND vectors without inserts. The levels of activity observed for the two fusion genes in combination were corrected for increases in background caused by individual components, and the results are presented as fold induction over basal. The 2nd bar indicates the result when positive control fusion genes supplied by the manufacturer of the kit (pACTMyo and pBindId) were used. There was a robust interaction between pAC220 and pCBP451 which codes for the CREB binding domain of CBP (100-fold over background), with virtually no interaction occurring with the E1A domain of CBP. These experiments were repeated in NIH 3T3 cells with very similar results and suggest that the effects of NFI may be mediated by competition for the CREB binding domain of CBP.

CBP Fragments Containing Binding Sites for Specific Transcription Factors Prevent Modulation of Transcription from the PEPCK Gene Promoter—Expression vectors coding for fragments of CBP were used to examine whether they alter the ability of well characterized transcription factors to modulate transcription from the PEPCK gene promoter. The effect of CREB and E1A was determined because the CBP domains involved in the signal transduction of these two transcription factors are well characterized. CREB binds to a region between amino acid residues 451 and 562, whereas E1A binds to a region between amino acids 1805 and 1854 (37), and both proteins are known to alter transcription from the PEPCK gene promoter. Hep G2 cells were co-transfected with plasmids for PEPCK-CAT and E1A in the presence and absence of expression vector CBP451, which corresponds to the CREB binding domain of CBP (amino acid residues 451–682) or an expression vector CBP1678, which encompasses the E1A binding domain of CBP (amino acid residues 1678–2441). CBP451 and CBP1678 stimulated expression of the PEPCK-CAT gene 5- and 12-fold, respectively (Fig. 5A, lanes 2 and 5). When E1A was co-transfected along with the individual CBP fragments, the results differed. E1A caused a substantial inhibition of transcription from the PEPCK gene promoter in the presence of CBP451 (compare Fig. 5A, lanes 2 and 3) but had no effect on

2 R. M. Gronostajski, unpublished results.
transcription in the presence of CBP1678 (the CBP fragment that contains the E1A binding domain; compare Fig. 5A, lanes 5 and 6). This effect was observed even when the amount of transfected E1A plasmid used in the transfection was increased 4-fold (Fig. 5A, lanes 4 and 7).

Complementary results were observed when a plasmid coding for CREB was transfected into HepG2 cells along with vectors encoding the different binding domains of CBP. CREB is a transcription factor that binds to the CRE of the PEPCK gene promoter and interacts with the CREB binding of CBP (Fig. 5B). When co-transfected with the PEPCK-CAT gene, CBP451 and CBP1678 caused a similar, approximately 8-fold increase in CAT activity. When CREB was co-transfected with CBP451 (the fragment that contains the CREB-binding domain of CBP), no effect was seen (compare Fig. 5B, lanes 3 and 4). However, when CREB was co-transfected with CBP1678, the CAT activity dropped from that seen with CBP1678 alone to a level almost identical to that observed with CREB alone (compare Fig. 5B, lanes 5, 6, and 7). CREB alone caused a modest increase in PEPCK-CAT expression (lane 2). CREB produces approximately this same level of activity in the presence of CBP1678 but not in the presence of the CBP fragment which contains the CREB-binding site (CBP451). Thus, these two fragments of CBP may be used to distinguish which region of the CBP molecule is involved in the transduction of signal from these two well characterized transcriptional regulatory factors.

One model consistent with all these data is that the protein products of the co-transfected CBP fragment expression vectors sequester transcription factors that normally bind to endogenous CBP. When CBP451 was co-transfected alone, we observed a strong increase in transcription. The two simplest explanations for this is that CBP451 transactivated the
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PEPCK-CAT reporter gene or that it removed endogenous inhibitors by sequestration. Since CBP451 lacks a transactivation domain, we suggest the latter the more likely to be the case. Hence, the protein products of overexpressed CBP451 most likely sequestered CREB and prevented the modulation of the PEPCK-CAT transcription. The protein products of CBP451 do not bind E1A and so, in the presence of overexpressed CBP451, E1A still functioned to block transcription from the PEPCK gene promoter.

**NFI Isoforms Are Prevented from Acting on Transcription from the PEPCK Gene Promoter by the Protein Product of Overexpressed CBP451 That Codes for the CREB Binding Domain of CBP**—In parallel experiments, NFI-A or NFI-C were co-transfected with PEPCK-CAT into HepG2 cells in the presence and absence of expression vectors E1A (0.05 and 0.2 g per plate) was determined 48 h later. The results were similar for both steroid and thyroid hormone stimulation of transcription from the PEPCK gene promoter. Thyroid hormone stimulated transcription from the PEPCK promoter approximately 8-fold (Fig. 6, lane 2) whereas NFI-A alone produced a 6-fold stimulation (lane 2). NFI-A in combination with thyroid hormone produced an additive effect (approximately 20-fold; compare Fig. 7, lanes 2–4). Similar results were observed in experiments in which NFI-A and dexamethasone were combined. Individually they stimulated PEPCK-CAT transcription by 6- and 3-fold respectively; in combination their effect on PEPCK-CAT activity in the HepG2 cells was additive (approximately 10-fold; compare Fig. 7, lanes 2, 5, and 6).

In a second series of experiments, NFI-A was co-transfected with PEPCK-CAT into HepG2 cells in the presence of expression vectors for CREB or PKA-c. Individually, NFI-A and CREB increased transcription from the PEPCK-CAT gene 8- and 3-fold, respectively (Fig. 8, lanes 2 and 3). When NFI-A and CREB were co-transfected together, the increase in transcription was not additive but was not significantly different from that found with NFI-A alone (Fig. 8, lane 4). A similar pattern was observed when NFI-A was co-transfected along with an expression vector PKA-c, a potent stimulator of PEPCK-CAT transcription whose actions are mediated through the CREB binding domain of CBP. PKA-c alone produced a 10-fold induction (Fig. 8, lane 5). When PKA-c was co-transfected with NFI-A, we observed again that the resulting induction was not significantly different from that produced by NFI-A alone (Fig. 8, lane 6). It thus appears that NFI-A blocks PKA-c and CREB-mediated activation of transcription from the PEPCK gene promoter by successfully competing for the CREB binding domain of CBP. These studies demonstrate that CBP has the capability of coordinating the
transcriptional response of the PEPCK-CAT gene to multiple stimuli simultaneously.

**DISCUSSION**

The Role of NFI in Regulating PEPCK Gene Transcription—
The family of four NFI genes give rise to four distinct isoforms (NFI-A, -B, -C, and -X (38, 39), each of which may give rise to splice variants by post-transcriptional processing (16, 38, 40). NFI isoforms bind as dimers (41), raising the possibility of subtle and complex interactions with DNA through heterodimerization. NFI was initially studied for its role in the replication of adenovirus (42). Later it was reported to be important for transcription of mouse mammary tumor virus (43). More recently, NFI has been implicated in transcriptional control of genes of physiological importance. For example, NFI is involved in glucocorticoid receptor-mediated transcriptional response of the gene for mouse mammary tumor virus (44) and aspartate aminotransferase (45). It is also a negative modulator of transcription of the gene for L-type pyruvate kinase (46).

The PEPCK gene promoter contains an NFI-binding element that maps immediately adjacent to the CRE (3). Mutating this element resulted in a marked increase in basal gene expression in the livers of transgenic mice that contained a chimeric PEPCK-bovine growth hormone (PEPCK-bGH) gene (20). In addition, there was premature expression of the transgene in 19-day-old fetal mice in utero (20). Recently, Crawford et al. (14) demonstrated that expressing any of the four isoforms of NFI in hepatoma cells inhibits the normally robust induction of transcription from the PEPCK gene promoter caused by cAMP. Understanding the role of NFI in the regulation of PEPCK gene transcription is complicated by the fact

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**FIG. 6.** The effects of NFI-A and NFI-C on PEPCK-CAT transcription in the presence of specific fragments of CBP. **A,** an expression vector for NFI-A (2.5 μg per plate) was tested for its ability to stimulate transcription of the PEPCK-CAT gene (2.5 μg) in the presence of 20 μg per plate of expression vector CBP451 (containing the CREB binding domain of CBP) or CBP1678 (containing the E1A binding domain of CBP). Cells were later assayed for CAT activity as described under “Experimental Procedures.” **B,** an expression vector for NFI-C was tested for its ability to inhibit PEPCK-CAT transcription in the presence of CBP protein fragments. The amount of plasmids transfected were the same as in **A** with NFI-C substituted for NFI-A. Values for CAT activity were normalized for protein content and expressed as the mean ± S.E. for 3 replicates. Results were analyzed using a one-tailed t test and were significant at p ≤ 0.05.

**FIG. 7.** The effects of NFI-A, dexamethasone, and thyroid hormone on transcription from the PEPCK gene promoter. **A,** HepG2 cells were co-transfected with 2.5 μg of PEPCK-CAT, along with a similar amounts of expression vectors for NFI-A, the human glucocorticoid receptor, and human thyroid hormone receptor, individually and in combination, as indicated in the figure. After 24 h, the cells were treated overnight with 10 nM dexamethasone (DEX) or 1.0 μM triiodothyronine (T3) and assayed for CAT activity as described under “Experimental Procedures.” All values for CAT activity were normalized for protein content and expressed as the mean ± S.E. for 3 replicates. Results were analyzed using a one-tailed t test and were significant at p ≤ 0.05.

**FIG. 8.** The combined effects of NFI-A with CREB or PKA-c on transcription from the PEPCK gene promoter. **A,** HepG2 cells were co-transfected with 2.5 μg of PEPCK-CAT together with a similar amount of expression vector for either NFI-A or the catalytic subunit of PKA-c individually and in combination. Cells were assayed 2 days later for CAT activity. **B,** as in **A** except that an expression vector for CREB was substituted for that of PKA-c. All values for CAT activity were normalized for protein content and expressed as the mean ± S.E. for 2 to 3 replicates. Results were analyzed using a one-tailed t test and were significant at p ≤ 0.05.
that the major isoforms of NFI exert different effects on the level of basal transcription (NFI-A and NFI-B increase basal transcription and NFI-C and NFI-X decrease this process). The results of the present study show that NFI does not alter PEPCK gene transcription by binding to an unoccupied site on the PEPCK gene promoter. This suggests that NFI exerts its effect by differential expression of one of the four NFI genes in response to different stimuli, and differences in modulation of PEPCK expression would result from an exchange of NFI occupying the P1 site. Alternatively, the NFI bound to the P1 site could be covalently modified by phosphorylation or glycosylation (47, 48) which would alter its ability to affect gene transcription. Toohey et al. (49) showed that although NFI binding to the mouse mammary tumor virus promoter sequences could be enhanced by mutating specific bases, the effect of NFI on steroid-induced transcription from the mouse mammary tumor virus gene promoter did not change. This suggests that affinity to the NFI-binding site is not the principal determinant of transcriptional activation by NFI protein.

From the results of Faber et al. (30) and those published in this paper (see Fig. 1B), it seems likely that both the CRE and NFI sites of the PEPCK gene promoter are occupied by transcription factors even in the absence of cAMP or glucocorticoid stimulation of transcription. Christ et al. (50) reported similar results that showed complex formation between protein nuclear extracts and an oligonucleotide containing an NFI-binding site was not altered when cells were pretreated with glucagon, which stimulates PEPCK gene transcription. From the available data it seems reasonable to assume that NFI acts to maintain a low level of basal transcription from the PEPCK promoter and that it interacts with protein(s) binding to the CRE to regulate PEPCK gene transcription. The proximity of the NFI-binding site and the CRE on the PEPCK gene promoter suggests that there is a functional interaction between NFI and the protein(s) that bind to the CRE. These include CREB, C/EBP, and Jun/Jun homodimers, all of which have been shown to bind to the CRE of the PEPCK gene promoter. However, Crawford et al. (14) demonstrated that the proximity of the CRE to the NFI binding domain was not required for the negative effect of NFI on transcription from the PEPCK gene promoter. In the present paper we show that the NFI-binding site is not required for NFI-C to block cAMP induction of PEPCK gene transcription in transfected HepG2 cells. This suggests that NFI interacts with a co-activator protein, such as CBP/p300, to block the normal induction of PEPCK gene transcription caused by cAMP.

The Role of CBP in the Regulation of PEPCK Gene Transcription—A role for CBP/p300 in the regulation of PEPCK gene transcription was suggested by a previous report by Kalvakolanu et al. (36) which demonstrated that the adenoviral early protein, E1A, totally inhibited the cAMP induction of PEPCK gene transcription in hepatoma cells, even though E1A itself does not bind to the PEPCK promoter. Subsequently it was established that the mechanism of E1A action involves its binding to CBP/p300. CBP/p300 has a number of discrete functions in regulating gene transcription. It is considered by some to be a subunit of RNA polymerase II and can integrate regulatory signals from transcription factors and chromatin by virtue of its intrinsic acetyltransferase activity (51). CBP/p300 can itself be phosphorylated on serine and threonine residues during retinoic acid-induced differentiation of F9 embryonal carcinoma cells (52), suggesting that phosphorylation of the protein by a cyclin-dependent kinase can control its transcriptional activity. At the present time there are more than 30 proteins known to bind to CBP/p300, and the list is growing (33). Nakajima et al. (53) have suggested a model in which the cAMP regulatory element-binding protein (CREB) is phosphorylated by PKA and then binds to CBP resulting in an association of CBP/p300 with TFIIB and RNA polymerase II.

The PEPCK gene promoter has binding domains for a large variety of transcription factors so that it is critical that the effect of these factors on transcription be integrated and coordinated with the transcriptional machinery at the TATA box. Based on the results of the current paper, we propose that CBP controls the interaction of the various effector molecules and acts to regulate the rate of PEPCK gene transcription. CBP has specific binding sites for many of the proteins that are known to be involved in the control of PEPCK gene transcription, including thyroid hormone and glucocorticoid receptors, CREB, Jun, and C/EBP (33). There are several lines of evidence to support a role for CBP in the regulation of PEPCK gene transcription. First, CBP in the absence of any other extrinsic factors can stimulate transcription of a chimeric PEPCK-CAT gene (Fig. 5). Second, E1A strongly inhibits cAMP-induced transcription from the PEPCK gene promoter by binding to CBP/p300. In support of the effects of E1A on PEPCK gene transcription is the report of Arany et al. (37) demonstrating a similar effect of E1A on cAMP-induced transcription from the somatostatin gene promoter. Third, transcription factors such as C/EBP, which play a key role in the response of the PEPCK gene promoter to cAMP (54–56), also bind to CBP/p300. Recently, Mink et al. (57) reported that the N terminus of C/EBP inter-
acts with the E1A binding domain of CBP, suggesting that the cAMP-induced effect of C/EBP isoforms on PEPCK gene transcription may be exerted at the E1A domain of CBP. A direct competition between C/EBP and E1A for binding to the same domain on CBP could explain the mechanism of the dominant negative effect of E1A on cAMP-induced PEPCK gene transcription (36). This possibility is reinforced by the fact that E1A totally inhibits the stimulatory effects of Jun/Jun homodimers on PEPCK gene transcription (36); Jun also binds to the E1A domain of CBP, suggesting a common mechanism.

A complicating factor in this interpretation of the action of E1A on PEPCK gene expression is a paper by Klemm et al. (58), which reported that infection of HepG2 cells with an adenovirus expressing E1A resulted in an increase rather than a decrease in transcription from the PEPCK gene promoter. It is not clear why there is such a marked difference in the response of PEPCK gene transcription in the two studies. One notable difference is that Klemm et al. (58) used whole virus as a source of E1A and reported viral titers of 100–200 infectious particles per cell. Their cells were maintained for 20 h after adenoviral infection, so that viral-induced transcription factors could participate in the regulation of PEPCK gene transcription. Kalgavakalanu et al. (36) infected HepG2 cells with adenovirus but isolated nuclei for transcription measurements within 2.5 h. In addition, Kalgavakalanu et al. (36, 58) determined the direct effect of E1A, introduced into the HepG2 cells via an expression plasmid, on transcription from the PEPCK gene promoter. The most likely explanation for the differences between the two studies is that the long term expression of the adenoviral genes in hepatoma cells could alter PEPCK gene expression in an indirect manner due to the expression of viral genes in the late stage of infection.

Control of PEPCK Gene Transcription Requires Coordination of the Activity of Transcription Factors Through Their Interaction with CBP—The integration of the numerous signals that control transcription of the gene for PEPCK is critical to ensure the appropriate expression of the gene in response to physiological stimuli. CBP is an ideal co-activator, since it contains binding sites for multiple transcription factors, has intrinsic histone acetylase activity (51), and interacts with RNA polymerase (53). Shikama et al. (33) have suggested that “CBP/pp30 may provide a transcriptional scaffold that enables regulatory molecules, such as kinases, acetyltransferases, and perhaps other enzymatic activities, to be recruited to and assembled at relevant sites for transcriptional activity.”

Fig. 9 presents our model for the proposed interaction of CBP and the transcription factors that control the expression of the gene for PEPCK. NFI binds to the CRE-binding site of CBP (Figs. 4 and 5), whereas C/EBP, which is critical for the cAMP regulation of PEPCK gene transcription, binds to the E1A site of CBP (57). The transcriptional activation domain of CBP is brought into contact with the TATA binding factors of the PEPCK gene promoter by first binding to transcription factors such as C/EBP; this in turn aligns CBP properly on the promoter to activate gene transcription. There are a number of lines of evidence that indicate that C/EBP rather than CREB is involved in the hepatic cAMP-induced transcription of the PEPCK gene (these are reviewed in detail in Ref. 59). However, since both CREB and C/EBP bind to the CRE of the PEPCK gene promoter, it is also possible that CREB mediates the cAMP effect on PEPCK gene transcription.

Since the CRE and NFI sites of the PEPCK gene promoter are always occupied by transcription factors (30), it is likely that CBP is recruited to bind to C/EBP, most likely through the E1A or CREB binding domains of CBP. Cyclic AMP control of PEPCK gene transcription would involve PKA-c phosphorylation of C/EBP (or CREB), resulting in an interaction of these transcription factors and CBP, aligning it appropriately to allow for interaction with RNA polymerase II and increased gene transcription. According to this model (Fig. 9), NFI blocks cAMP-induced transcription from the PEPCK gene promoter by binding to the CREB site on CBP and preventing the appropriate alignment of CBP or by blocking the binding of C/EBP (or CREB) to CBP. The strong negative effect of E1A on PEPCK gene transcription is a prototype of the negative control exerted by proteins that bind to critical sites of CBP. Our model predicts that intermediate molecules activated in the insulin signaling cascade will act by blocking the interaction of CBP with critical proteins bound to the PEPCK promoter, thus interfering with the appropriate alignment of CBP with the transcriptional machinery. The finding that the mitogen-regulated S6 kinase pp90Bob, which binds to the E1A domain of CBP, is responsible for inactivating the cAMP-inducible gene transcription caused by CREB (53) supports a model in which an intermediate in a signaling cascade is able to interact with CBP to control gene transcription. CBP can also coordinate the effects of more than one transcription factor simultaneously and can distinguish between a positive and a negative effector molecule. As an example, NFI-A, which inhibits cAMP-induced transcription from the PEPCK gene promoter, causes an additive increase in PEPCK gene transcription in the presence of glucocorticoids (Fig. 7). This indicates that CBP can coordinate a response to two signals simultaneously and has a high degree of selectivity in orchestrating the response of the PEPCK gene promoter to various signals. Further work is required to more fully delineate the role played by CBP in coordinating the dietary and hormonal control of PEPCK gene transcription.

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