CREB-binding Protein Is a Transcriptional Coactivator for Hepatocyte Nuclear Factor-4 and Enhances Apolipoprotein Gene Expression*

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Hepatocyte nuclear factor-4 (HNF-4) is a liver-enriched transcription factor that is crucial in the regulation of a large number of genes involved in glucose, cholesterol, and fatty acid metabolism and in determining the hepatic phenotype. We have previously shown that HNF-4 contains transcription activation functions at the N terminus (AF-1) and the C terminus (AF-2) which work synergistically to confer full HNF-4 activity. Here, we show that HNF-4 recruits the CREB-binding protein (CBP) coactivator on promoters of genes that contain functional HNF-4 sites. HNF-4 interacts with the N-terminal region of CBP (amino acids 1–771) and the C-terminal region of CBP (amino acids 1812–2441). The two activating functions of HNF-4, AF-1 and AF-2, interact with the N terminus and the N and C terminus of CBP, respectively. In addition, we show that in contrast to the other nuclear hormone receptors the interaction between HNF-4 and CBP is ligand-independent. Recruitment of CBP by HNF-4 results in an enhancement of the transcriptional activity of the latter. CBP does not activate gene expression in the absence of HNF-4, and dominant negative forms of HNF-4 prevent transcriptional activation by CBP, suggesting that the mere recruitment of CBP by HNF-4 is not sufficient for enhancement of gene expression. These findings demonstrate that CBP acts as a transcriptional coactivator for HNF-4 and provide new insights into the regulatory function of HNF-4.

The hepatocyte nuclear factor-4 (HNF-4) is a member of the superfamily of nuclear hormone receptor (NHR) proteins that control transcription in diverse metabolic pathways. Originally identified by its importance in the regulation of liver-specific genes, it is also expressed in the pancreas, kidney, stomach, skin, and intestine (1–3). There are currently three family members HNF-4α, β, and γ (4, 5), and seven splice variants of HNF-4α have been identified from human, rat, and mouse cDNAs (6–11). HNF-4 is a crucial regulator of several metabolic pathways, including those for glucose and lipid homeostasis. Mutations in HNF-4α impair insulin secretion and cause type 2 diabetes (12). In addition, the promoters of apolipoproteins apoAI, apoAII, apoAIV, apoB, apoCII, and apoCIII all contain binding sequences for HNF-4 (13–17). HNF-4α also plays a vital role in development. In mice, HNF-4α transcripts have been detected as early as day 4.5 and its knock-out impairs gastrulation and is embryonic-lethal (3, 18).

In a recent study, we performed a systematic analysis of the functional domains of HNF-4 that are involved in DNA binding, dimerization, and transactivation (19). We have found that HNF-4 contains two activation functions (AFs), designated AF-1 and AF-2, that are located in the A/B and D/E regions, respectively, and activate transcription in a cell type-independent manner. In most cell types examined, AF-1 and AF-2 synergize for full HNF-4 activity. The AF-1 consists of the extreme N-terminal 24 amino acids and functions as a constitutive autonomous activator of transcription. The AF-2 transcriptional activator is more complex, spanning the region between amino acids 128 and 366. The 360–366 region of HNF-4 (AF-2 AD) contains a motif that is highly conserved among transcriptionally active nuclear receptors and is essential for AF-2 activity. Unlike the AF-2 domains of retinoid X receptor, retinoic acid receptor, and thyroid receptor, the corresponding AF-2 AD region does not exhibit an autonomous function, although it can adopt a similar amphipathic α-helical conformation (20–24). For AF-2 activity in HNF-4, the entire 128–366 region is required, representing a new subclass of activation domains defined by the requirement of an intact D/E region for activity. The F region, which is unique to HNF-4, has a negative effect on AF-2 activity. It was recently shown that long chain fatty acids directly modulate the transcriptional activity of HNF-4α by binding as their acyl-CoA thioesters to HNF-4. Depending on the chain length and the degree of saturation of the fatty acyl-CoA ligand, HNF-4 can either activate or repress transcription of the target genes (25). However, the involvement of the different HNF-4 domains in ligand-mediated transcriptional activation has yet to be defined.

Recent studies have shown that the ligand binding domains (LBDs) of certain nuclear hormone receptors interact strongly with the coactivator CBP and its functional homolog p300 in a ligand-dependent manner (26, 27). CBP is a multi-faceted protein involved in multiple signal transduction pathways with different activators and in interactions with components of the basal transcription machinery like TATA-binding protein, TFIIB, and RNA helicase (28–31). It has intrinsic histone...
acetylase (HAT) activity (32, 33) and interacts with the nuclear hormone receptor coactivators SRC-1, P/CAF, and p/CIP, which have their own HAT activity (27, 34–39). Together these proteins form a putative coactivator complex, the components of which appear to be specific for different transcriptional activators (40). Current theory suggests that transcription factors bound to their DNA response elements associate with CBP or other coactivators and direct acetylation of histones in their vicinity. The consequent restructuring of the chromatin leads to enhanced assembly of the basal transcription machinery, possibly recruited by CBP, to form a stable preinitiation complex. However, chromatin disruption by histone acetylation is not sufficient for NHR-dependent transcription (41). In addition, p300 can acetylate the general transcription factors TFIEβ and TFII F (42) suggesting that modification of core proteins by CBP or other coactivators may have a role in transcriptional activation.

In the present study, we show that HNF-4 interacts in vitro with CBP and that HNF-4 can recruit CBP while bound to the DNA. CBP targets both the AF-1 and the AF-2 domains of HNF-4. The AF-1 domain interacts weakly only with the N-terminal region of CBP, and the AF-2 domain of HNF-4 interacts with both the N- and C-terminal regions of CBP, and this interaction is both AF-2 AD and ligand-independent. In co-transfection assays, CBP functions as a coactivator and stimulates the transcriptional activity of HNF-4. CBP does not activate gene expression in the absence of HNF-4, and dominant negative forms of HNF-4 prevent transcriptional activation by CBP. Although both the N- and C-terminal regions of CBP associate with HNF-4, transcriptional coactivation may be mediated by the C-terminal region. These findings demonstrate that CBP acts as a transcriptional coactivator for HNF-4 and provide new insights into the regulatory function of HNF-4.

MATERIALS AND METHODS

Plasmids—The wild type apoCIII promoter [apoCIII(−890/+24)/CAT] plasmid has been described previously (43). The reporter plasmid [apoCIII(−890/+24)/CAT(1)] was prepared by transfection into 3T3-L1 adipocytes for three times with Tyrode’s solution containing 10 mM sodium acetate, 0.1 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride (GST-CBP1, GST-CBP2, and GST-CBP3), or of 50 mM NaCl, 20 mM Tris-HCl, pH 8.0, 0.05% Nonidet P-40, 1 mg/ml BSA, 1 mg DTT, and 1 mg phenylmethlysulfonyl fluoride (GST-CBP4). Equal amounts of in vitro translated and [35S]methionine-labeled HNF-4 proteins were added, and binding was allowed to proceed for 2 h at 4 °C. The beads were washed with the same buffer and eluted with the same buffer lacking BSA. The bound proteins were eluted with 50 μl of 2× SDS-PAGE loading buffer and were resolved by electrophoresis. The proteins were visualized by autoradiography.

Electrophoretic Mobility Shift Assay (EMSA)—HNF-4 CDNA was cloned in the bacterial pET15b vector under the control of the T7 promoter and was expressed in E. coli BL21(DE3) strain. The expression and purification of HNF-4 protein has been previously described (45). A double-stranded oligonucleotide corresponding to the B regulatory element of the apoCIII promoter (CHIB) which binds HNF-4 was used as a probe (15). Bacterially expressed HNF-4 protein was incubated with the 32P-labeled probe for 15 min at 4 °C in the presence of 25 mM Hepes, pH 7.6, 40 mM KCl, 1 mM DTT, 5 mM MgCl2, 0.1% Nonidet P-40, 5 μg/ml BSA, and 50 μg of purified HNF-4. Where indicated, the reactions also included 0.25 μg of either GST-CBP1, GST-CBP2, or GST-CBP4 proteins that had been eluted from the agarose beads.

Cell Culture and DNA Transfection—HepG2, HeLa, or CV1 cells were maintained as stocks in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. Fifty to 60% confluent 35-mm dishes were transfected using the calcium-phosphate coprecipitation method, as described previously (46). The transfection mixture contained 3 μg of the CAT reporter plasmid, 50 ng of the HNF-4 or HNF-4 mutant expression plasmids (shown in Fig. 5a), or 100 ng of GAL-HNF-4 fusion plasmid, 1 μg of CMV-β-galactosidase plasmid, and where appropriate, 1.25 μg of CBP or CBP mutant plasmid. In every case vector DNA (pcDNAI/Amp) was added as necessary to achieve a constant amount of transfected DNA (5.75 μg). Forty hours post-transfection the cells were washed, solubilized, and collected in TEN solution (0.04 μM Tris-HCl, pH 7.4, 1 mM EDTA, pH 8.0, 1 mM DTT, 1 mM phenylmethlysulfonyl fluoride, 0.1 mM benzamidine, 2 μg/ml aprotinin, 1 μg/ml pepstatin, and 1 μg/ml leupeptin) and were broken by sonication on ice. Cell debris was removed by centrifugation for 10,000 rpm for 20 min at 4 °C, and the supernatant was incubated for 2 h with glutathione-agarose beads (SIGMA G4510) equilibrated to the same buffer. The coupled proteins were washed several times with the bovine serum albumin solution and were stored at 4 °C covered by an equal volume of buffer. The purity of proteins bound to the GST-agarose beads was identified by SDS-PAGE. When necessary, the pure GST-CBP proteins were eluted from the agarose beads by resuspending the pelleted beads in a half-volume of cold phosphate resuspension buffer containing 2.5 mM glutathione. The suspension was incubated for 2–3 min on ice, the beads pelleted by centrifugation, and the eluate reserved. The beads were washed 2–3 more times, and the protein content in each fraction of the eluate was observed by SDS-PAGE.

Protein Binding Assays Using GST Fusion Proteins—For the binding assays 5–50 μl of glutathione beads containing approximately 0.5 μg of the fusion GST-CBP protein were incubated at 4 °C in 0.35 ml of 150 mM sodium acetate, pH 7.0, 25 mM HEPES, pH 7.2, 2 mM EDTA, 0.25% BSA, 0.1% Nonidet P-40, 5 μg DTT, and 1 mg phenylmethlysulfonyl fluoride (GST-CBP1, GST-CBP2, and GST-CBP3), or of 50 mM NaCl, 20 mM Tris-HCl, pH 8.0, 0.05% Nonidet P-40, 0.25% BSA, 1 mg DTT, and 1 mg phenylmethlysulfonyl fluoride (GST-CBP4). Equal amounts of in vitro translated and [35S]methionine-labeled HNF-4 proteins were added, and binding was allowed to proceed for 2 h at 4 °C. The beads were washed with the same buffer and eluted with the same buffer lacking BSA. The bound proteins were eluted with 50 μl of 2× SDS-PAGE loading buffer and were resolved by electrophoresis. The proteins were visualized by autoradiography.

In Vitro Transcription and Translation—[35S]Met-methionine-labeled HNF-4 proteins were produced from cDNA templates cloned downstream of T7 RNA polymerase promoter using the TNT T7 Quick-coupled reticulocyte lysate system (Promega).

Preparation and Expression of GST Fusion Proteins—Escherichia coli strain BL21 was transformed with the GST-CBP fusion proteins GST-CBP1, GST-CBP2, GST-CBP3, and GST-CBP4. Fresh transformant colonies were grown in LB media supplemented with ampicillin (50 μg/ml) at 37 °C until an A600 = 0.5 was reached. Isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.5 mM. After incubation for 3 h cells were harvested by centrifugation at 4,000 × for 10 min. Cell pellets were resuspended in 1/20 of the original culture volume in ice-cold phosphate resuspension buffer (phosphate-buffered saline containing 20% glycerol, 1% Nonidet P-40, 100 mM EDTA, pH 8.0, 1 mM DTT, 1 mM phenylmethlysulfonyl fluoride, 0.1 mM benzamidine, 2 μg/ml aprotinin, 1 μg/ml pepstatin, and 1 μg/ml leupeptin) and were broken by sonication on ice. Cell debris was removed by centrifugation at 10,000 rpm in 20 min at 4 °C, and the supernatant was incubated for 2 h with glutathione-agarose beads (SIGMA G4510) equilibrated to the same buffer. The coupled proteins were washed several times with the bovine serum albumin solution and were stored at 4 °C covered by an equal volume of buffer. The purity of proteins bound to the GST-agarose beads was identified by SDS-PAGE. When necessary, the pure GST-CBP proteins were eluted from the agarose beads by resuspending the pelleted beads in a half-volume of cold phosphate resin suspension buffer containing 2.5 mM glutathione. The suspension was incubated for 2–3 min on ice; the beads pelleted by centrifugation, and the eluate reserved. The beads were washed 2–3 more times, and the protein content in each fraction of the eluate was observed by SDS-PAGE.

GST-CBP fusion constructs in pGEX-3T (Amersham Pharmacia Biotech) and the various CBP mutants cloned in vector pRC/RSV (Invitrogen) (44) were a generous gift from Dr. Dimitris Thanos (Columbia University).
CBP Enhances HNF-4-dependent Transactivation

**RESULTS**

**HNF-4 Interacts with CBP in Vitro**—To investigate whether there is an interaction between HNF-4 and CBP, we used a protein-protein interaction assay with glutathione S-transferase (GST)-CBP fusion proteins. Several fragments of CBP cloned in frame with the GST gene (Fig. 1A) were expressed, purified, and immobilized on glutathione-agarose beads before incubation with in vitro translated, [35S]methionine-labeled HNF-4. As shown in Fig. 1B, HNF-4 associates with CBP in vitro. Both the N-terminal fragment of CBP (GST-CBP1, lane 3) and the C-terminal fragment of CBP (GST-CBP4, lane 6) were found to interact with HNF-4. These interactions were specific as CBP regions 2 and 3 (lanes 4 and 5) did not associate with HNF-4 nor did GST alone (lane 2). The interaction of HNF-4 with the C terminus of CBP appeared to be weaker than that with the N terminus, since they retained approximately 10 and 20% of the HNF-4 input (lane 1), respectively.

To investigate whether HNF-4 can recruit CBP to the promoter, we carried out EMSA experiments using recombinant HNF-4 along with the GST-CBP fusion proteins. The CIIIB element of the apoCIII promoter that binds HNF-4 with strong affinity (15) was used as a probe. Bacterially expressed purified HNF-4 binds strongly to CIIIB (Fig. 2, lane 2). Interestingly, the same N-terminal fragment of CBP-(1–771) that specifically interacts with HNF-4 forms a ternary complex on the DNA with HNF-4 (Fig. 2, lane 3). GST-CBP4-(1892–2441) does not appear to bind to the DNA-HNF-4 complex (Fig. 2, lane 5). This is in agreement with the observation from the protein-protein interaction assay that the N terminus of CBP forms a stronger interaction with HNF-4 than the C terminus (Fig. 1B, lane 3 versus lane 6). The specificity of the HNF-4-CBP complex formed in the EMSA was demonstrated by the inability of GST-CBP2-(706–1069) to supershift the HNF-4-DNA complex (Fig. 2, lane 4), confirming the result (Fig. 1, lane 4) that this region of CBP does not associate with HNF-4.

**CBP Is a Transcriptional Coactivator for HNF-4**—Based on the interaction between HNF-4 and CBP, we sought next to evaluate the role of CBP in HNF-4-dependent transcription. Although CBP is widely expressed, its low levels are rate-limiting (27, 30), which permitted the use of cotransfection experiments. To test for transcriptional coactivation by CBP, we utilized the apoCIII promoter in combination with HNF-4. We showed previously that HNF-4 binds with high affinity to regulatory elements CIIIB and CII-1, present in the proximal promoter and in the enhancer region of the apoCIII gene, respectively (14, 15), and that it strongly activates the transcription of the apoCIII promoter (19). To examine the role of CBP in the HNF-4-dependent transactivation of the apoCIII gene, we performed transient transfection experiments in three different cell types (HepG2, HeLa, and CV1) using the wild type apoCIII promoter along with mammalian expression vectors for HNF-4 and/or CBP (Fig. 3A). We chose HepG2 cells, because they support the expression of liver-specific genes like apoCIII, and HeLa and CV1 cells in which the expression of apoCIII gene is totally dependent on the expression of cotransfected HNF-4. In HepG2 cells, HNF-4 enhanced apoCIII promoter activity by 4.5-fold and CBP by 6.5-fold in the absence of transfected HNF-4 (Fig. 3A). However, when the CBP expression vector was cotransfected along with HNF-4, apoCIII promoter activity levels were increased from 4.5- to 6.5–25-fold. The increase in apoCIII promoter activity by CBP alone ob-
CBP is a transcriptional co-activator for HNF-4. A, transient transfection experiments were performed in HepG2, HeLa, and CV1 cells with the apoCIII promoter construct (shown at the top of the panel) and plasmids HNF-4 and CBP expressing their corresponding cDNAs. Typical CAT assays for each cell type are shown. The CAT activity achieved with the reporter alone was set to 1, and other activities are presented relative to this value. B, transient transfection experiments in HeLa and HepG2 cells were performed with the (BA1)5CAT reporter plasmid (shown in the schematic diagram) and plasmids HNF-4 and CBP expressing their corresponding cDNAs. The bar graphs are mean values of CAT activity of at least three independent transfections, each carried out in duplicate, and show the relative transcription of the reporter in the absence (−) and presence (+) of expression plasmids CBP and HNF-4. C, transient transfection experiments in HepG2 cells were performed with the pG5CAT reporter (shown schematically at the top of the panel) and expression plasmids for CBP and/or a fusion of GAL4 DNA binding domain (residues 1–147) with HNF-4 (GAL-HNF-4). The bar graphs are mean values of CAT activity of at least three independent transfections, each carried out in duplicate, and show the relative transcription of the reporter in the absence (−) and presence (+) of expression plasmids CBP and HNF-4.
in HepG2 cells is probably attributable to the interaction of transfected CBP with endogenous HNF-4. Therefore, CBP functions as a coactivator for HNF-4-induced apoCIII gene transcription. This enhancement of HNF-4 transcription by CBP is more profound in HeLa and CV1 cells where the expression of CBP alone had no effect on the levels of activation of the reporter in the absence of cotransfected HNF-4 (Fig. 3A). Remarkably, a significant stimulation of the reporter above the levels observed with HNF-4 alone was induced by coexpression of full-length CBP. Specifically, cotransfection of CBP and HNF-4 increased apoCIII promoter activity by 4-fold (HeLa cells) and 2-fold (CV1 cells) over the activation by HNF-4 alone (Fig. 3A), implying that the synergism occurs through HNF-4.

The apoCIII promoter is complex, containing binding sites for various transcriptional activators. To discern whether the synergism between HNF-4 and CBP observed is entirely due to HNF-4, two simpler promoter plasmids were used in transfection experiments, one containing five HNF-4-binding sites ([BA1]5CAT) and the other with a heterologous promoter [pG5CAT] (19). As seen in Fig. 3B, the transcriptional activity of [BA1]5CAT was increased approximately 2-fold by overexpression of CBP and HNF-4 as compared with HNF-4 alone, both in HeLa and HepG2 cells. In agreement with the results presented in Fig. 3A, this effect appears to be due to the interplay between CBP and HNF-4 rather than merely the presence of CBP, since the expression of CBP alone does not increase basal activity of the reporter. The 2-fold increase observed in HepG2 cells is probably due to the interaction of CBP with endogenous HNF-4. Similarly, cotransfection with GAL-HNF-4 and CBP resulted in a 3-fold enhancement in the transcriptional activity of the reporter pG5CAT over its activity in the presence of GAL-HNF-4 alone (Fig. 3C).

These results clearly indicate that CBP-mediated transcriptional enhancement is dependent on the presence of HNF-4, since in the absence of HNF-4 (HeLa and CV1 cells), CBP has no effect on basal transcription.

**CBP Recruitment to the ApoCIII Promoter Is Mediated by HNF-4 Binding Either to the Enhancer or the Proximal Promoter Site**—To examine whether recruitment of CBP to the apoCIII promoter requires synergism between the two HNF-4 sites, we carried out cotransfection experiments in HepG2 cells. CBP recruitment to the apoCIII promoter is mediated by HNF-4 binding either to the enhancer or the proximal promoter site. Transient transfection experiments were performed in HepG2 cells with mutant apoCIII promoter templates (shown at the top of the panel) and plasmids HNF-4 and CBP expressing their corresponding cDNAs. Typical CAT assays for each promoter mutant are shown. The CAT activity achieved with the reporter alone was set to 1, and other activities are presented relative to this value and are the means of at least three independent experiments each carried out in duplicate.

HNF-4 and CBP responsiveness was studied by analyzing two promoter constructs with mutations in the proximal HNF-4-binding site, apoCIII(−890/+24)BM5CAT, or the distal HNF-4-binding site, apoCIII(−890/+24)M4CAT. We have previously shown that mutations in the proximal and enhancer HNF-4-binding sites severely compromise the apoCIII promoter strength to 36 and 20%, respectively (43, 48). As seen in Fig. 4, transcription by HNF-4 or CBP alone results in a modest 2–4-fold transactivation of the mutant apoCIII promoter templates. However, the simultaneous expression of CBP and HNF-4 led to a 12–20-fold stimulation of transcription, corresponding to a 2–4-fold synergism (Fig. 4). Therefore, we conclude that CBP can act in synergy with HNF-4 at either of these elements.

**CBP Targets Both the AF-1 and the AF-2 Domains of HNF-4**—To identify the regions of HNF-4 involved in protein-protein interactions with CBP, we carried out in vitro binding experiments using the N- (CBP1) and C (CBP4)-terminal regions of CBP, immobilized on glutathione-agarose beads and several in vitro translated and [35S]methionine-labeled HNF-4 deletion mutants. As seen in Fig. 4A, both the N- and C-terminal fragments of CBP form complexes with mutants HNF-4-(1–370) and HNF-4-(1–360), lacking the F domain of HNF-4 or the core AF-2 AD motif, respectively. In addition, removal of the AF-1 domain in mutants HNF-4-(48–455) or HNF-4-(48–370) does not affect the interaction of CBP and HNF-4. Interestingly, deletion of the entire AF-2 domain in mutants HNF-4-(1–174) or HNF-4-(1–128) does not abolish the interaction with HNF-4, thus unmasking a second region in HNF-4 that interacts with CBP. This region encompasses the AF-1 domain of HNF-4, which appears to interact with CBP weakly, since the amount of AF-1 retained by the GST-CBP1 decreases from approximately 20 to 10% of the input. Furthermore, the association between AF-1 and GST-CBP4 is abolished when the AF-2 domain is deleted. Therefore the AF-2 domain, in particular the E region between 174 and 360, is essential for interaction between HNF-4 and the C terminus of CBP. Surprisingly, interaction with both the N- and C-terminal regions of CBP is completely lost with mutants HNF-4-(128–370) and HNF-4-(174–370) encompassing the entire AF-2 domain or lacking the D domain of HNF-4, respectively (Fig. 5A). Since removal of the AF-1 does not affect the CBP interactions, and the AF-2 alone...
does not form a complex with either GST-CBP1 or GST-CBP4, it seemed possible that the DNA binding domain (DBD) of HNF-4 might be involved in these interactions. To examine the role of HNF-4 DBD in interactions with CBP, we prepared mutant HNF-4-(45–142), which encompasses the DBD of HNF-4 alone, and two other mutants containing regions C, D, and E of HNF-4, but with mutations in one or the other of the two zinc fingers, in an attempt to disrupt the structure of that region. This involved a double mutation changing C68G/C71G and or C103G/C106G. As seen in Fig. 5B, there is no interaction between the HNF-4 DBD and GST-CBP1. Furthermore, mutation of either zinc finger does not affect the interaction of GST-CBP1 and GST-CBP4 with HNF-4. Assuming that the mutations to the zinc fingers do disrupt the DNA binding domain, it would seem that this region does not mediate the interaction of HNF-4 with CBP per se.

This analysis identified two domains of HNF-4 that interact with CBP. The AF-1 domain interacts only weakly with the N-terminal region of CBP, and the AF-2 domain interacts with both the N- and C-terminal regions of CBP, and this interaction is both AF-2 AD and ligand-independent.

**HNF-4 Mutated in the AF-2 Domain Does Not Allow CBP to Activate Transcription**—To examine whether there is a correlation between the interacting domains of HNF-4 that associate with CBP and the ability of CBP to enhance transcription, we carried out cotransfection experiments using the (BA1)5CAT reporter construct. The deletion mutants of HNF-4 that were used are shown in Fig. 5A, with the exception of HNF-4-(Δ128–175), containing an internal deletion from amino acids 128 to 175. Wild type HNF-4 and its deletion mutants were shown to express in comparable amounts with insufficient variations to account for the observed differences in their transcriptional activation potential (19). Fig. 6 shows the transcriptional activity of the (BA1)5CAT reporter transfected into HepG2 and HeLa cells with expression vectors for the HNF-4 deletion mutants in the presence and absence of CBP.

HNF-4 with deletion of the F domain (HNF-4-(1–370)) activated the promoter in both HepG2 and HeLa cells 12- and 70-fold, respectively (Fig. 6). However, addition of CBP increased the (BA1)5 promoter activity to approximately 24- and 125-fold, respectively, indicating that the absence of the F domain does not interfere with the functional interaction between HNF-4 and CBP and that CBP enhances HNF-4 transactivation by 2-fold. Mutant HNF-4-(48–455) lacking the AF-1 domain activated the (BA1)5 promoter by 2-fold in HepG2 and by 18-fold in HeLa cells. In each of these cases, in both cell types, the presence of CBP increased HNF-4 transactivation by 2-fold. As shown previously (19), the dominant negative HNF-4 mutants, HNF-4-(1–360) and HNF-4-(Δ128–175), abolished transcriptional activation of the (BA1)5 promoter. These mutants also showed very little or no increase in transcriptional potential in the presence of CBP (Fig. 6). These results indicate that CBP does not activate gene expression in the absence of HNF-4, and HNF-4 mutated in the AF-2 domain does not allow CBP to activate expression, suggesting that the mere recruitment of CBP is not sufficient for enhancement of gene expression.

**Effect of N- and C-terminal Mutants of CBP on the Transcriptional Potential of HNF-4**—The two regions of CBP at the N and C termini that interact with HNF-4 in vitro were tested
DISCUSSION

This work describes the functional association of HNF-4 with CBP and the recruitment of CBP to the HNF-4-DNA complex. The ability of HNF-4 to recruit CBP appears to be essential for enhancement of its function and is consistent with previous studies demonstrating the importance of CBP in nuclear receptor function (27).

We observed a physical association of HNF-4 with CBP in protein-protein binding assays (Fig. 1), and we have also shown that CBP can complex with HNF-4 whereas the HNF-4 is bound to DNA (Fig. 2), implying a role for CBP in HNF-4-mediated transcriptional activation. HNF-4 binds to the N-terminal and also the C-terminal regions of CBP. Both CBP and SRC-1 family members contain the nuclear receptor (NR) box, a short leucine-rich motif (LXXLL, where L is leucine and X is any amino acid) that appears to mediate the interactions between these proteins and the NR receptors (27). It has been suggested that the amino acids within this motif may determine the particular interactions between nuclear hormone receptors and cofactors (37, 49). The three leucine-rich motifs of CBP are located at amino acids 65, 356, and 2964 (shown as asterisks in Fig. 1A), and interestingly they are contained within the two GST-CBP fusion proteins that were identified in our study as interacting with HNF-4. The CBP N terminus between amino acids 1 and 771 encompasses the two receptor interacting domains that were previously described to interact in a ligand-dependent manner with retinoic acid receptor-α and other nuclear receptors (27). The C terminus between amino acids 1892 and 2441 includes the glutamine-rich region and the NR box shown previously to interact with the transcriptional coactivators SRC-1 and p/CIP (35, 37, 39).

Transient transfection analyses show transcriptional enhancement of HNF-4 by CBP using the apoCIII promoter and HNF-4 homopolymeric templates (Fig. 3). In these systems, CBP functions cooperatively with HNF-4 to enhance transcription. CBP does not activate gene expression in the absence of HNF-4, implying that HNF-4 functions as a high affinity site for the entry of CBP to the transcription initiation complex. Previously CBP has been shown to interact with a number of nuclear hormone receptors in a ligand-dependent and dose-dependent manner (26, 27, 35). In our studies, CBP enhanced HNF-4 transactivation by 2–5-fold, which is in good agreement with the previous studies. The natural apoCIII promoter was approximately twice as responsive to CBP as the heterologous promoter pG5CAT and the homopolymeric promoter (BA1)5CAT (Fig. 3). This may be due to the presence of two HNF-4-binding sites in the apoCIII promoter that are known to act synergistically together and with Sp1 and other transcription factors to activate the transcription of the apoCIII gene (14, 19, 50).

It was previously shown that CBP synergistically activates the human interferon-β promoter and that transcriptional synergy requires recruitment of CBP via a novel surface assembled from the activation domains of all the activators of the enhancerome (44). Our studies suggest that although CBP can act in synergy with HNF-4 (Fig. 4), recruitment of CBP to the apoCIII promoter is mediated by either of the HNF-4-binding sites and so does not appear to require such an assembly.

We have also shown that CBP targets both the AF-1 and the AF-2 domains of HNF-4. The N terminus of CBP-(1–771) interacts strongly with the A/B/C and D/E regions of HNF-4, but not with the C (DBD) region alone (Fig. 5). This suggests that the N terminus of CBP can contact both the AF-1 and AF-2 domains of HNF-4 and can bind, with varying strength, to each in the absence of the other. This fits with previous data showing that, when tethered to a promoter via a GAL4 DBD, both the AF-1 (amino acids 1–24) and the AF-2 (amino acids 128–370) domains of HNF-4 are capable of activating transcription

in cotransfection experiments to determine whether these regions alone can coactivate HNF-4. HeLa cells were transfected with the reporter (BA1)5CAT and expression vectors coding for HNF-4, CBP, or one of the CBP mutants depicted on the left of Fig. 7. The results indicate that only the mutants that contain the C-terminal region of CBP can coactivate HNF-4 (Fig. 7). Specifically, the C-terminal mutants CBP-(468–2441) and CBP-(1892–2441) were able to coactivate at a level equal to that induced by the wild type CBP. Remarkably, the N-terminal mutant CBP-(1–771) was not able to coactivate, although this region of CBP interacts strongly with HNF-4 in in vitro assays. Finally, the mutant CBP-(1–771 + 1892–2441) that contains both of the HNF-4 interacting domains is able to coactivate at a level similar to that elicited by CBP, indicating that coactivation may be mediated through the C domain (Fig. 7).

CBP Enhances HNF-4-dependent Transactivation

**Fig. 6. The effect of mutation of HNF-4 on synergistic transcriptional activation by CBP.** Transient transfection experiments were performed in HeLa and HepG2 cells using the (BA1)5CAT reporter plasmid and effector plasmids expressing HNF-4 and the indicated deletion mutants. Transfections were carried out with (black bars) or without (gray bars) an expression vector for CBP. The bar graphs are mean values of CAT activity of at least three independent transfections, each carried out in duplicate and show the relative transcription of the reporter in the absence (−) and presence (+) of expression plasmids CBP and HNF-4.
CBP Enhances HNF-4-dependent Transactivation

Effect of N- and C-terminal mutants of CBP on the transcriptional potential of HNF-4. HeLa cells were transiently transfected with the (BA1)5CAT reporter construct and expression vectors for HNF-4 and CBP. The bar graphs show the relative transcription of the (BA1)5CAT reporter transfected with HNF-4 in the absence (−) and presence (+) of CBP. The values depicted are mean values of CAT activity of at least three independent transfections, each carried out in duplicate. The relative CAT activity of the reporter in the presence of HNF-4 alone is significantly different from that with HNF-4 + CBP-(468–2441), HNF-4 + CBP-(1892–2441), and HNF-4 + CBP-(1–771 + 1892–2441) (p = 0.012, 0.023, and 0.032, respectively, as determined by a Student’s t-test). The relative CAT activity with HNF-4 alone is not significantly different from that with HNF-4 + CBP-(1–771) (p > 0.1).

In addition, although a minimal transcription activation domain has been defined between aa 344 and 451 of CBP (63), cotransfection of CBP-(1–771) failed to enhance transcriptional activation by HNF-4. Therefore, we concluded that the interaction of HNF-4 with the N terminus of CBP is not sufficient to direct transcriptional activation. Interestingly, in the presence of HNF-4 CBP mutants containing only aa 1892–2441 or a deletion of aa 772–1891 are both capable of activating transcription to levels approximating those of full-length CBP. The HAT activity of CBP is associated with aa 1099–1758 (32, 33) and is not contained within either of these deletion mutants. However, SRC-1 has been shown to interact with CBP in the region between aa 2058 and 2163 (35, 39). We propose that although HNF-4 associates with CBP, it can also associate with other coactivator(s) and can utilize the HAT activity from this putative coactivator. This is supported by our finding that recruitment of CBP by HNF-4 is not sufficient to activate transcription. In addition, since the AF-2 AD helix is not involved in interactions with CBP, it would be available to contact other coactivators. In this scenario, CBP may be functioning as a platform for the assembly of a coactivator complex.

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To similar extent (19). If contact with a coactivator complex is the major route by which transcription is activated, then it is necessary for both of these regions to be able to interact with coactivators independently. CBP has also been observed to enhance the ligand-dependent interaction between the N- and C-terminal activation domains of the androgen receptor (AR) (51), and the coactivator SRC-1 also acts to enhance cooperation between the AF-1 and AF-2 functions of the progesterone receptor (52).

The AF-2 domain of HNF-4 is very complex spanning residues 128–366 (19). It contains a short α-helical motif at residues 360–366 termed AF-2 AD, which is conserved between nuclear hormone receptors and is required for ligand-dependent transactivation (20–22). The association of CBP with HNF-4 AF-2 occurs outside the AF-2 AD region (Fig. 5A), and none of the studies presented here included a ligand. This is in agreement with yeast two-hybrid system where CBP interacted with HNF-4 in the absence of ligand (53). We conclude that the interaction of HNF-4 AF-2 and CBP is AF-2 AD and ligand-independent. Deletion or mutation of this region in HNF-4 causes complete loss of transcriptional activity (19, 54), and addition of CBP fails to activate transcription (Fig. 6). The mere recruitment of CBP by HNF-4, therefore, is not sufficient for transcriptional enhancement of gene expression.

In vitro, the C-terminal region of CBP associates only with the C/D/E region of HNF-4 and does not interact with the A/B/C region (Fig. 5A). Hence, the interaction domain appears to be located within the AF-2 region of HNF-4. However, HNF-4-(128–370) and HNF-4-(174–370) were not capable of interacting with the C-terminal region of CBP, implying that the presence of HNF-4 DBD is necessary for this interaction to occur. Mutagenesis studies have localized dimerization domains in both the DBD and the LBD of NHRs (55–57). The dimerization domain in the HNF-4 DBD is required for high affinity, cooperative, specific binding to an HNF-4 response element (58). The LBD dimerization domain in HNF-4 appears to be important, specific binding to an HNF-4 response element (59–61). We propose that the interaction of the C terminus of CBP with HNF-4 occurs at a surface formed when HNF-4 dimerizes. The HNF-4-(128–370) and HNF-4-(174–370) mutants that cannot interact with the C terminus of CBP exclude part, or all, of the DBD dimerization domain and therefore may not be able to form dimers in solution. The mutation of the zinc fingers of the HNF-4 DBD does not affect the interaction with CBP, suggesting that the region that is important for HNF-4/CBP association lies outside this area. It is interesting to note that studies on the interaction of CBP with other nuclear receptors have either used a heterologous dimerization domain or constructs that included the DBD and/or hinge regions and, therefore, the DBD dimerization domain of the receptors in addition to the LBD (27, 51). Moreover, the association of the corepressors SMRT and NCoR with certain receptors is dependent on dimerization of the receptor (62).
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