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Chicken Ovalbumin Upstream Promoter Transcription Factors Act as Auxiliary Cofactors for Hepatocyte Nuclear Factor 4 and Enhance Hepatic Gene Expression

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Chicken ovalbumin upstream promoter transcription factors (COUP-TFs) strongly inhibit transcriptional activation mediated by nuclear hormone receptors, including hepatocyte nuclear factor 4 (HNF-4). COUP-TFs repress HNF-4-dependent gene expression by competition with HNF-4 for common binding sites found in several regulatory regions. Here we show that promoters, such as the HNF-1 promoter, which are recognized by HNF-4 but not by COUP-TFs are activated by COUP-TFI and COUP-TFII in conjunction with HNF-4 more than 100-fold above basal levels, as opposed to about 8-fold activation by HNF-4 alone. This enhancement was strictly dependent on an intact HNF-4 E domain. In vitro and in vivo evidence suggests that COUP-TFs enhance HNF-4 activity by a mechanism that involves their physical interaction with the amino acid 227 to 271 region of HNF-4. Our results indicate that in certain promoters, COUP-TFs act as auxiliary cofactors for HNF-4, orienting the HNF-4 activation domain in a more efficient configuration to achieve enhanced transcriptional activity. These findings provide new insights into the regulatory functions of COUP-TFs, suggesting their involvement in the initial activation and subsequent high-level expression of hepatic regulators, as well as in the positive and negative modulation of downstream target genes.

The nuclear hormone receptor superfamily encodes a diverse set of transcription factors, including receptors for steroids, retinoids, and thyroid hormones. Ligand-dependent activation of the different members of this superfamily modulates the transcription of a large number of target genes whose coordinate expression plays important roles in the control of vertebrate differentiation, development, and cellular homeostasis (20, 33, 34). Besides the classical receptors, the nuclear receptor superfamily comprises many orphan receptors which are structurally and functionally related but whose ligands have not been identified (10). The orphan receptors possess highly homologous DNA binding and ligand binding domains, and the DNA sequences with which they interact are common to the response elements of other hormone receptors. These characteristics give the orphan receptors the potential to participate in the different hormone signaling pathways through promoter element occupancy or heterodimerization with other ligand-dependent receptors. Two of the most studied orphan receptor families are COUP-TF (chicken ovalbumin upstream promoter transcription factor) and HNF-4 (hepatocyte nuclear factor 4).

COUP-TFI (also called ear3) (52) and COUP-TFII (also called Arp-1) (26) are closely related transcription factors which have been implicated in the regulation of several important biological processes, such as early embryonic development (39) and neuronal cell fate determination (38). Although COUP-TFI was identified as an activator of the ovalbumin (42) and the rat insulin II (18) promoters, recent studies have shown that COUP-TFs are potent repressors of retinoic acid receptor-, thyroid receptor-, vitamin D_3 receptor-, peroxisome proliferator-activated receptor-, estrogen receptor-, and steroidogenic factor 1-mediated transcription (3, 10, 11, 29, 30, 39, 49).

HNF-4 is a liver-enriched transcription factor which plays a pivotal role in the regulation of a very large number of liver-specific genes whose expression pattern is characteristic of terminally differentiated hepatocytes (43, 44). HNF-4 is an important activator of the HNF-1 gene (21, 24, 48), establishing a hierarchical pathway of transcriptional activation in the liver. HNF-4 is an important developmental regulator, as evidenced by its temporal and spatial expression pattern during drosophila (54) and early mouse development (13, 47) and by gene disruption studies demonstrating the importance of HNF-4 expression in the visceral endoderm for embryonic ectoderm survival and normal gastrulation (9). Most of the promoter elements that interact with HNF-4 can also recognize COUP-TFs (23, 25, 35, 43). COUP-TFs strongly repress HNF-4-mediated transcription from these promoters by a mechanism that involves competition with HNF-4 for the common binding site (25, 35). This antagonistic effect has led to the prevailing view that the expression of HNF-4-dependent liver-specific genes is fine tuned by the actual intracellular balance of these positive and negative regulators.

In this report, we present evidence that COUP-TFI and COUP-TFII act as auxiliary cofactors for HNF-4 on promoters that can be directly recognized by HNF-4 but not by COUP-TFs. We demonstrate that COUP-TFs, through protein-protein interaction with HNF-4, dramatically increase HNF-1 promoter activity, suggesting that they may play an important role in the activation of a wide range of liver-specific genes.

MATERIALS AND METHODS

Plasmid constructions. pMT2 vectors expressing full-length HNF-4, COUP-TFI, and COUP-TFII, as well as the promoter constructs HNF-1-CAT and apoCIII-CAT, have been described previously (21, 23, 46). Chimeric reporter constructs were generated by ligating double-stranded kinased oligonucleotides encompassing the nucleotide (nt) −70 to −41 region of the rat HNF-1 promoter ("1×A"TK-CAT), the nt −96 to −61 region of the human apoC-III promoter

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("1×CIIIB"TK-CAT), the nt −128 to −99 region of the human α1 antitrypsin promoter ("1×LFA1"TK-CAT), and the nt −151 to −130 region of the human transthyretin promoter ("2×TTR"TK-CAT) into the *Sal*I site of previously described plasmid TK85-CAT (21). A G4-CAT reporter containing four copies of the 17-mer Gal4 binding site in front of the β-globin minimal promoter was a generous gift from I. Papamatheakis. pMT-HNF-4 (1-368) was constructed by PCR by using pMT-HNF-4 FL as the template and *Eco*RI adapter oligonucleotide primers corresponding to the nt 1 to 20 (sense) and 345 to 368 (antisense) regions of the rat HNF-4 cDNA. The PCR products were cleaved by *Eco*RI and ligated to the same site of a pMT2 vector. To obtain pMT-HNF-4 (1-354), the HNF-4 cDNA was cut with *Eco*RI and *Msc*I, filled in with the Klenow enzyme, and ligated to the blunt-ended *Eco*RI site of pMT2. Gal4 HNF-4 (E), Gal4 HNF-4 (AF2h), and Gal4 HNF-4 (337-455) were generated by subcloning of the PCR-amplified fragment corresponding to the nt 391 to 1102, 1009 to 1102, or 1009 to 1356 region of HNF-4 into the *Eco*RI site of a pBxG1 vector. This vector is derived from pSG424 (41) expressing the amino acid (aa) 1 to 147 region of the yeast Gal4 cDNA (Gal4 DBD) with an altered polylinker region (provided by I. Papamatheakis). Gal4 HNF-4 (E354) was obtained by deleting the *Msc*I/*Bam*HI fragment from Gal4 HNF4 (E). Gal4 HNF-4 (227-455) was generated by deletion of the N-terminal aa 1 to 227 region of pMT-HNF-4 by *Nco*I digestion, followed by religation, and the *Eco*RI fragment of the resulting plasmid was subcloned into a pBxG1 vector. The C-terminal activation domain of VP16 (40) was excised from a pSJT 1193 CRF1 vector (kindly provided by D. Tzamarias) by digestion with *Bgl*II and *Bam*HI and ligated into the *Bam*HI site of a pBxG1 vector to obtain Gal4 VP16. pGex-COUP-TFI was constructed by subcloning the *Eco*RI fragment of pMT-COUP-TFI, containing the complete open reading frame of COUP-TFI, into the *Eco*RI site of a pGex-1 vector (Pharmacia). Plasmid pGex-TFIIB was kindly provided by D. Thanos. All constructs were verified by nucleotide sequencing with T7 polymerase (Sequenase).

Transfections and CAT assays. Cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (for Cos-1 and HepG2), 20% (for Caco2), or 7% (for C33) heat-inactivated fetal calf serum. At 24 h before transfection, the cells were seeded at 50 to 60% confluence. A mixture of 2 μg of a reporter construct, 0.5 μg of an expression vector, and 2 μg of pSVβgal (pCH110; Pharmacia) was introduced to the cells by the calcium phosphate DNA precipitation method as previously described (21–23). At 48 h later, the cells were harvested and chloramphenicol acetyltransferase (CAT) activities were measured by using constant amounts of protein extracts as previously described (21). The protein concentrations and incubation times were carefully selected by titration and kinetic experiments to ensure linear conversion of chloramphenicol to the acetylated form. β-Galactosidase activity in the cell extracts was measured as previously described (15), and the values were used to normalize variations in transfection efficiency.

Electrophoretic mobility shift assays (EMSAs). Whole cell extracts from transfected Cos-1 cells were prepared by three freeze-thaw cycles in a buffer containing 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.9), 0.1 M KCl, 2 mM dithiothreitol, 0.5% Nonidet P-40 (NP40), 0.1 M phenylmethylsulfonyl fluoride (PMSF), 10 μg of aprotinin per ml, and 1 μg each of pepstatin and leupeptin per ml. DNA binding assays were performed essentially as described previously (21, 22).

The oligonucleotides used in this study were CIIIB (TCGAGGTCAGCAGG TGACCTTTGCCCAGCG-67) and HNF-1 "A" (TCGAGGCTGAAGTCCAA AGTTCAGTCCCTTCGC-41).

In vitro protein-protein interaction assays. Growth and expression of glutathione *S*-transferase (GST) fusion proteins in bacterial strain JM109 were performed as previously described (45). ^{35}S-labeled recombinant HNF-4 derivatives were synthesized in vitro from the corresponding constructs in Bluescript KS (Stratagene) by using the TNT coupled reticulocyte lysate system (Promega) in accordance with the manufacturers instructions. A 2-μg sample of each fusion protein was loaded into a glutathione-Sepharose column (Pharmacia) preequilibrated with 0.5% bovine serum albumin (BSA) containing phosphate-buffered saline (PBS). After binding, the columns were washed three times with PBS and then three times with a wash buffer containing 100 mM KCl, 20 mM HEPES (pH 7.9), 0.1% NP40, 5 mM $MgCl_2$, 0.1 M PMSF, and 10 μg of aprotinin per ml. The beads were washed once with interaction buffer (100 mM KCl, 20 mM HEPES [pH 7.9], 0.1% NP40, 5 mM $MgCl_2$, 0.2% BSA, 10% glycerol, 0.1M PMSF, 10 μg of aprotinin per ml) and incubated with ^{35}S-labeled proteins for 1.5 h at 4°C with constant agitation. After extensive washing with excess amounts of wash buffer, the beads were resuspended in 20 μl of sodium dodecyl sulfate (SDS) loading buffer and analyzed by SDS-polyacrylamide gel electrophoresis.

Indirect immunofluorescence assays. Transfected Cos-1 cells on coverslips were washed twice with PBS and fixed for 10 min at −20°C with methanol. After fixation, the coverslips were washed once with PBS and incubated with PBS containing 1% BSA (BSA-PBS) for 30 min at room temperature. HNF-4 antiserum diluted 1:200 in BSA-PBS was applied to the cells, which were then incubated for 45 min. After washing with BSA-PBS, the cells were incubated with diluted (1:100) fluorescein isothiocyanate-conjugated anti-rabbit secondary antibodies to immunoglobulin G (Jackson Laboratories) for 30 min at room temperature. The coverslips were washed with BSA-PBS, rinsed in water, mounted on glass slides with Mowiol (Polyscience), and observed in a Leitz Dialux 20 EB microscope equipped with epifluorescence optics.

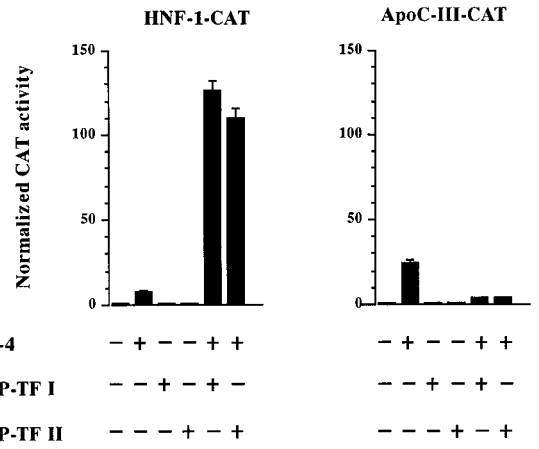

FIG. 1. Enhancement by COUP-TFI and COUP-TFII of HNF-4-dependent activation of the HNF-1 promoter. Cos-1 cells were cotransfected with 2 μg of the HNF-1-CAT and apoC-III–CAT reporter plasmids, together with 0.5 μg of the indicated expression vectors. The columns and error bars represent mean values and standard errors of β-galactosidase-normalized CAT activity from at least six independent experiments.

RESULTS

COUP-TFI and COUP-TF-II enhance HNF-4-mediated activation of the HNF-1 promoter. The A element of the HNF-1 promoter contains a DR-1 (direct repeat) motif that has been previously shown to interact with and be transactivated by HNF-4 in either a homologous or heterologous promoter context (21, 24, 48). Since DR-1 sequences are potential binding sites for other members of the hormone receptor family, including COUP-TFs, we investigated their possible involvement in HNF-1 gene expression. Cos-1 cells devoid of endogenous HNF-4 or COUP-TFs were transfected with HNF-1 promoter-CAT reporter and expression vectors for HNF-4, COUP-TFI, or COUP-TFII. As shown in Fig. 1, the HNF-1 promoter was transactivated eightfold above the basal levels by HNF-4, while COUP-TFI or COUP-TFII alone did not affect it. Surprisingly, when cotransfections were performed with COUP-TFI and COUP-TFII together with HNF-4, we observed 15- and 13-fold enhancement of HNF-4-mediated transactivation. As a control, we performed the same experiments with the apoC-III–CAT reporter, which is known to be repressed by COUP-TFs (25, 35, 46). As expected, overexpression of HNF-4 increased apoC-III promoter activity about 25-fold, and this activation was repressed by both COUP-TFI and COUP-TFII, excluding the possibility of certain experimental artifacts. Additional control experiments showed that COUP-TFs did not influence the amounts of HNF-4 protein produced by the expression vectors used or the activities of unrelated promoters that lack HNF-4 binding sites (data not shown). Cotransfection experiments with a "1×A"TK-CAT reporter, which contains one copy of the DR1 motif of the HNF-1 promoter in front of the nt −85 to +51 region of the herpes simplex virus thymidine kinase gene linked to CAT cDNA, produced results very similar to those obtained in the homologous promoter context. HNF-4 transactivated this promoter construct 7-fold, and COUP-TFI and COUP-TFII further enhanced this activation 16- and 17-fold, respectively (Fig. 2). No change was observed when COUP-TFs were transfected alone. As a control, we tested the TK85-CAT reporter, which was unaffected by any combinations of the above factors. In addition, the "1×CIIIB"TK-CAT construct, containing one copy of the apoC-III hormone response element, was transactivated by HNF-4 and

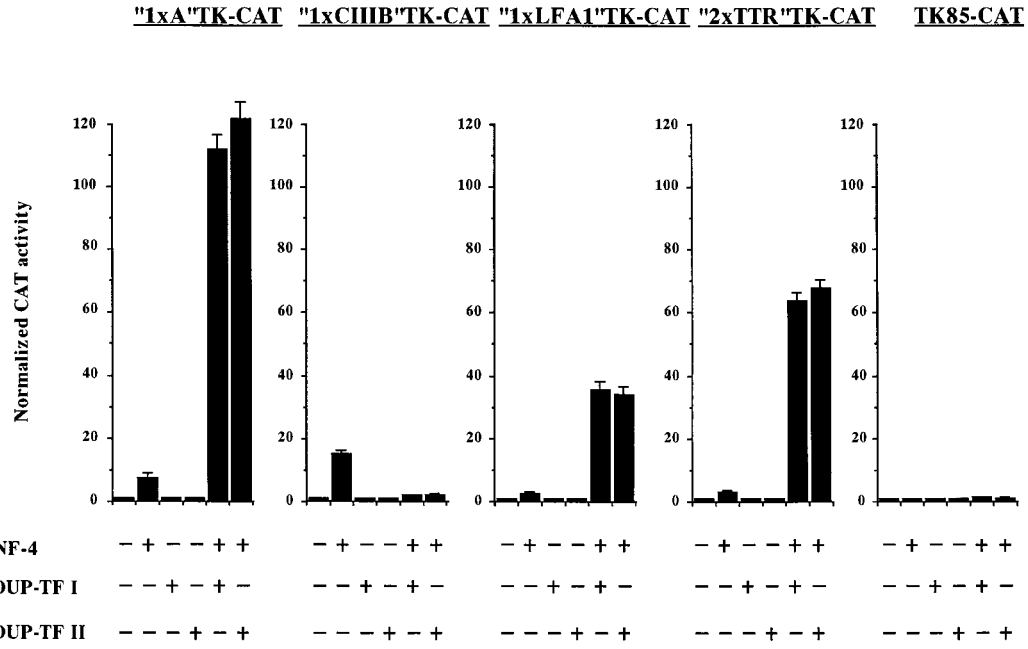

FIG. 2. Effects of COUP-TFI and COUP-TFII on HNF-4-activated transcription of chimeric promoter constructs. Cos-1 cells were cotransfected with 2 μg of the indicated chimeric reporter constructs, together with 0.5 μg of the indicated expression vectors. The columns and error bars represent mean values and standard errors of β-galactosidase-normalized CAT activity from at least four independent experiments.

repressed by COUP-TFs as expected (Fig. 2). Two other heterologous reporter constructs, containing HNF-4 binding sites from the α1-antitrypsin ("1×LFA1"TK-CAT) and transthyretin ("2×TTR"TK-CAT) genes, were affected by HNF-4 and COUP-TFs in the same direction as "1×A"TK-CAT. HNF-4 transactivated both constructs 2.7- and 3.2-fold respectively. COUP-TFs alone had no effect on the promoter activities, while coexpression of COUP-TFI or COUP-TFII with HNF-4 resulted in activation 13- and 12-fold ("1×LFA1"TK-CAT) or 19- and 21-fold ("2×TTR"TK-CAT) above HNF-4-activated transcription (Fig. 2). Taking into consideration the different natures of these *cis*-acting elements with respect to hormone receptor binding abilities (e.g., the CIIIB site is known to bind both HNF-4 and COUP-TFs with similar affinities [25, 35], while the LFA1 and TTR sites are known to bind HNF-4 but not COUP-TFs [25, 26]), the above data indicate that enhancement of HNF-4-induced transcription by COUP-TFs may be achieved through protein-protein interaction.

COUP-TFI and COUP-TFII do not bind directly to site A of the HNF-1 promoter. To assess the possible direct or indirect interactions of COUP-TFs with the site A element of the HNF-1 promoter, EMSA experiments were carried out by using extracts from Cos-1 cells transfected with HNF-4, COUP-TFI, COUP-TFII, or combinations of them. As shown in Fig. 3, DNA-protein complex formation could be detected with HNF-4 but not with COUP-TFI or COUP-TFII, confirming previous observations that COUP-TFs do not directly recognize the HNF-1 promoter (21; data not shown). When extracts from cells cotransfected with HNF-4 and COUP-TFI or COUP-TFII were used in the assay, only one major band corresponding to HNF-4 was observed. A faint supershifted band that may correspond to an HNF-4–COUP-TF protein-protein complex was also observable; however, this band was not reproducibly seen at all times when the same experiment was repeated and could not be eliminated by either COUP-TF antibodies or COUP-TF-specific competitor oligonucleotides (data not shown). The same extracts were also tested by EMSA using the CIIIB binding site (Fig. 3). As expected, all three factors formed a complex with this probe, excluding the possibility of artifacts generated during transfection or extract preparation. The possibility of heterodimer formation between HNF-4 and COUP-TFs, which may have escaped detection in the previous experiment due to their similar running positions, was tested by using a 3′ truncated derivative of HNF-4 [HNF-4 (1-368)]. No heterodimer formation was observed when either site A or CIIIB was used as the probe (Fig. 3), confirming

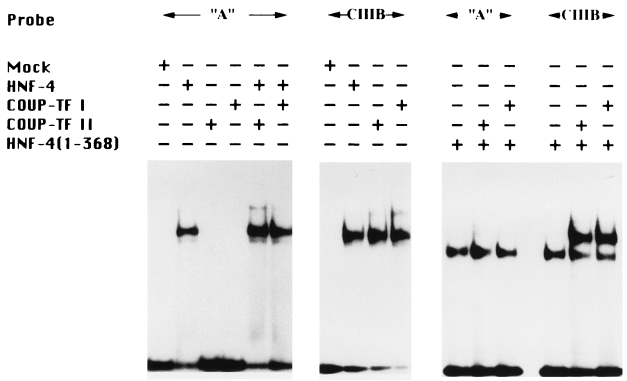

FIG. 3. Failure of COUP-TFI and COUP-TFII to interact with the DR-1 element of the HNF-1 promoter as homodimers or by heterodimerization with HNF-4. EMSA experiments were performed with cellular extracts from Cos-1 cells transfected with the indicated expression vectors and radiolabeled double-stranded oligonucleotides corresponding to site A of the rat HNF-1 promoter ("A") and the proximal hormone response element of the human apoC-III promoter (CIIIB). Each binding reaction was performed with an extract containing 5 μg of total protein. The same results were obtained when individual extracts were mixed in the binding reaction.

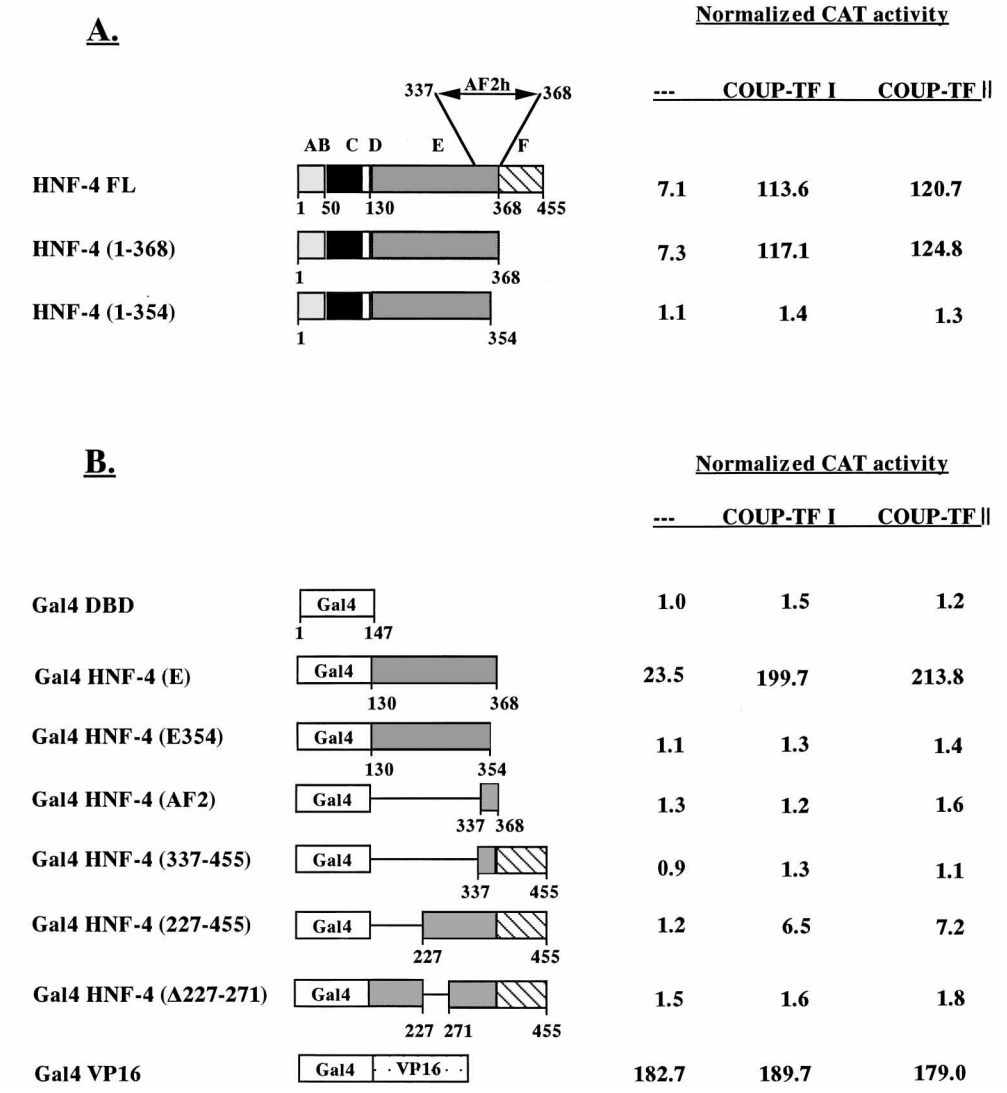

FIG. 4. Requirement of the intact E region of HNF-4 for COUP-TF-mediated enhancement. (A) Cos-1 cells were cotransfected with 2 μg of a "1×A"TK-CAT reporter together with 0.5 μg of a pMT vector expressing the indicated HNF-4 deletion mutant and COUP-TFI or COUP-TFII. The N-terminal AB region, the Zn finger DNA binding domain (C), the hinge region (D), the ligand-binding–dimerization domain (E), and the carboxy-terminal region (F) of the HNF-4 protein are depicted by different shadings. (B) Cos-1 cells were cotransfected with 2 μg of a G4-CAT reporter together with 0.5 μg of the indicated Gal4 fusion construct and pMT-COUP-TFI or pMT-COUP-TFII. The values are mean normalized CAT activities, with standard deviations of less than 10%, from at least five independent experiments. A value of 1.0 corresponds to the activity obtained by cotransfection of an empty pMT2 vector. DBD, DNA binding domain.

previous studies defining HNF-4 as a factor that forms exclusively homodimers (19).

In vivo and in vitro interactions of COUP-TFs with HNF-4. Although we could not detect in vitro heterodimerization or protein-protein interaction between HNF-4 and COUP-TFs by EMSAs, potential weak interactions that do not survive the electrophoretic conditions used cannot be ruled out as an explanation for the observation that COUP-TFI and COUP-TFII enhanced HNF-4-mediated activation of the promoters containing *cis*-acting elements exclusively binding HNF-4. To test this possibility, we attempted to map the HNF-4 protein domains necessary for COUP-TF-mediated enhancement by using truncated mutant forms of HNF-4. COUP-TFs enhanced activation mediated by HNF-4 (1-368), a mutant construct lacking the C-terminal F region, to the same levels as wild-type HNF-4. This construct includes a 30-aa sequence (between aa 337 and 368) highly homologous to the AF2 (activation function 2) region of other hormone receptors (1, 14) and named AF2h (AF2 homolog). Deletion of part of the AF2h domain [HNF-4 (1-354)] resulted in loss of activity and COUP-TF enhancement (Fig. 4A). Further deletions resulted in loss of dimerization and, consequently, DNA binding and were unaffected by COUP-TFs (data not shown). These results indicate that enhancement of HNF-4 activity by COUP-TFs requires an intact AF2h-region-containing HNF-4 protein. Next, we investigated the effects of COUP-TFs on chimeric protein constructs containing dissected parts of HNF-4 fused to the DNA binding domain of the yeast Gal4 protein. These chimeras bind to the Gal4 response element as dimers through the Gal4 DNA binding domain (4), and their expression levels are not affected by COUP-TFs (data not shown), eliminating complications arising from altered dimerization and DNA binding activity. As shown in Fig. 4B, Gal4 HNF-4 (E), containing the complete E domain of HNF-4, is a potent activator of the

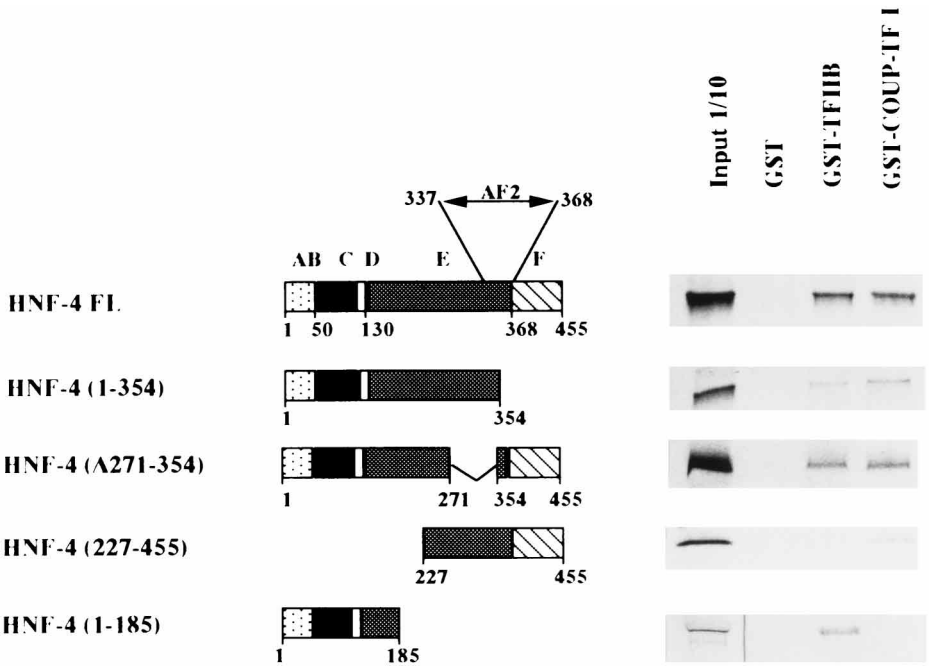

FIG. 5. In vitro interaction of wild-type HNF-4 and mutant HNF-4 derivatives with COUP-TFI and TFIIB. The ^{35}S-labeled HNF-4 proteins on the left were produced by in vitro translation and incubated with bacterially expressed GST fusion proteins bound to glutathione-Sepharose beads. The beads were washed extensively, and specific interactions were assessed by SDS-polyacrylamide gel electrophoresis. The input lanes represent 10% of the total in vitro-translated proteins used in the binding reaction, except in case of HNF-4 (1-185), where 20% of the input is shown.

Gal4-responsive reporter (G4-CAT), and this activation was enhanced by COUP-TFI and COUP-TFII 8.5- and 9-fold respectively. Deletion of either the distal [Gal4 HNF-4 (E354)] or the proximal [Gal4 HNF-4 (AF2h), Gal4 HNF-4 (337-455), Gal4 HNF-4 (227-455), or Gal4 HNF-4 (Δ221-271)] part of the E domain abolished transcriptional activity. Superactivation by COUP-TFs was observed only when all or the aa 227 to 368 part of the E domain was present in the chimeras, but not with other mutants or Gal4 VP16, which was used as an unrelated control (Fig. 4B). These data indicate that the AF2h domain of HNF-4 can function only in the context of the intact E domain and that COUP-TF-mediated superactivation may involve protein-protein interactions between COUP-TFs and the aa 227 to 271 region of HNF-4. In vitro evidence of such interactions was provided by pull-down experiments employing a GST–COUP-TFI fusion protein and ^{35}S-labeled HNF-4 derivatives. As shown in Fig. 5, appreciable binding of full-length HNF-4 to GST–COUP-TFI could be detected. The amount of bound protein recovered in this assay was comparable to the amount recovered by GST-TFIIB, which was used as a control. Interestingly, HNF-4 (1-354) and HNF-4 (Δ271-354), which lack different parts of the AF2 domain, could interact with both COUP-TFI and TFIIB while some of the N-terminally truncated HNF-4 (227-455) could be recovered by the GST–COUP-TFI, but not by the GST-TFIIB, column. On the other hand, interaction with TFIIB, but not with COUP-TFI, was observed when HNF-4 (1-185) containing the N-terminal part of the protein was tested (Fig. 5). Direct interactions between HNF-4 and COUP-TFs in intact cells were determined by nuclear cotranslocation assays with a mutant form of HNF-4 [HNF-4 (227-455)] that lacks specific nuclear localization signals but contains the domain required for in vitro interaction with COUP-TFs. This mutant was detected by in situ immunofluorescence exclusively in the cytoplasm of transfected Cos-1 cells (Fig. 6A). Coexpression of either COUP-TFI or COUP-TFII, but not the full-length Gal4-800 protein, resulted in translocation of the cytoplasmic mutant form of HNF-4 to the nucleus, demonstrating HNF-4–COUP-TF interaction in vivo (Fig. 6). Taken together, the above results indicate that COUP-TFs interact with the HNF-4 protein outside the AF2h domain, somewhere between aa 227 and 271 of the E domain. For superactivation, the intact AF2h domain of HNF-4 is also required.

COUP-TFs enhance HNF-4-mediated transcription in HepG2 and Caco2 cells but not in C33 cells. The biological importance of the above observations prompted us to reexamine the effects of COUP-TFs in cell lines of hepatic (HepG2) and intestinal (Caco2) origin which express both HNF-4 and COUP-TFs. As shown in Fig. 7, COUP-TFI and COUP-TFII enhanced the activation obtained by the Gal4 HNF-4 (E) fusion protein in both cell types. The extent of activation was 3.7- to 3.9-fold in HepG2 cells and 4.3- to 5.1-fold in subconfluent Caco2 cells, significantly lower than that observed in Cos-1 cells. This difference should be due to the high concentrations of endogenous COUP-TFs in these cell lines (35), suggesting that the values obtained with Gal4 HNF-4 (E) alone represent an already superactivated state. Surprisingly, COUP-TFI and COUP-TFII did not enhance Gal4 HNF4 (E)-mediated activation of the Gal4 reporter (G4-CAT) in the human epithelial carcinoma-derived C33 cell line, although they were able to inhibit HNF-4-activated apoC-III activity (Fig. 7 and data not shown). This finding suggests that for cofactor function, either an additional factor(s) or an intracellular ligand(s) that is missing from C33 cells may be required.

DISCUSSION

Early studies describing promoter context-dependent activities and transcriptional cross-interference effects for the AFs of different hormone receptors raised the concept that these

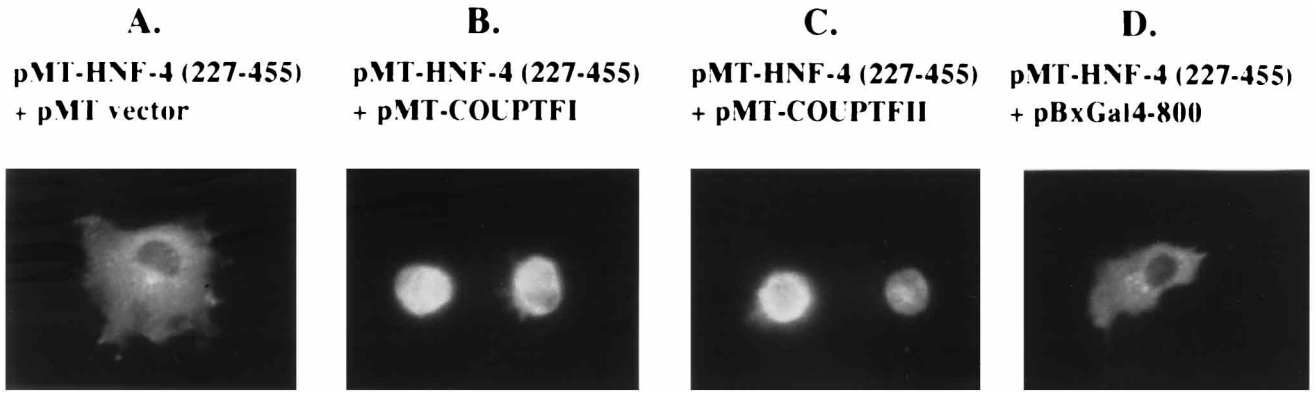

FIG. 6. Interaction of HNF-4 with COUP-TFI and COUP-TFII in intact cells. Cos-1 cells were transfected with 500 ng of nuclear localization signal-deficient mutant HNF-4 (271-455) alone (A) or together with 1 μg of a COUP-TFI (B) or COUP-TFII (C) expression vector, transferred to coverslips, and stained with a polyclonal peptide antibody raised against the C-terminal 11-aa epitope of HNF-4. Cotransfection with Gal4-800 was used as an unrelated control (D). Immunofluorescence images are shown at a magnification of about ×316.

proteins may interact with transcriptional intermediary factors (TIFs) distinct from the basal transcription factors (1, 5, 14). Several such mediators have since been identified, including RIP140 (6), TIF1 (28), TIF2 (51), SRC-1 (36), TRUP (2), N-CoR (17), and SMRT (8), which positively or negatively modulate the activities of specific hormone receptors via protein-protein interactions. Here we show that two known orphan receptors, COUP-TFI and COUP-TFII, function as transcriptional intermediary cofactors for HNF-4, another orphan member of the hormone receptor superfamily. Our results suggest that COUP-TFs do not form heterodimers with HNF-4 on DNA. On the other hand, we provide in vitro and in vivo evidence that COUP-TFI and COUP-TFII interact with HNF-4 homodimers in either a homologous or a heterologous context. This interaction occurs in promoters that are recognized by HNF-4 but not by COUP-TFs (such as the HNF-1 promoter) and results in dramatic enhancement of HNF-4-mediated activation. We have mapped the COUP-TF interaction domain of HNF-4 to the E region of the molecule, between aa 227 and 271, outside the main activation domain of HNF-4 situated between aa 337 and 368, which contains a sequence motif highly homologous to the AF2 region of other hormone receptors (1, 14). Unlike the AF2 domains of RXR, RaR, and TR, the corresponding region of HNF-4 (named AF2h) does not exhibit an autonomous activation function when fused to the DNA binding domain of Gal4, although it

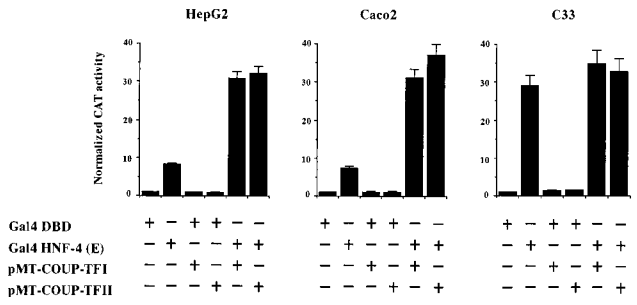

FIG. 7. Enhancement of HNF-4 activation by COUP-TFs in HepG2 and Caco2, but not in C33, cells. HepG2, Caco2, and C33 cells were transfected with 2 μg of a G4-CAT reporter together with 0.5 μg of the indicated expression vectors. The columns and error bars represent mean values and standard deviations of β-galactosidase-normalized CAT activity from at least three independent experiments. Due to the different transfection efficiencies, the absolute activity values can be compared only within one cell line.

can adopt a similar amphipathic α-helical conformation. This finding implies that, functionally, the HNF-4 AF2h region may represent a new subclass of activation domains defined by the requirement of an intact E region for activity. This is rather interesting, because the E regions of the classical hormone receptors have been found to confer ligand dependency on the AF2 domain, while HNF-4 does not require a ligand to function (31). Mutations disrupting the AF2h region abrogated both HNF-4 activity and COUP-TF-mediated enhancement, showing that even the enhanced transcription rate was absolutely dependent on AF2h. This finding argues against a mechanism presuming a classical coactivator function of COUP-TFs, which by definition should exhibit transcriptional activation when the coactivator itself is tethered to the promoter via interaction with another protein. COUP-TFs are unable to activate transcription not only when recruited to the promoter through interaction with mutant HNF-4 proteins disrupted in the AF2 region but also when they are brought in by fusion to the Gal4 DNA binding domain or allosteric interaction with RXR or TR (5, 29). In fact, in both of the latter cases, COUP-TFs exhibit a transcriptional repression effect. The possibility of increased DNA binding of HNF-4 when complexed with COUP-TFs as a potential mechanism can also be ruled out, since COUP-TFs efficiently enhanced transcription driven by the Gal-4 HNF-4 (E) fusion protein. In addition, we have not observed increased binding or any change in the in vitro association and dissociation kinetics or the affinity of HNF-4 to site A when extracts from Cos-1 cells coexpressing HNF-4 and COUP-TFs were analyzed or by mixing individual factor-containing extracts in the binding assays (data not shown). A more intriguing possibility is that COUP-TFs might function as "bridging factors" between the E region of HNF-4 and another component(s) of the core transcription machinery, facilitating or stabilizing the formation of the preinitiation complex. A good candidate for the downstream partner is TFIIB, since both HNF-4 and COUP-TFs can physically interact with this factor (31, 32). Although we were unable to detect increased in vitro interaction of HNF-4 with TFIIB in the presence of COUP-TFI (data not shown), we cannot exclude the possibility that, in vivo, this type of stabilization may occur. A further complication of this idea arises when one considers the results obtained with Gal4 HNF-4 (E) and Gal4 HNF4 (227-455). Both of these constructs lack the TFIIB binding domain of HNF4; nevertheless, their activity was enhanced by COUP-TFs. The possibility that, in the HNF-4 context,

COUP-TFs may acquire a configuration that induces a productive conformation state of TFIIB also seems unlikely, since the enhancing effect was strictly dependent on the AF2h domain. Furthermore, direct interactions of COUP-TFs with TFIIB, and the subsequent freezing of the preinitiation complex in an inactive state, have been speculated to be a potential mechanism for their repressor function (29). The above considerations led us to propose a model for COUP-TF-mediated enhancement of HNF-4 activity that involves a conformational change of the HNF-4 protein induced by COUP-TF binding to its E region. According to this model, HNF-4 adopts a suboptimal configuration of its activation surface when bound to DNA. Associations with COUP-TFs lead to a more efficient orientation of the HNF-4 AF2h domain to facilitate the entry of other components of the preinitiation complex. These components may be either coactivators that transduce the AF2 activity to the transcription machinery (e.g., TIF2) or general transcription factors which enter the preinitiation complex downstream of TFIIB, since TFIIB and COUP-TFs can interact with HNF-4 independently on domains distinct from AF2h. In this respect, it is interesting that COUP-TFs did not enhance HNF-4-mediated activation in all cell types. Our failure to detect the enhancing effect in C33 cells may reflect the requirement of an additional cofactor(s) that interacts either with COUP-TFs or the HNF-4 AF2h domain and is missing from this cell line. An alternative but not mutually exclusive possibility is that for the activation function, COUP-TFs require an unidentified, cell type-specific intracellular ligand or a posttranslational modification such as phosphorylation.

Although COUP-TFs have been shown to possess an intrinsic transcriptional activation potential and have been implicated as activators of some genes (18, 32, 42), the findings presented in this work are rather unexpected, since COUP-TFs are known to repress the activities of other hormone receptors, including RXR, RAR, T3R, PPAR, and ER (10, 11). More importantly, COUP-TFs were shown to be strong repressors of all of the HNF-4-dependent promoters studied so far (43). The main mechanism by which COUP-TFs repress transcription involves competition of COUP-TF homodimers with HNF-4 and other hormone receptors for a common binding site (35). However, recent studies indicated that in RXR and TR, other mechanisms, such as transrepression, may also be involved (29). The diverse effects of COUP-TFs on different genes suggest that they represent a multifunctional transcription factor family with highly important biological roles. They inhibit transcription driven by a wide range of hormone receptor (including HNF-4)-dependent promoters through promiscuous binding to their DR1 to DR5 elements, while they activate another set of HNF-4-dependent promoters which they do not recognize directly. Our finding that HNF-1, another important liver-enriched transcription factor which regulates a different set of genes (50), belongs to this second class adds a new dimension to the complexity of the network involved in liver-specific gene expression. HNF-4 has been postulated to be at the top of the hierarchy of transcriptional factors involved in the specification of the hepatic phenotype (16, 24). However, other events or factors may also play important roles in the initial activation of this cascade, such as the activin A signaling pathway, which was shown to induce HNF-4 activity during early *Xenopus* development (53), possibly by triggering phosphorylation of HNF-4, which is important for its transcriptional activity (22). Our results suggest that COUP-TFs may also participate in the initial activation of the transcriptional cascade responsible for hepatic differentiation by the enhancement of HNF-1 expression. Consistent with this idea is the parallel appearance of COUP-TFs and HNF-1 during early endodermal differentiation (7). In mouse embryos, both HNF-1 and COUP-TFs can first be detected at 8.5 days and reach maximum levels at 14 to 15 days postconception (7), much later than the initial appearance of HNF-4 (4.5 to 5.5 days postconception) (13). In addition, it was recently shown that COUP-TFs positively regulate the expression of variant HNF-1 (also called LFB3) (37), a factor thought to be involved in the early differentiation stages of polarized epithelial cells of endodermal and mesodermal origin, such as those of the liver, kidneys, and digestive tract (12, 27, 50). COUP-TFs activate variant HNF-1 promoter activity by an indirect mechanism via octamer factor binding sites (37), suggesting that besides their DNA binding properties, their ability to interact with other proteins is also highly promiscuous. Taking these findings into consideration, together with the potential role of COUP-TFs during neural development (38), it is tempting to speculate that COUP-TFs may function as global regulators in the early differentiation processes of all three germ layers.

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