

Transcription Activation by the Orphan Nuclear Receptor, Chicken Ovalbumin Upstream Promoter-Transcription Factor I (COUP-TFI)

DEFINITION OF THE DOMAIN INVOLVED IN THE GLUCOCORTICOID RESPONSE OF THE PHOSPHOENOLPYRUVATE CARBOXYKINASE GENE*

(Received for publication, November 10, 1999)

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Chicken ovalbumin upstream promoter-transcription factors (COUP-TFs), orphan members of the nuclear receptor superfamily, play a key role in the regulation of organogenesis, neurogenesis, and cellular differentiation during embryonic development. COUP-TFs are also involved in the regulation of several genes that encode metabolic enzymes. Although COUP-TFs function as potent transcription repressors, there are at least three different molecular mechanisms of activation of gene expression by COUP-TFs. First, as we have previously shown, COUP-TF is required as an accessory factor for the complete induction of phosphoenolpyruvate carboxykinase gene transcription by glucocorticoids. This action is mediated by the binding of COUP-TF to the glucocorticoid accessory factor 1 (gAF1) and 3 (gAF3) elements in the phosphoenolpyruvate carboxykinase gene glucocorticoid response unit. In addition, COUP-TF1 binds to DNA elements in certain genes and transactivates directly. Finally, COUP-TF1 serves as a coactivator through DNA-bound hepatic nuclear factor 4. Here we show that the same region of COUP-TF1, located between amino acids 184 and 423, is involved in these three mechanisms of transactivation by COUP-TF1. Furthermore, we show that GRIP1 and SRC-1 potentiate the activity of COUP-TF1 and that COUP-TF1 associates with these coactivators *in vivo* using the same region required for transcription activation. Finally, overexpression of GRIP1 or SRC-1 does not convert COUP-TF1 from a transcriptional repressor into a transcriptional activator in HeLa cells.

orphan receptors. COUP-TFI (also termed EAR3) and COUP-TFII (also termed ARP-1) are closely related transcription factors that are expressed ubiquitously and are involved in the regulation of several important biological processes, such as neurogenesis, organogenesis, cell fate determination, and metabolic homeostasis (4–6). Targeted disruptions of mouse COUP-TFI or COUP-TFII result in perinatal and embryonic lethality, respectively (7, 8). COUP-TFs are also involved in the regulation of the transcription of genes that encode various metabolic enzymes such as phosphoenolpyruvate carboxykinase (PEPCK; EC 4.1.1.32), cholesterol 7 α -hydroxylase (CYP7A; EC 1.14.13.17) (9), and mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase (EC 4.1.3.5) (10).

COUP-TFs homodimerize or heterodimerize with retinoid X receptor and a few other nuclear receptors and bind to a wide variety of response elements that contain imperfect AGGTCA direct repeats separated by a variable number of nucleotides (7, 11). Although COUP-TF was initially identified as an activator of the chicken ovalbumin gene, COUP-TFs generally serve as negative regulators in conjunction with other nuclear hormone receptors such as retinoic acid receptor, thyroid hormone receptor, vitamin D receptor, peroxisome proliferator-activated receptor, and hepatocyte nuclear factor 4 (HNF-4) (11–13). Several mechanisms account for the repressive effects of COUP-TFs. COUP-TFs bind to a number of nuclear hormone receptor response elements and, thus, compete with receptors for these DNA elements. COUP-TFs may also repress transcription by forming nonproductive complexes with retinoid X receptor, the essential heterodimer partner of a number of nuclear hormone receptors. In addition, at least two direct mechanisms of repression have been described. In one example, termed active repression, COUP-TFs bind to specific DNA elements and repress by forming a direct interaction with corepressors such as nuclear receptor corepressor and silencing mediator for retinoic acid and thyroid hormone receptor (SMRT) (14). Finally, COUP-TFs can repress transcription by directly binding to the ligand binding domain of nuclear hormone receptors, a process called transrepression (13, 15).

COUP-TFs also activate transcription in various promoter contexts (9, 10, 16–21). COUP-TFII stimulates the transcriptional activity of the rat CYP7A promoter by binding to the nucleotide sequence located between –74 and –54 (relative to the transcription start site), which contains a direct repeat of two hormone response element half-sites separated by 4 nucleotides

The nuclear hormone receptor superfamily is composed of many diverse subsets of transcriptional factors, including receptors for steroids, retinoids, and thyroid hormones. Also included are a large number of structurally and functionally related transcription regulatory proteins termed orphan receptors, so named because their natural ligands have not been identified (1–3). Chicken ovalbumin upstream promoter-transcription factor (COUP-TF)¹ is one of the most studied of the

* This work was supported by National Institutes of Health Grant DK 35107 and the Vanderbilt Diabetes Research and Training Center Grant DK 20593). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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¹ The abbreviations used are: COUP-TF, chicken ovalbumin upstream promoter-transcription factor; PEPCK, phosphoenolpyruvate carboxykinase; CYP7A, cholesterol 7 α -hydroxylase; HNF, hepatocyte nuclear factor; SMRT, silencing mediator for retinoic acid and thyroid

hormone receptor; GRU, glucocorticoid response unit; AF, accessory factor; gAF1, gAF2, and gAF3, glucocorticoid AF binding sites; GR, glucocorticoid receptor; GR1 and GR2, GR binding sites; GRIP1, GR-interacting protein-1; SRC-1, steroid receptor coactivator-1; CAT, chloramphenicol acetyltransferase.

(a DR4) (9). Furthermore, COUP-TFs function as accessory factors for the hormonal response of some genes, such as the glucocorticoid response of the PEPCK and the estrogen response of the trout estrogen receptor gene (19). Finally, COUP-TFs also can function as coactivators. For instance, COUP-TFs potentiate HNF-4-mediated transactivation in the hepatocyte nuclear factor 1 α (HNF-1 α) gene promoter through direct interaction with HNF-4 and without a requirement for DNA binding (17). A similar coactivator function has been described in the vHNF (also termed HNF-1 β) gene promoter, where COUP-TFs do not bind DNA directly but potentiate the transcriptional activity of the vHNF gene promoter through a DNA-independent interaction with Oct-1 (20).

Our interest is directed to the role COUP-TF plays in the hormonal regulation of the PEPCK gene. PEPCK catalyzes the conversion of oxaloacetate to phosphoenolpyruvate, a rate-controlling step in hepatic gluconeogenesis. PEPCK gene transcription is positively regulated by glucocorticoids, glucagon (cAMP) and retinoic acid, whereas insulin inhibits the transcription of the PEPCK gene (22–24). Induction of transcription of the PEPCK gene by glucocorticoids is achieved through a complex glucocorticoid response unit (GRU) (see Fig. 1). The GRU includes, as a linear array from 5' to 3', two glucocorticoid accessory factor binding sites, gAF1 and gAF2,² two glucocorticoid receptor (GR) binding sites, GR1 and GR2, and a third accessory factor binding site, gAF3 (25, 26). An intact cAMP response element (located between -93 and -86) is also required for a full glucocorticoid response and, thus, is part of the GRU (see Fig. 1). The proteins that mediate the accessory activities through gAF1, gAF2, and gAF3 have been identified. Both HNF-4 and COUP-TF bind the gAF1 element and act as accessory factors for the glucocorticoid response (27). A number of factors bind to gAF2, although only binding of hepatic nuclear factor 3 (HNF-3) specifically correlates with the ability of the gAF2 element to induce the glucocorticoid response (28). COUP-TF also binds to gAF3 and acts as the accessory factor through this element (26). Thus, COUP-TF serves as an accessory factor required for the induction of PEPCK gene transcription by glucocorticoids through both the gAF1 element and the gAF3 element (see Fig. 1).

The goal of this study is to begin to elucidate the mechanism by which COUP-TF acts as a transactivator of transcription. We first identify the minimal region of the molecule (amino acids 184–423) that confers the transactivation function of COUP-TFI. This is distinct from the previously described repression domain of COUP-TFI (13). The activation domain mediates gAF1 and gAF3 accessory activity of the glucocorticoid response of the PEPCK gene, is responsible for transactivation through the CYP7A COUP-TFII binding site, and enables COUP-TFI to function as a coactivator for HNF-4. GRIP1 and SRC-1 potentiate the transactivation function of COUP-TFI. However, in a system wherein COUP-TF is repressive, these coactivators cannot convert COUP-TFI into an activator. GRIP1 and SRC-1 interact with COUP-TFI *in vivo*, and these interactions require the intact activation domain defined in the function experiments.

MATERIALS AND METHODS

Plasmid Construction—The preparation of the PEPCK promoter-CAT reporter construct (pPL32) has been described previously (29). The

plasmids pGAL4gAF1 and pGAL4gAF2 were derived from pPL32 by the polymerase chain reaction megaprimer method. The plasmids pGAL4gAF3, pGALgAF1-gAF3, and a series of site-directed mutations of pABGAL4-COUP-TFI and pRSV-COUP-TFI were constructed using the Quick Change site-directed mutagenesis kit (Stratagene). The sequences of the oligonucleotides used in this study are shown in Table I. Expression plasmids for full-length human COUP-TFI (pRSV-hCOUP-TFI) (11) and pRSV-hCOUP-TFD15 (13) were gifts of Dr. Ming-Jer Tsai (Baylor College of Medicine, Houston, TX). Plasmids that encode GAL4-COUP-TFI chimeras, including GAL4-human COUP-TFI (pABGAL147-hCOUP-TFI) (11), pABGAL147-COUP-TFD15, and pABGAL147-COUP-TFD35 (13) were also provided by Dr. Tsai.

A *KpnI/HindIII* fragment of the pLcat62' (30) was inserted into the *KpnI/HindIII* sites of the pGL3-Basic vector (Promega, Madison, WI) to generate the LPK-LUC reporter plasmid. Three copies of the COUP-TF binding site from the CYP7A promoter (positions -74 to -54, relative to the transcription start site) were placed upstream of the minimal L-pyruvate kinase promoter fragment within the LPK-LUC reporter plasmid to generate the plasmid CYP7A(DR4)₃LPK-LUC using oligonucleotides CYP7Aa and CYP7Ab. The 5' overhangs of the *XhoI/BamHI* fragment of the (GAL4)₅E1b-CAT plasmid (31) were filled-in using the Klenow reaction and subcloned into the *SmaI* sites of the pGL3-Basic vector (Promega) to generate (GAL4)₅E1b-LUC.

To generate constructs for the expression of proteins in yeast, cDNAs that encode the wild type protein or a mutation of the C-terminal region of COUP-TFI were amplified by polymerase chain reaction using the primers COUP-TFIa and COUP-TFIb, and COUP-TFIa and COUP-TFIc, respectively, that generate an *EcoRI* and a *BamHI* site. Each polymerase chain reaction product was digested with *EcoRI* and *BamHI* and then subcloned into the pGBT9 plasmid (CLONTECH). The resulting plasmids were named pGBT9-COUP-TFI156/423 and pGBT9-COUP-TFI156/408, respectively. Yeast plasmids pGBD424-GRIP1/FL (32) and pGAD424 SRC-1a (33) were gifts from Dr. Michael Stallcup (University Southern California, Los Angeles, CA).

The DNA sequence of all constructs was verified by dideoxy sequencing. All oligonucleotides were produced on a Perceptive Biosystems Expedite 8909 DNA synthesizer located in the Vanderbilt University Diabetes Research and Training Center DNA chemistry core laboratory.

Transient Transfection—The maintenance and transfection of H4IIE, HepG2, and HeLa cells and the measurement of CAT activity have been described previously (25, 34, 35). In the transfection experiments using LUC reporters, firefly luciferase activity was normalized to *Renilla* luciferase activity using the Promega dual luciferase kit to control for transfection efficiency. The mammalian expression vector, pSV2GR, was provided by Dr. Keith Yamamoto (University of California, San Francisco, CA).

Yeast Two Hybrid Assays—*In vivo* protein-protein interaction assays were performed using the Matchmaker yeast two hybrid system (CLONTECH). A yeast plasmid, pGBT9-COUP-TFI156/423 or pGBT9-COUP-TFI156/408, was transformed into the yeast strain SFY526, and transformants were selected on synthetic dropout plates that contained medium lacking tryptophan. Yeast plasmids pGAD424 GRIP1/FL and pGAD424 SRC-1a were then transformed into yeast strain SFY526 that contained either pGBT9-COUP-TFI156/423 or pGBT9-COUP-TFI156/408. These transformants were selected on synthetic dropout plates with medium lacking leucine and tryptophan. The colonies that grew in these selection media were subjected to liquid assays by the expression of β -galactosidase, as described in the technical manual of the Matchmaker yeast two-hybrid system (CLONTECH).

RESULTS

GAL4-COUP-TFI Confers Both gAF1 and gAF3 Activity—We have previously demonstrated that the accessory factor activity of COUP-TF is required for a maximal induction of the PEPCK gene by glucocorticoids. COUP-TF accomplishes this by binding to both the gAF1 and gAF3 elements (Refs. 26 and 27 and see Fig. 1). We first examined whether COUP-TFI can mediate accessory activity while tethered to the GAL4 DNA binding domain (GAL4DBD) to establish a system that would allow us to map the domain required for the accessory factor activity of COUP-TF. Constructs were made wherein either gAF1 or gAF3 alone (pGAL4gAF1 and pGAL4gAF3, respectively) or both gAF1 and gAF3 together (pGAL4gAF1-AF3) were replaced by a yeast GAL4 DNA binding site(s) in the context of the wild type PEPCK promoter-CAT fusion gene construct (pPL32) (Fig. 2A).

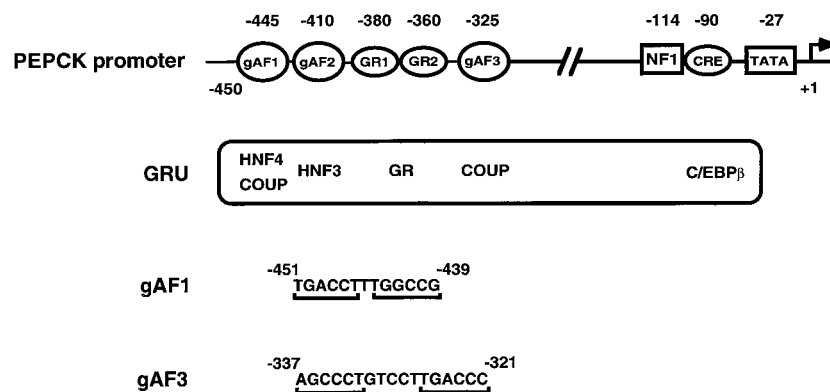
² We originally designated the accessory factor elements AF1, AF2, and AF3 in order of their discovery. The subsequent definition of transactivation domains of the nuclear receptor subfamily as AF1 and AF2 (48) has created a potentially confusing situation. To avoid having to discuss the interaction of the AF1 (or AF2) transcription domain of the glucocorticoid receptor with the AF1 element (or with the AF2 and AF3 elements) in the GRU, we now designate these elements as gAF1 to denote the glucocorticoid accessory factor 1 element, etc.

TABLE I
Oligonucleotides Used in This Study

All sequences are written in the 5' to 3' direction.

Name	Sequence
156/183a	TGT ATC GCC GGA ATT CTA CCT GTC CGG CTA C
156/183b	GGC CGT CGA CGC TCC GGT TCC CAA CGA TC
156/240a	TGT ATC GCC GGA ATT CCC GGA TCT GCA GAT C
156/240b	GAT CTG CAG ATC CGG GAA TTC CGG CGA TAC A
156/281a	TGT ATC GCC GGA ATT CCT GCA TGC CTC GCC C
156/281b	GGG CGA GGC ATG CAG GAA TTC CGG CGA TAC A
156/388a	TGT ATC GCC GGA ATT CTT CTT CGT CCG TTT G
156/388b	CAA ACG GAC GAA GAA GAA TTC CGG CGA TAC A
156/408a	TGT ATC GCC GGA ATT CTC TGG GAG CAG CTT C
156/408b	GAA GCT GCT CCC AGA GAA TTC CGG CGA TAC A
167/183a	TCC AAC CCA GCC CTA CCT GTC CGG CTA C
167/183b	GTA GCC GGA CAG GTA GGG CTG GGT TGG A
184/388a	TCA ACG GCC ACT GCT TCT TCG TCC GTT TGG
184/388b	CCA AAC GGA CGA AGA AGC AGT GGC CGT TGA
184/281a	TCA ACG GCC ACT GCC TGC ATG CCT CGC CC
184/281b	GGG CGA GGC ATG CAG GCA GTG GCC GTT GA
282/388a	TGG CCG CCG CCG GCT TCT TCG TCC GTT TGG
282/388b	CCA AAC GGA CGA AGA AGC CGG CGG CGG CCA
184/240a	TCA ACG GCC ACT GCC CGG ATC TGC AGA TC
184/240b	GAT CTG CAG ATC CGG GCA GTG GCC GTT GA
241/281a	AAC ATC CCC TTC TTC CTG CAT GCC TCG CCC
241/281b	GGG CGA GGC ATG CAG GAA GAA GGG GAT GTT
282/345a	TGG CCG CCG CCG GCG AGA AGT CGC AGT GC
282/345b	GCA CTG CGA CTT CTC GCC GGC GGC GGC CA
346/388a	TCG AGA GCC TGC AGT TCT TCG TCC GTT TGG
346/388b	CCA AAC GGA CGA AGA ACT GCA GGC TCT CGA
389/408a	TCC GTC ATC GAG CAG CTC TCT GGG AGC AGC TTC
389/408b	GAA GCT GCT CCC AGA GAG CTG CTC GAT GAC GGT
409/415a	ATA TGT TAC TGC CTT ACA TGT CC
409/415b	GGA CAT GTA AGG CAG TAA CAT AT
389/423a	TCC GTC ATC GAG CAG CTC TAG ACC TTG GGC GCT TCC
389/423b	GGA AGC GCC CAA GGT CTA GAG CTG CTC GAT GAC GGA
CYP7A-DR4a	TTG GTC ACT CAA GTT CAA GTA C
CYP7A-DR4b	TTG AAC TTG AGT GAC CAA GTA C
COUPa	CCG GAA TTC GTT CAG CGA GGA AGA ATG CC
COUPb	CGC GGA TCC GGA GCA CTG GAT GGA CAT G
COUPc	CGC GGA TCC CAG TAA CAT ATC GCG GAT GAG

FIG. 1. Schematic diagram of the PEPCK gene promoter GRU. The *cis*-acting elements and associated *trans*-acting factors required for a complete glucocorticoid response are shown. The location of each *cis*-element with respect to the transcription start site is shown above the schematic of the PEPCK promoter. Also shown are the sequences of the gAF1 and gAF3 elements and their relative locations. C/EBP, CAAT/enhancer-binding protein.



The substitution of gAF1 or gAF3 (or both) by the GAL4 DNA binding element resulted in a 70 to 80% reduction of the glucocorticoid response when compared with pPL32 (compare lines 2–4 with line 1 in Fig. 2A). This loss of function is equivalent to that obtained from mutations that abolish gAF1 or gAF3 activity (25–27, 36). Cotransfection of a vector that encodes the GAL4DBD with the pGAL4gAF1, pGAL4gAF3, or pGAL4gAF1·AF3 reporter constructs had very little, if any, effect on the glucocorticoid response (lines 1, 3, 5, Fig. 2B). By contrast, cotransfection of each reporter gene and a plasmid that encodes a chimeric protein consisting of a region of COUP-TFI and the GAL4DBD (GAL4-COUP-TFI156/423) restored the glucocorticoid response to the level found in cells transfected with pPL32 (compare lines 2, 4, and 6 in Fig. 2B with line 1 in Fig. 2A). Indeed, in the presence of GAL4·COUP-TFI156/423, the pGAL4gAF3 construct provided a glucocorticoid response

that was significantly greater than that provided by pPL32. This may be explained by the fact that COUP-TF binds with relatively low affinity to gAF3 (26). Thus the GAL4DBD provides a higher affinity binding site for COUP-TF in the context of the pGAL4gAF3 construct than is present in the context of the wild type promoter. The gAF1 and gAF3 elements cannot function as accessory factor elements when either is placed in the position normally occupied by gAF2 (37). GAL4-COUP-TFI156/423 was cotransfected with the pGAL4gAF2 reporter plasmid to determine whether the accessory activity provided by GAL4-COUP-TFI156/423 is sensitive to its location within the PEPCK gene promoter GRU. GAL4-COUP-TFI156/423 was unable to provide accessory factor activity to the glucocorticoid response when bound to the gAF2 element (compare line 8 in Fig. 2B with line 1 in Fig. 2A). These results indicate that GAL4-COUP-TFI confers accessory activity through both gAF1

FIG. 2. A GAL4-COUP-TFI chimeric protein confers accessory activity to the PEPCK glucocorticoid response through both gAF1 and gAF3. Mutations were made in the context of the full-length wild type (WT) PEPCK gene promoter construct, pPL32, so that gAF1 or gAF3 (or both) were replaced with the DNA binding site for the yeast transcription factor, GAL4. As shown in panel A, the ability of the mutant constructs (lines 2–4) to confer a glucocorticoid response was compared with that of the wild-type construct (line 1). H4IIE cells were transfected with 10 μ g of the reporter plasmid and 5 μ g of the pSV2GR expression vector and, where indicated, an additional 5 μ g of a vector that expresses either the GAL4DBD or a GAL4-COUP-TF chimeric protein. The cells were treated overnight with or without 0.5 μ M dexamethasone (Dex), and CAT activity was measured in the cell extracts. A schematic representation of the domains of COUP-TFI is shown at the top of panel B. Vectors that express the GAL4DBD or a GAL4-COUP-TFI fusion protein were cotransfected with the mutant reporters as described above to assess whether GAL4-COUP-TFI can restore a complete glucocorticoid response. The data represent the mean (\pm S.E., $n = 3$ –8) of the fold induction (dexamethasone-treated response divided by the untreated response) of CAT activity. None of the mutations depicted affects basal expression from the reporter gene.

and gAF3. Furthermore, GAL4-COUP-TFI activity is restricted by its location within the promoter in a manner similar to the authentic COUP-TF (26).

The E/F Region of COUP-TFI Confers Accessory Activity to the Glucocorticoid Response through Both gAF1 and gAF3—A number of constructs were made wherein portions of the D and E/F regions of COUP-TFI (see diagram in Fig. 2B) were fused to the GAL4DBD to map the domains of COUP-TFI that confer accessory activity to the glucocorticoid response. These constructs were cotransfected with the pGAL4gAF1, pGAL4gAF3, or pGAL4gAF1-gAF3 reporter plasmids, and the ability of the various GAL4-COUP constructs to confer accessory activity to the glucocorticoid response was monitored. The results of these experiments are summarized in Fig. 3. In all tests, the expression from these three reporter plasmids was identical. Deletion of the extreme C terminus (residues 409 to 423) resulted in a complete loss of accessory activity (Fig. 3, line 2). However, deletion of the D, or hinge, region (located between amino acids 156 and 183) did not affect the glucocorticoid response (Fig. 3, line 4). Interestingly, all of the other constructs, which represent a variety of deletions of COUP-TF, resulted in complete loss of accessory activity (Fig. 3, lines 2, 3, and 5–17). Gel shift analyses confirmed the equivalent expression of all of the GAL4-COUP-TFI mutants described in Fig. 3, as well as the ability of these constructs to bind DNA (data not shown). Thus, the E/F region of COUP-TFI, located between amino acids 184 and 423, is critical for the accessory factor activity provided through either gAF1 or gAF3.

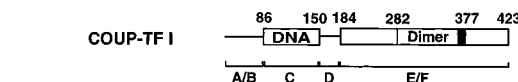
The E/F Region of COUP-TFI Also Confers the Transactivation Activity of COUP-TFI—The enzyme CYP7A catalyzes the first and rate-controlling step in the pathway that converts cholesterol to bile acids in the liver (9). COUP-TF transactivates through the rat CYP7A gene promoter by binding to a

A

Designation	Reporter Constructs	Fold Induction by Dex
1. pPL32 (WT)		16.6 \pm 2.9 (n=8)
2. pGAL4gAF1		3.7 \pm 1.2 (n=7)
3. pGAL4gAF3		4.2 \pm 0.7 (n=5)
4. pGAL4gAF1-gAF3		3.0 \pm 0.8 (n=4)

B

Designation	Reporter Constructs	Coexpressed	Fold Induction by Dex
1. pGAL4gAF1			4.1 \pm 0.7 (n=7)
2. pGAL4gAF1			19.41 \pm 5.6 (n=7)
3. pGAL4gAF3			5.7 \pm 1.1 (n=6)
4. pGAL4gAF3			31.1 \pm 9.6 (n=7)
5. pGAL4gAF1-gAF3			3.3 \pm 0.7 (n=5)
6. pGAL4gAF1-gAF3			13.3 \pm 1.4 (n=5)
7. pGAL4gAF2			5.2 \pm 0.7 (n=3)
8. pGAL4gAF2			5.5 \pm 0.9 (n=3)



sequence TGGTCActcaAGTTCA, which is comprised of two imperfect direct repeat nuclear hormone receptor half-sites separated by 4 base pairs (DR4) (9). A construct was made wherein three copies of the CYP7A COUP-TF binding site (positions -74 to -54) were placed upstream of the minimal L-pyruvate kinase promoter fragment, which in turn was positioned 5' from the luciferase reporter gene (CYP7A(DR4)₃LPK-LUC). This construct was used to test whether COUP-TFI provides direct activation of transcription in the context of this heterologous promoter. Surprisingly, the full-length pRSV-COUP-TFI construct and the construct that has a deletion of the hinge region (pRSV-COUP-TFID167/183) increased expression from the LPK-LUC vector when cotransfected into HeLa cells (Fig. 4). This vector does not contain any known COUP-TF binding sites, but there could be weak or cryptic COUP-TF binding sites, particularly since COUP-TF binds to such a wide range of sequences (7, 11). Most importantly, the pRSV-COUP-TFI and the pRSV-COUP-TFID167/183 reporter gene vectors potentiated expression (to a much greater degree than the empty vector) of luciferase activity from the CYP7A(DR4)₃LPK-LUC reporter construct when cotransfected into HeLa cells. In contrast, cotransfection with the C-terminal deletion mutant, pRSV-COUP-TFID15, or the internal ligand binding domain deletion mutant, pRSV-COUP-TFID184/281, did not affect expression from this reporter plasmid (Fig. 4). Thus, the E/F region of COUP-TFI, which is required for accessory factor activity in the PEPCK gene promoter (Fig. 3), is also important for the activating function of COUP-TFI through the CYP7A gene hormone response elements. Furthermore, this activity does not appear to be cell-specific, as equivalent results were obtained in HeLa and H4IIE cells (H4IIE cell data not shown).

The E/F Region of COUP-TFI Potentiates HNF-4 Activity

FIG. 3. The E/F region of COUP-TFI is required for a complete induction of the PEPCK gene by glucocorticoids. Ten μg of the reporter constructs that contain mutations wherein gAF1 or gAF3 (or both) were replaced with the GAL4 DNA binding site were cotransfected into H4IIE cells with 5 μg of the plasmids that express various GAL4-COUP-TFI fusion proteins and 5 μg of the pSV2GR expression vector. The cells were treated overnight with or without 0.5 μM dexamethasone (*Dex*), and CAT activity was measured in the cell extracts. A *plus* represents a complete restoration of the glucocorticoid response conferred by the GAL4-COUP-TFI construct depicted in *line 1*. A *minus* indicates that the fusion protein was unable to confer any accessory activity to the glucocorticoid response. Each transfection was performed at least three times. The basal rate of transcription from all of the reporter genes was similar in these experiments. *a.a.*, amino acids.

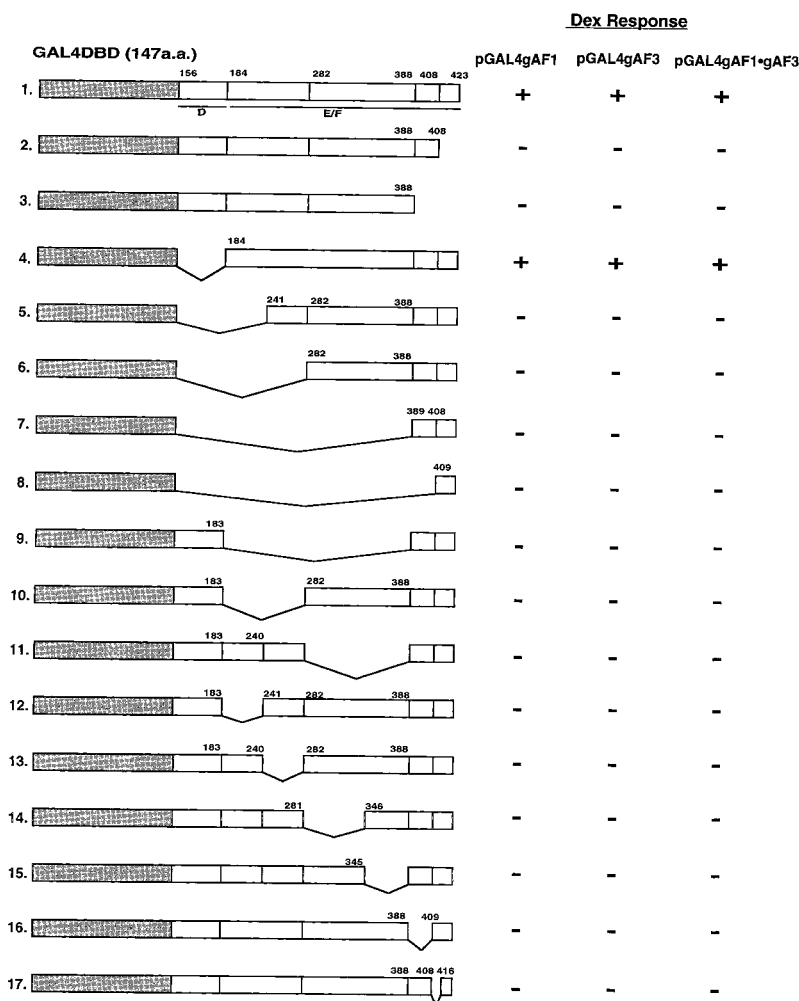
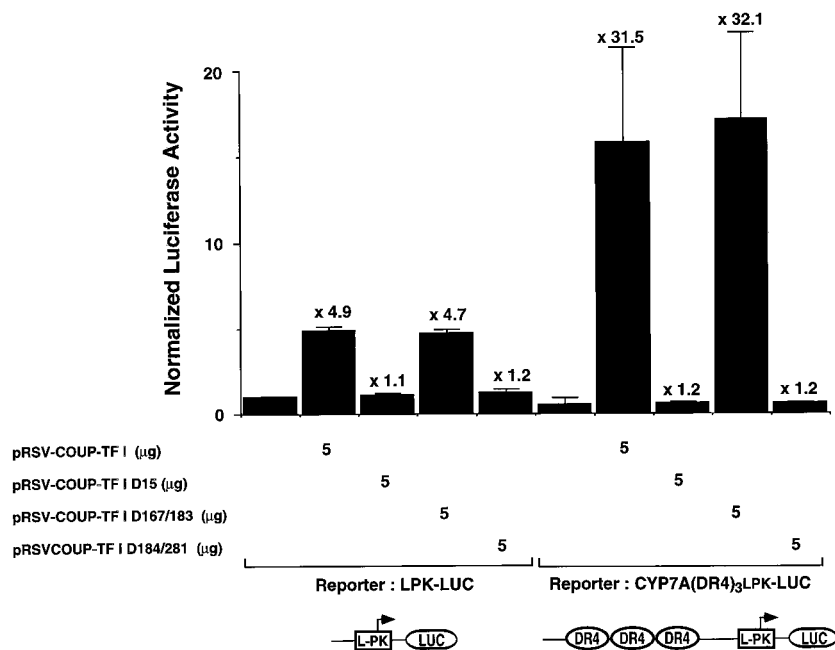


FIG. 4. The E/F region of COUP-TFI is required for transactivation from the DR4 element of the CYP7A gene promoter. A reporter construct was made wherein three copies of the DR4 element of the CYP7A gene promoter were ligated upstream of a minimal pyruvate kinase gene promoter-luciferase reporter gene (CYP7A(DR4)₃LPK-LUC). HeLa cells were cotransfected with 10 μg of the LPK-LUC and CYP7A(DR4)₃LPK-LUC reporter constructs with or without 5 μg of vectors that express COUP-TFI and various mutations of that protein along with 5 μg of the pSVGR1 expression vector and 0.1 μg of the pRL-SV40 vector (*Renilla* luciferase). Empty expression vectors were also transfected so that the total amount of DNA in each transfection experimental group was identical. The luciferase activities were measured in cell lysates 18 h after the transfection. The fold increase over the basal activity of LPK-LUC is indicated above each bar. The data represent the mean firefly luciferase activity normalized to *Renilla* luciferase activity (\pm S.E.) and represent at least three independent experiments. *L-PK*, L-pyruvate kinase.

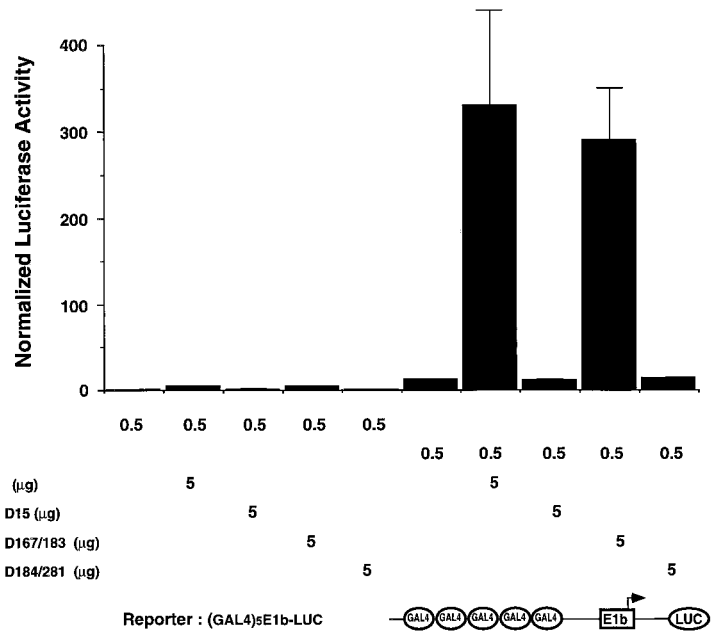


through Its Activity as a Coactivator—COUP-TFs act as coactivators for HNF-4-mediated transactivation through elements that can bind HNF-4 but not COUP-TFs (17). A series of expression plasmids that encode the wild type COUP-TFI or various mutations of COUP-TFI were cotransfected into HeLa

cells with GAL4-HNF-4 or GAL4DBD and a reporter gene, (GAL4)₅E1b-LUC, which contained five copies of the GAL4 binding site, to define the regions required for the coactivator activity of COUP-TFI. The activity of GAL4-HNF-4 was increased by about 30-fold when cotransfected into HeLa cells

FIG. 5. The E/F region of COUP-TFI is required for coactivator activity with the orphan receptor, HNF-4.

HeLa cells were cotransfected with a reporter construct that contains five GAL4 DNA binding sites positioned upstream of the minimal E1b promoter ligated to the luciferase reporter gene, 0.5 μ g of a construct that expresses either the GAL4DBD or the GAL4DBD ligated to HNF-4, and 5 μ g of a construct that expresses COUP-TF or various mutations of that protein and 0.1 μ g of the pRL-SV40 vector. Empty expression vectors were also transfected so that the total amount of DNA was constant for each experimental group. Luciferase activities were measured in the cell lysates 18 h after the transfection. The data represent the mean of firefly luciferase activity normalized to *Renilla* luciferase activity \pm S.E. from at least three separate transfections.



with the full-length pRSV-COUP-TFI or the pRSV-COUP-TFID167/183 constructs. By contrast, pRSV-COUP-TFID15 and pRSV-COUP-TFID184/281 were no more effective than the GAL4DBD construct (Fig. 5). These observations, considered with the results described above, suggest that the functional domain(s) of COUP-TFI, and required for all three mechanisms of COUP-TF activation, is located between amino acids 184 and 423. Furthermore, this functional property does not appear to require a specific cellular environment, since qualitatively similar results were observed when H4IIE cells were transfected (data not shown).

The Coactivators GRIP1 and SRC-1 Potentiate Transactivation by COUP-TFI—Many nuclear hormone receptors activate transcription through an interaction with coactivators (38). We tested whether the coactivators SRC-1 or GRIP1, known to interact with a variety of nuclear hormone receptors, could potentiate COUP-TF activation. Expression plasmids that encode either GRIP1 or SRC-1 were cotransfected into HeLa cells with the reporter plasmid, CYP7A(DR4)₃LPK-LUC, with or without a plasmid that encodes full-length COUP-TFI. As shown in Fig. 6, neither GRIP1 nor SRC-1 potentiated the reporter gene in the absence of expressed COUP-TFI, whereas both GRIP1 and SRC-1 potentiated the transactivation mediated by COUP-TFI expression by 2- to 3-fold. The effects of the two coactivators were not additive when plasmids that encode them were cotransfected together with the reporter construct (data not shown). These results suggest that the coactivators, GRIP1 and SRC-1, coactivate COUP-TFI-mediated transactivation. This response is also not cell type-specific, as similar results were obtained when H4IIE cells were transfected (data not shown).

COUP-TFI Interacts with GRIP1 and SRC-1 in Vivo—We used the yeast two-hybrid system to examine whether COUP-TFI interacts with either GRIP1 or SRC-1 and, if so, to define the regions of COUP-TFI required for this interaction. β -Galactosidase activity was induced when a chimeric fusion protein that encodes the GAL4 activation domain with GRIP1 or SRC-1 was coexpressed with GAL4DBD-COUP156/423. In contrast, the GAL4 activation domain alone did not induce β -galactosidase activity (Fig. 7). These data indicate that the E/F region of COUP-TFI interacts with SRC-1 and GRIP1. There was no detectable β -galactosidase activity when the same experiment was performed with GAL4-COUP-TFI156/408 (Fig. 7), a result

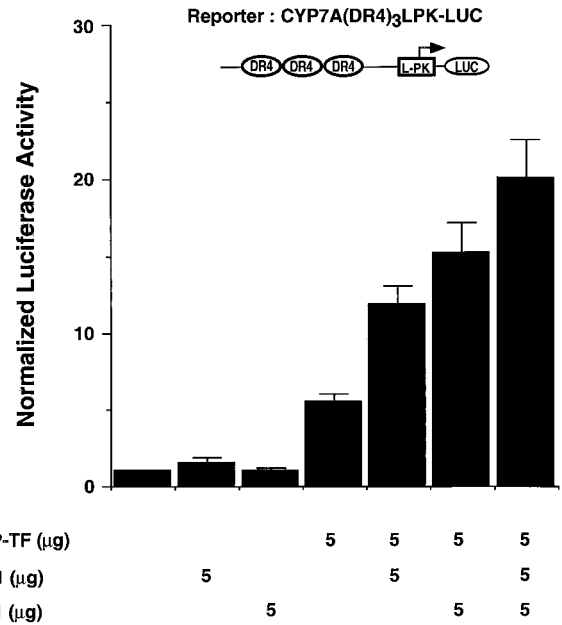


FIG. 6. GRIP1 and SRC-1 potentiate the transactivation activity of COUP-TFI. HeLa cells were cotransfected with 10 μ g of the CYP7A(DR4)₃LPK-LUC reporter construct, 5 μ g of vectors that express either GRIP1 or SRC-1 (pGBD424-GRIP/FL and pGAD424SRC-1a, respectively (32, 33)), and 0.1 μ g of the pRL-SV40 vector. Luciferase activities were measured in cell lysates 18 h after the transfection. The data represent the mean firefly luciferase activity normalized to *Renilla* luciferase activity (\pm S.E.; $n = 3$). L-PK, L-pyruvate kinase.

that demonstrates that the C terminal 15 amino acids are required for the protein-protein interaction between COUP-TF and the coactivators. These results establish a correlation between the activation function of COUP-TF and the ability of COUP-TF to bind to SRC-1 and GRIP1, since the same 15 amino acid region is required for both activities.

The C-terminal 15-Amino Acid Segment of COUP-TFI Is Required for Activation but Is Dispensable for Active Repression—Shibata *et al.* (14) demonstrate that the C terminus of COUP-TFI interacts with the corepressors nuclear receptor corepressor and SMRT, an interaction that is postulated to cause COUP-TF to function as an active repressor in a DNA-dependent manner. Leng *et al.* (13) demonstrate that the

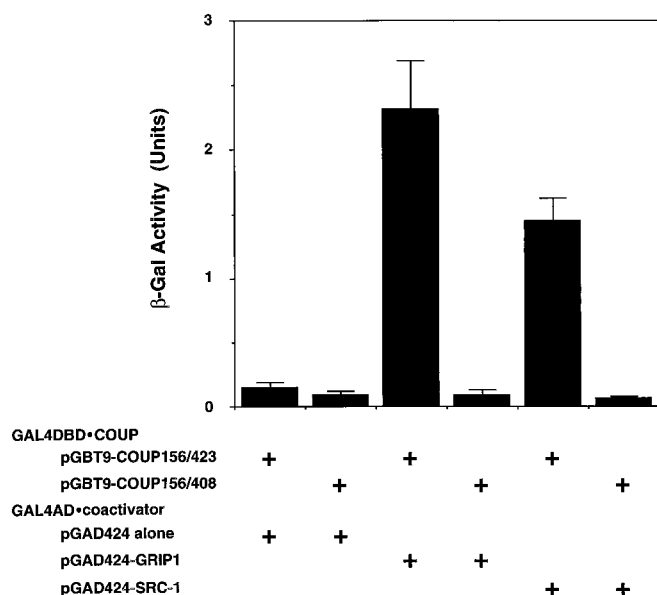


FIG. 7. COUP-TFI interacts with both GRIP1 and SRC-1 *in vivo*. Yeast cells were co-transformed with the plasmids pGBT9-COUP156/423, pGBT9-COUP156/408, pGAD424, pGAD424-GRIP1, and pGAD424-SRC-1, as described under "Materials and Methods." Colonies were grown on synthetic dropout plates lacking Leu and Trp and were subjected to a liquid assay for β -galactosidase (β -Gal) activity. The data represent the mean units of β -galactosidase activity (\pm S.E.) from at least three separate determinations.

C-terminal 15-amino acid segment could be deleted with only a slight reduction in the ability of COUP-TF to actively repress basal transcription through the thymidine kinase minimal promoter. Thus, with the exception of the C-terminal 15 amino acids, the region required for repression overlaps with the activation region of COUP-TFI that we defined. We next tested whether GAL4-COUP-TFI156/423 or GAL4-COUP-TFID15 could repress basal transcription from the (GAL4)₅E1b-LUC reporter gene, which contains five GAL4 DNA binding sites positioned upstream of the E1b minimal promoter. Cotransfection of both GAL4-COUP-TF constructs repressed the basal transcriptional activity provided by the reporter (compare lane 1 with lanes 2 and 5, Fig. 8). Therefore, the C-terminal 15 amino acids are not required for repression, a result consistent with previous reports (13, 14). These data demonstrate that the repression and activation domains of COUP-TF are different, at least by the C-terminal 15 amino acids.

Overexpression of Coactivators Does Not Reverse the Repressive Effect of COUP-TFI—The bifunctional activity of COUP-TF could be explained by a context-dependent equilibrium between corepressors and coactivators. By this view, the overexpression of coactivators could allow COUP-TF to switch from a transrepressor to a transactivator (14). As a test of this hypothesis, GRIP1 (Fig. 8, lanes 3 and 6) or SRC-1 (Fig. 8, lanes 4 and 7) were cotransfected into HeLa cells with GAL4-COUP-TFI156/423 and GAL4-COUP-TFID15 and the (GAL4)₅E1b-LUC reporter gene. Overexpression of the coactivators did not override the active repressive effect of COUP-TF.

DISCUSSION

COUP-TF, extensively studied as a repressor of transcription, also activates transcription by 1) binding to a nuclear receptor DNA response element and directly activating gene expression, as in the CYP7A gene promoter (9), 2) binding to a DNA element and indirectly influencing expression in the context of several other transcription factors, as in the PEPCK gene GRU (26, 27), and 3) by forming a protein-protein interaction with a DNA-bound factor, such as with HNF-4 in the

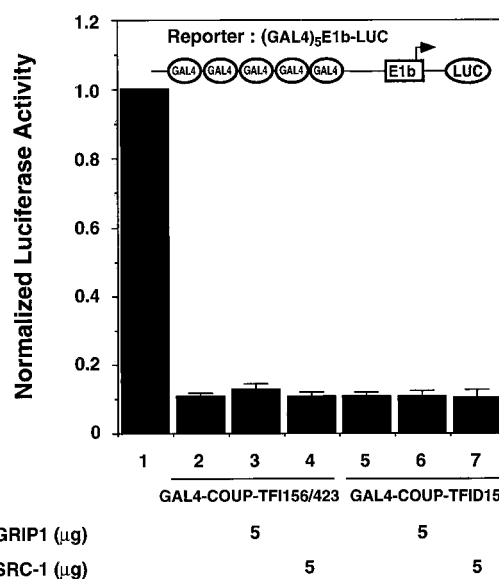


FIG. 8. The overexpression of coactivators fails to alter the active repression activity of COUP-TFI. HeLa cells were cotransfected with 10 μ g of the (GAL4)₅E1b-LUC reporter construct, 0.1 μ g of the pRL-SV40 vector, 5 μ g of the vectors that express either the GAL4-COUP-TFI or GAL4-COUP-TFID15 chimeric proteins, and 5 μ g of vectors that express either GRIP1 or SRC-1, as indicated. Empty expression vectors were cotransfected so that the total DNA concentration was identical for each group. Eighteen hours after the transfection, luciferase activities were measured in cell lysates. The data represent the mean firefly luciferase activity normalized to *Renilla* luciferase activity (\pm S.E.; $n = 3$).

HNF-1 α gene promoter (17). We show here by analyzing a number of deletion mutations that span the COUP-TFI molecule that a broad region located between amino acids 184–423 is required for these three activation functions. Furthermore, this same broad region is required for the interaction of COUP-TFI with the coactivators SRC-1 and GRIP1. It seems likely that amino acids scattered throughout this segment form the surface(s) required for direct transactivation and for the interaction with other factors. Although all three functions are equally disrupted by these mutations, we cannot conclude that precisely the same domains are involved in each.

It is perhaps not surprising that the entire E/F domain, also referred to as the ligand binding domain, is required for activation since, in other members of the nuclear receptor superfamily, this domain must be intact for transactivation (39). In addition to transactivation, this domain provides other functions, including nuclear localization and interactions with various coactivators (40). Although COUP is an orphan receptor that does not bind a known ligand, the other functions of the ligand binding domain may have been compromised by the different mutations that were made in the E/F domain. The coactivators GRIP1 and SRC-1 interact with the activation function 2 (AF2) domain near the C terminus of many nuclear receptors (39). It is noteworthy that the C-terminal 15 amino acids of COUP-TFI that are required for transactivation are not within the domain of COUP-TFI that is homologous with the AF2 domain.

There is significant overlap in the amino acid segments of COUP-TFI that are involved in the opposing actions of transcription repression and activation. The C-terminal 15 amino acid segment of the protein, which is dispensable for repression (see Fig. 8 and Ref. 13), is essential for the activation functions. On the other hand, a deletion of the C-terminal 35 amino acids, which are apparently required for transactivation, also renders COUP-TFI incapable of serving as a repressor. The corepressors, nuclear receptor corepressor and SMRT, bind to this

35-amino acid segment and confer the repression activity of COUP-TFI (14). It is not clear whether other segments of the molecule are also involved in repression. By contrast, the E/F domains of COUP-TFII, including the C-terminal 15 amino acids, are required for active repression (15). We have not studied COUP-TFII as a transactivator, but the accumulated evidence suggests that some very subtle differences between the two COUP molecules account for quite different biologic responses.

Repression and transactivation are presumably facilitated through protein-protein interactions that occur on the E/F region of COUP-TFI. Given that both activation and repression require overlapping sequences, there must be a mechanism in place that allows different sets of proteins to interact with COUP-TFI. By this view, either a coactivator complex or a corepressor complex is bound to the activation or repression domains within the ligand binding domain of COUP-TFI. It is possible that an equilibrium exists between these two complexes so that an excess of one complex or the other determines the direction of the response. This explanation seems unlikely for several reasons. First, if an equilibrium between coactivator and the corepressor complexes determines the direction of the COUP-TFI response, then at a particular time in a given cell all the COUP-TFI binding sites should mediate either a positive or a negative response. This is obviously not the case (*e.g.* compare Figs. 5 and 8). Furthermore, we show here that overexpression of SRC-1 and GRIP1 fails to switch COUP-TFI from a repressor to an activator in at least one specific instance.

The local intranuclear environment may determine the direction of COUP-TFI activity. For example, a given DNA sequence can function as an allosteric effector and influence the activity of a bound transcription factor (41). Indeed, Cooney *et al.* (11) demonstrate that COUP-TFI exists in different conformations when bound to different DNA binding sites. Hence, a specific DNA sequence may put COUP-TFI into a conformation that exposes surfaces for binding to either coactivator or corepressor complexes. This plasticity would allow the COUP-TFI binding site from the CYP7A gene promoter, which was used in the present study, to function as a positive element, whereas another DR4 element, with a slightly different sequence, could serve as a negative element when it binds COUP-TFI (42).

Protein-protein interactions in the context of a specific promoter may also be an important determinant of the direction of COUP-TFI activity. Whether COUP-TFI acts as a coactivator for HNF-4 or as a transrepressor for nuclear hormone receptors such as retinoic acid receptor is determined by protein-protein interactions, which allow COUP-TFI to associate with either SRC-1/GRIP1 or nuclear receptor corepressor/SMRT. The interaction of COUP-TFI with HNF-4 is of interest in this regard. The domain required for the HNF-4 coactivator activity of COUP-TFI is also required for its interaction with SRC-1 and GRIP1 *in vivo* (Figs. 5 and 7). It is possible that COUP-TFI interacts with the E region of HNF-4 (amino acids 227–271) and thus provides a surface, either directly or indirectly through coactivators, that associates with the basal transcription machinery (17). Interestingly, this coactivator activity of COUP-TFI is only seen in DNA sequences that are recognized by HNF-4 but not by COUP-TFI (17). Thus, COUP-TFI may only serve as a coactivator when HNF-4 exists in a specific conformation induced by DNA binding. Thus, although GAL4-COUP-TFI acts as a repressor in the context of the (GAL4)₅E1bCAT reporter, it acts as a positive accessory factor in the context of the pGAL4gAF1 reporter (compare Figs. 2 and 8). Thus, the action of COUP-TFI is probably determined by the combinatorial effects of the DNA sequence to which it binds and by specific interactions with other proteins, as influenced by specific promoter contexts.

COUP-TF is an important component of the PEPCK gene GRU. In addition to glucocorticoids, PEPCK gene transcription is regulated by a number of other hormones. Each hormone response is mediated by a set of *cis*-elements termed hormone response units. Many of the elements within one hormone response unit are also components of another. In addition, a number of these pleiotropic elements bind different sets of protein complexes. For example, the gAF1 and gAF3 components of the GRU bind COUP-TF (gAF1 can also bind HNF-4) but are also retinoic acid response elements 1 and 2. In this case, these two elements bind retinoic acid receptor/retinoid X receptor heterodimers and together comprise the retinoic acid response unit. It is curious that, although very different combinations of proteins bind, the same DNA contact points are used (43, 44). Similarly, the insulin response unit and the cAMP response unit share elements with the GRU, and again the proteins that bind to these elements are specific for the hormone response (45, 46). This overlapping structure of the PEPCK promoter is termed a metabolic control domain, and we hypothesize that this structure allows for a complex, integrated response of the PEPCK gene, which encodes a protein essential for gluconeogenesis, to a wide variety of environmental signals (26, 37).

Our current view is that different hormone response signals result in the recruitment of different sets of proteins to multiple elements within the promoter, which presumably tether different combinatorial sets of coactivators to the promoter (37). For example, the glucocorticoid response requires the recruitment of the ligand-bound GR and associated accessory factors and coactivators to the promoter. The retinoic acid response requires the recruitment of retinoic acid receptor/retinoid X receptor and associated coactivators, presumably SRC-1-like coactivators. These processes are, however, exclusive. For example, in the case of the retinoic acid response, the GR and its coactivators are not recruited; hence, the set of proteins bound to the PEPCK promoter during a response to retinoic acid is different from the complexes recruited during a glucocorticoid response. Indeed, the structural requirements of these responses is different, since a 5-base pair insertion between gAF2 and GR1, which effectively rotates the helix by one-half turn, decreases the glucocorticoid response but has no effect on the retinoic acid response (37).

The binding (or absence of binding) of AFs to gAF1, gAF2, and gAF3 has no effect on basal activity (26–28, 37), so it was of great interest to determine whether other proteins interact with COUP-TF, HNF-4, and HNF-3 to confer accessory activity to the glucocorticoid response, particularly since these transcription factors are specifically required to confer accessory factor activity from their particular location within the promoter. We have recently demonstrated that HNF-4 binds to both SRC-1 and GRIP1 and that these proteins act as coactivators for HNF-4-mediated transactivation in heterologous promoters and as co-accessory factors for the glucocorticoid response in the PEPCK gene promoter (47). Here we show that the same coactivators bind to COUP-TFI and potentiate activation by COUP-TF. Hall *et al.* (27) demonstrate that either HNF-4 or COUP-TF can act as an accessory factor through the gAF1 element. This observation may now be explained by the observations that both COUP-TF and HNF-4 utilize the same coactivators to promote their transactivation activities (this study and Ref. 47).

In summary, we have characterized the domain required for COUP-TF-mediated transcriptional activation in three different systems and have determined that SRC-1 and GRIP1 bind to this domain and serve as coactivators. This takes us a step

closer to understanding how the complex PEPCK gene GRU is assembled and how it functions.

Acknowledgements—We thank Cathy Caldwell for her excellent technical assistance, Deborah Caplenor Brown for preparation of the manuscript, and Kazuya Yamada for his invaluable advice.

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