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The Glucocorticoid Receptor and the Orphan Nuclear Receptor Chicken Ovalbumin Upstream Promoter-Transcription Factor II Interact with and Mutually Affect Each Other's Transcriptional Activities: Implications for Intermediary Metabolism

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Glucocorticoids exert their metabolic effect via their intracellular receptor, the glucocorticoid receptor (GR). In a yeast two-hybrid screening, we found the chicken ovalbumin upstream promoter transcription factor II (COUP-TFII), an orphan nuclear receptor that plays important roles in glucose, cholesterol, and xenobiotic metabolism, as a partner of GR. In an *in vitro* glutathione-S-transferase pull-down assay, COUP-TFII interacted via its DNA-binding domain with the hinge regions of both GRα and its splicing variant GRβ, whereas COUP-TFII formed a complex with GRα, but not with GRβ, in an *in vivo* chromatin immunoprecipitation and a regular immunoprecipitation assay. Accordingly, GRα, but not GRβ, enhanced COUP-TFII-induced transactivation of the simple COUP-TFII-responsive 7α-hydroxylase promoter through the transcriptional activity of its activation function-1 domain, whereas COUP-TFII repressed GRα-induced transactivation of the glucocorticoid-responsive promoter by attracting the silencing mediator for retinoid and thyroid hormone receptors. Importantly, mutual protein-protein interaction of GRα and COUP-TFII was necessary for glucocorticoid-induced enhancement of the promoter activity and the endogenous mRNA expression of the COUP-TFII-responsive phosphoenolpyruvate carboxykinase, the rate-limiting enzyme of hepatic gluconeogenesis. We suggest that COUP-TFII may participate in some of the metabolic effects of glucocorticoids through direct interactions with GRα. These interactions influence the transcription of both COUP-TFII- and GRα-responsive target genes, seem to be promoter specific, and can be in either a positive or negative direction. (*Molecular Endocrinology* 18: 820–833, 2004)

GLUCOCORTICOIDS EXERT MOST of their physiological and pharmacological actions through the intracellular glucocorticoid receptor (GR), a member of the steroid/thyroid/retinoic acid/orphan/nuclear receptor superfamily (1, 2). The GR gene, located in chromosome 5, produces two isoforms, GRα and GRβ, by alternative use of exon 9 α or 9 β, respectively (3). Human (h) GRα, a 777-amino acid polypeptide, is the classic receptor that binds glucocorticoids and mediates most of their known effects (1). In contrast, GRβ, a 742-amino acid molecule with a unique C-terminal 15-amino acid domain, does not bind glucocorticoids, has dominant negative activity on the transcriptional effects of GRα, and its physiological and pathological roles are as yet unclear (3–5).

GRα in the nonligand-bound condition is located in the cytoplasm, where it forms heterocomplexes with several heat shock proteins (6, 7). Upon binding to its ligand through its C-terminal ligand-binding domain (LBD), GRα dissociates from the heat shock proteins and enters the nucleus through the nuclear pore, using energy-dependent importin-mediated mechanisms (8). GRα homodimerizes and binds to specific DNA sequences, the glucocorticoid-responsive elements (GREs), in the enhancer regions of the glucocorticoid-responsive genes (8, 9). GRE-bound GRα homodimers

Abbreviations: AF-1, Activation function 1; C/EBP, CAAT/enhancer-binding protein; ChIP, chromatin Immunoprecipitation; COUP-TF, chicken ovalbumin upstream promotor-transcription factor; CYP7A, cholesterol 7α-hydroxylase; DEX, dexamethasone; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GR, glucocorticoid receptor; GRE, glucocorticoid response element; GST, glutathione-*S*-transferase; HNF, hepatocyte nuclear factor; LBD, ligand-binding domain; MMTV, mouse mammary tumor virus; PEPCK, phosphoenolpyruvate carboxykinase; SDS, sodium dodecyl sulfate; SMRT, silencing mediator for retinoid and thyroid hormone receptors; TAT, tyrosine aminotransferase.

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stimulate the transcription rate of responsive genes by facilitating the formation of a transcription initiation complex that includes the RNA polymerase II and its ancillary factors (10). In addition to these molecules, the GRE-bound GRα homodimers attract several proteins and protein complexes, such as transcription factor coactivators and chromatin modulators, via two transactivation domains termed activation function (AF)-1 and -2 (11, 12). AF-1 is located in the N-terminal domain of GRα and is ligand independent, whereas AF-2 is located in LBD and is ligand dependent (2). Through binding to these transactivation domains, these bridging factors transmit signals from the GR-ligand complex to the transcription initiation machinery, as well as loosen the chromatin structure to facilitate access and/or binding of other transcription factors and transcription machinery components to DNA.

Ligand-activated GRα also affects the transcriptional events of other signal transduction cascades via mutual protein-protein interactions with several transcription factors (1, 13). This activity may be more important than that exerted via direct binding to GRE DNA, because a mouse model harboring a mutant GR, which was active in protein-protein interactions but unable to homodimerize and bind GREs, was not lethal, in contrast to the neonatal lethality of mice in which the entire GR gene was deleted (14, 15). Transrepression or transactivation through protein-protein interactions of other transcription factors, such as nuclear factor-κB, activator protein-1, and signal transducers and activators of transcription, may be particularly important in the suppression of immune function and inflammation exerted by glucocorticoids (16–19). In addition, GRα influences the transcriptional activity of other transcription factors, such as cAMP response element-binding protein, CAAT/enhancer-binding protein (C/EBP), Nur77, p53, hepatocyte nuclear factor (HNF)-6, GATA-1, Oct-1 and-2, and nuclear factor 1 (20–27).

Glucocorticoids influence several metabolic pathways, including those of glucose, fatty acid, cholesterol, and xenobiotics (28–30). For example, glucocorticoids are potent inducers of hepatic gluconeogenesis (31). Through this activity, glucocorticoids may increase circulating glucose and insulin levels and cause the characteristic insulin resistance/hyperinsulinemia associated with glucocorticoid excess. Glucocorticoids stimulate gluconeogenesis by increasing the transcription rate of the key enzyme phosphoenolpyruvate carboxykinase (PEPCK), which catalyzes the conversion of oxaloacetate to phospoenolpyruvate (32). Ligand-activated GRα stimulates PEPCK promoter activity by cooperating with an orphan nuclear receptor, the chicken ovalbumin upstream promoter transcription factor II (COUP-TFII) and several other transcription factors by mechanisms that are as yet unclear (33).

COUP-TFII, along with its closely related protein COUP-TFI in humans, plays important roles in glucose, fatty acid, cholesterol, and xenobiotic metabolism and embryonic development (34–42). This transcription factor homodimerizes or heterodimerizes with several nuclear hormone receptors and binds a wide variety of response elements that contain imperfect AGGTCA direct repeats separated by a variable number of nucleotides (39, 43, 44). COUP-TFII suppresses the transcriptional activity of retinoic acid, thyroid hormone, vitamin D, and peroxisome proliferator-activated receptors, as well as that of HNF-4, via several postulated mechanisms, such as competition for DNA binding, formation of inactive heterodimers, or active repression by tethering the nuclear receptor corepressor SMRT (silencing mediator for retinoid and thyroid hormone receptors) via direct interaction mediated by its last 35 amino acids (39, 45). In addition to PEPCK, several other enzymes or regulatory molecules involved in intermediary metabolism, such as mitochondrial 3-hydroxy 3-methylglutaryl coenzyme A synthase, cholesterol 7α-hydroxylase (CYP7A), cytochrome p450 3A, and insulin, contain COUP-TFII-responsive elements in the promoter regions of their genes (38–40, 46).

To further elucidate the molecular mechanisms of glucocorticoid actions in metabolism, we performed a yeast two-hybrid screening assay, using components of the GR isoforms as baits. We found that GRα interacted with COUP-TFII in a ligand-dependent fashion *in vivo* and that GRα and COUP-TFII mutually affected each other's transcriptional activities in a promoter-specific fashion. This interaction may be biologically relevant in the regulation of intermediary metabolism.

RESULTS

Identification of COUP-TFII as a GR-Interacting Protein

To identify any protein(s) that interact(s) with GRβ and, possibly, GRα, we performed a yeast two-hybrid screening initially using the GRβ LBD, which also contains the hinge region, as a bait and the Jurkat cDNA library. From more than 80 interactors, we found two independent clones that contained the human COUP-TFII coding sequence. We confirmed the interaction of these clones with the GRβ and GRα LBDs in a mating assay (data not shown). To further examine the details of these interactions, we performed a glutathione-*S*-transferase (GST) pull-down assay using bacterially produced and purified GST-fused COUP-TFII together with *in vitro* translated and ^{35}S-labeled GRα and GRβ (Fig. 1A). Both GRα and GRβ bound GST-COUP-TFII in a ligand-independent fashion. Using a set of GST-COUP-TFII fragment fusions, GRα and GRβ bound the COUP-TFII region enclosed by amino acids 75–163, a portion corresponding to the DNA-binding domain of this transcription factor. On the other hand, using bacterially produced and purified GRα and GRβ with *in vitro* translated and ^{35}S-labeled COUP-TFII, we found

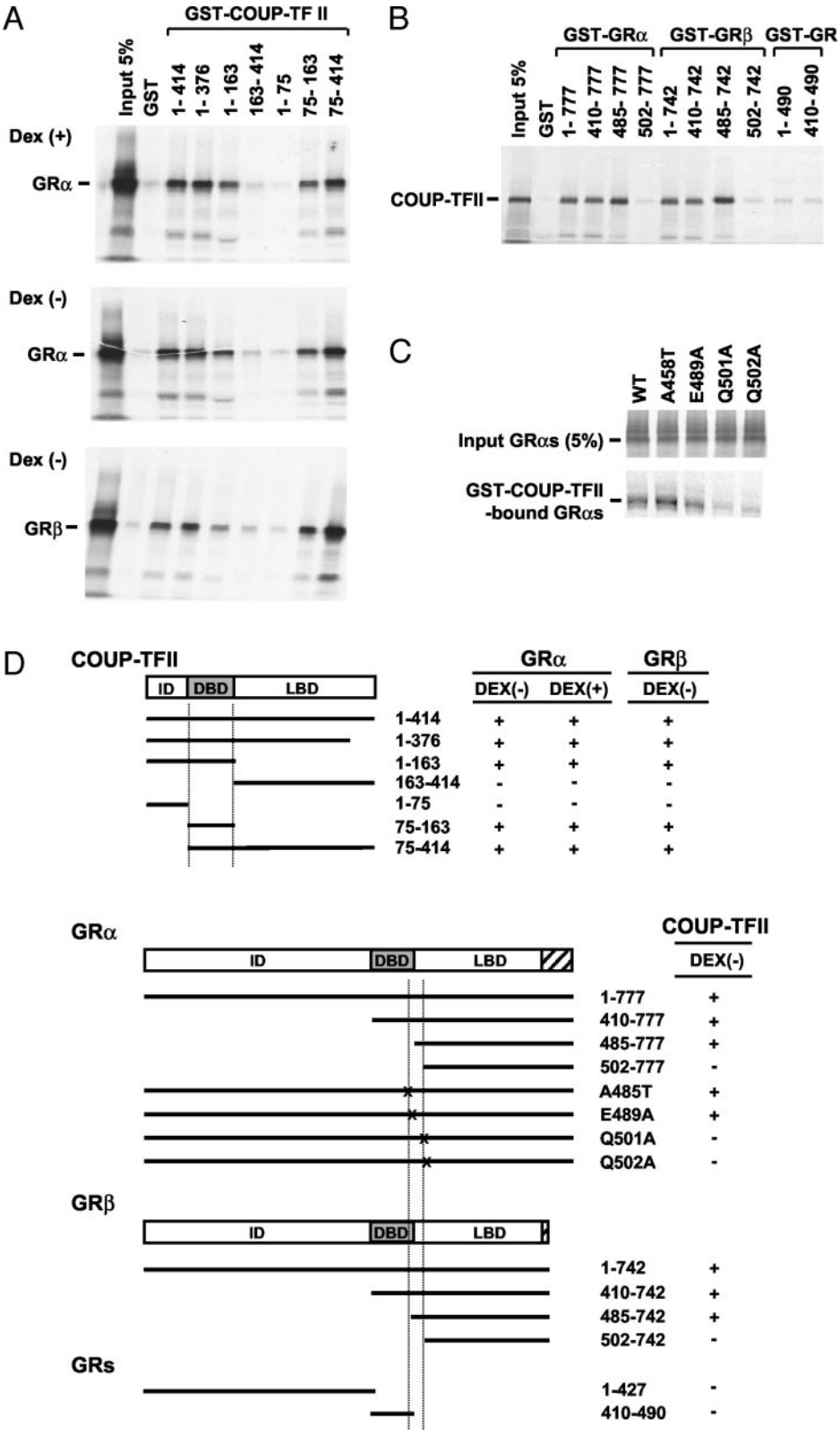

Fig. 1. Identification of Mutual Interaction Domains between GR Isoforms and COUP-TFII in GST Pull-Down Assays

In vitro translated and ^{35}S-labeled GRα and GRβ (A), COUP-TFII (B), and indicated mutants of GRα (C) were incubated with bacterially produced and purified full-length and fragments of COUP-TFII, GRα, and GRβ, respectively, in the presence or absence of 10^{-5} M DEX. Five percent of the total was spotted, and samples were run on 8% (GRα and GRβ), or 12% (COUP-TFII) SDS-PAGE gels. A summary of the results is shown in panel D.

that both GR isoforms bound COUP-TFII at the hinge region enclosed by amino acids 490–502 (Fig. 1B). Next, we used several GRα mutants, which have point mutations in the region 490–502, in the same GST pull-down system (Fig. 1C). GRαQ501A and Q502A, in which an aspartic acid at position 501 or 502 was replaced with alanine, respectively, was unable to bind COUP-TFII, whereas GRαE489A, which had a mutation replacing a glutamic acid at position 489 with alanine, preserved its binding capacity. GRαA458T, which has a threonine instead of an alanine at position 458 and, therefore, is unable to dimerize, also interacted with COUP-TFII. Results from GST pull-down assays are summarized in Fig. 1D.

To examine the *in vivo* binding of GRα or β and COUP-TFII in the context of a natural chromatin-bound promoter, we next performed a chromatin Immunoprecipitation (ChIP) assay (Fig. 2). The rat cholesterol 7α-hydroxylase (CYP7A) promoter, which is known to respond to COUP-TFII through two sets of the COUP-TFII-responsive elements, was used in this experiment. In H4IIE rat hepatoma cells that endogenously express GRα and COUP-TFII (data not shown), GRα was successfully coprecipitated with this promoter in a dexamethasone (DEX)-dependent fashion (Fig. 2A). In contrast, GRβ was not coprecipitated with this promoter, although it bound COUP-TFII in the *in vitro* GST pull-down assays. In a reciprocal ChIP assay using anti-COUP-TFII antibody, COUP-TFII was coprecipitated with the rat tyrosine aminotransferase (TAT) promoter, which contains tandem GREs approximately 2,500 bp upstream from its transcription start site (47) (Fig. 2B, *top panel*). In contrast, neither COUP-TFII nor GRα was precipitated with a proximal portion of the arginase promoter, which is devoid of GREs and COUP-TFII-responsive elements (Fig. 2B, *bottom panel*). The transcription of the arginase gene is stimulated by glucocorticoids through its enhancer region, located 11 kb downstream from the transcription start site and spanning the junction of intron 7 and exon 8. This region of the arginase gene has two C/EBPβ-responsive elements, and glucocorticoids

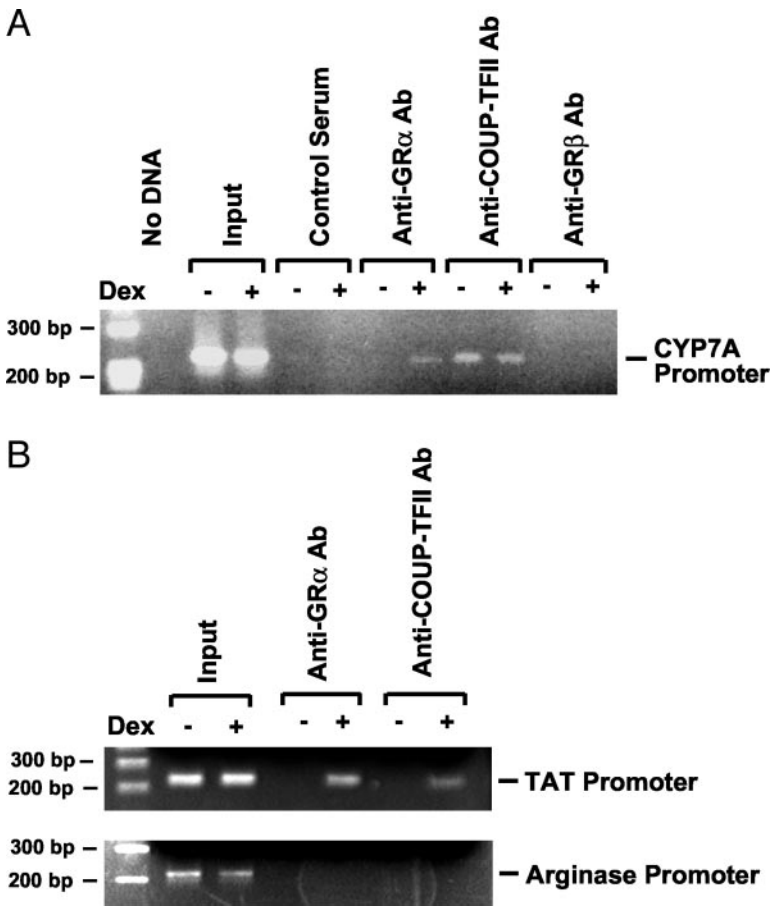

Fig. 2. COUP-TFII Forms a Complex with GRα, But Not with GRβ, on the COUP-TFII-Responsive CYP7A (A) and the GRE-Containing TAT (B) Promoters, But Not on the Control Arginase Promoter (B) in a ChIP Assay

H4IIE rat hepatoma cells were cultured in the presence or absence of 10^{-6} M DEX and treated with 1% paraformaldehyde, and their nuclei were harvested. DNA-bound GRα, GRβ, and COUP-TFII were immunoprecipitated with their specific antibodies, and a fragment of the CYP7A (A), TAT (B, *top panel*), and arginase (B, *bottom panel*) promoters were amplified by PCR. Mouse serum was used as a control. Five percent (B) or 10% (A) of the starting sample was used as input in the ChIP assay.

stimulate its activity by inducing the C/EBPβ protein and/or by promoting association between GRα and C/EBPβ (26, 48). These results indicate that GRα, but not GRβ, forms protein complexes with COUP-TFII in the presence of a ligand on both the COUP-TFII-responsive CYP7A and the GRE-containing TAT promoters *in vivo*. We also confirmed the interaction of GRα and COUP-TFII on a free GRE oligonucleotide probe in an EMSA (data not shown). The results of the chromatin immunoprecipitation assay indicated that COUP-TFII was coprecipitated with the TAT promoter through its interaction with GRα but not via independent binding to the promoter.

To further examine the *in vivo* association of GR and COUP-TFII, we performed regular coimmunoprecipitation assays in HepG2 and COS7 cells (Fig. 3). In HepG2 cells, which expressed endogenous GRα, GRβ, and COUP-TFII, COUP-TFII was coprecipitated with GRα but not with GRβ in a ligand-dependent fashion (Fig. 3A). In COS7 cells, overex-

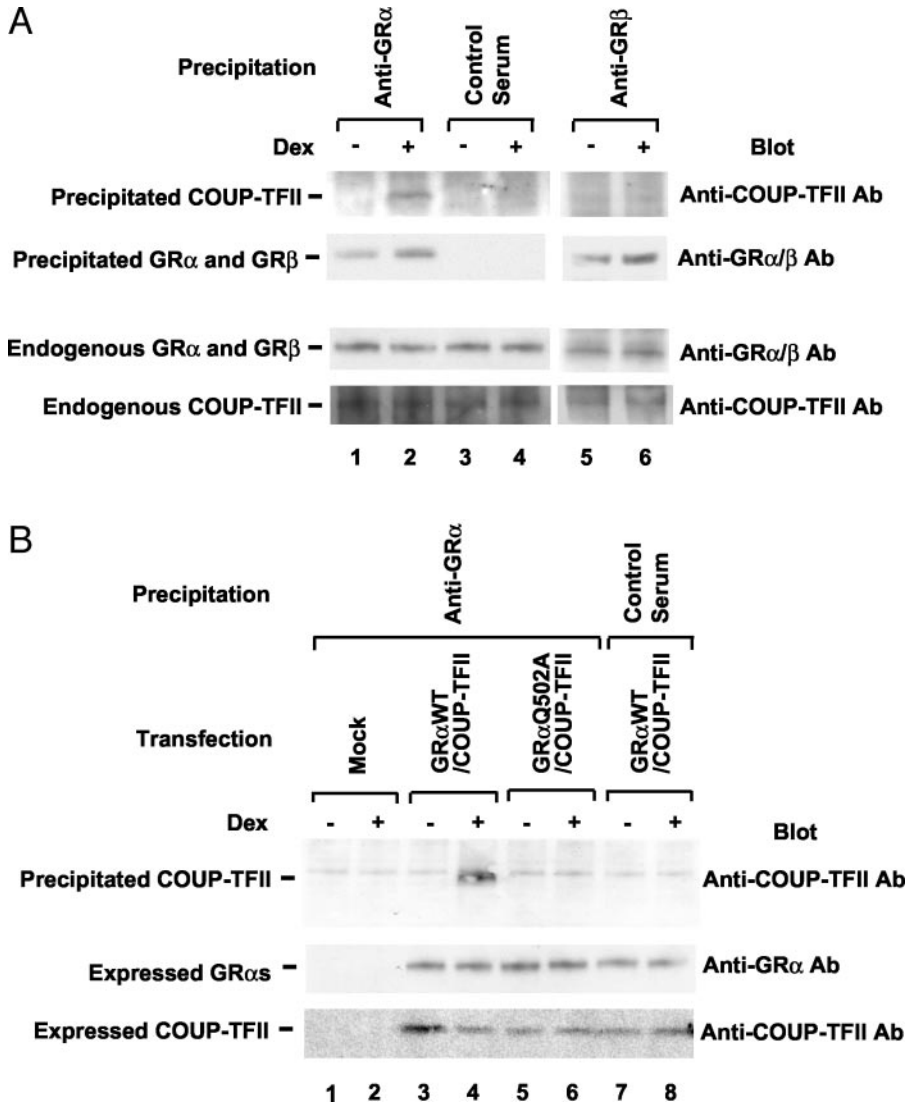

Fig. 3. COUP-TFII Forms a Complex with GRα, But Not with GRβ or GRαQ502A, *in Vivo*

A, HepG2 cells were treated with DEX, and protein complexes were precipitated with anti-GRα (lanes 1 and 2), rabbit control serum (lanes 3 and 4), or anti-GRβ (lanes 5 and 6) antibodies in the *top panel*. Precipitation of GRα or GRβ was confirmed in the same precipitation sample by blotting with anti-GRα (lanes 1–4) and -GRβ (lanes 5 and 6) in the *second top panel*. In the *lower two panels*, 10% of cell lysates were run on SDS-PAGE gels, and endogenous GRα (lines 1–4), GRβ (lines 5 and 6) (*third panel*), or COUP-TFII (*bottom panel*) were detected with anti-GRα (lanes 1–4), -GRβ (lanes 5 and 6) (*third panel*), or -COUP-TFII (*bottom panel*) antibodies, respectively. B, COS7 cells were transfected with COUP-TFII- and GRα wild type (WT)- or Q502A mutant-expressing plasmids, and protein complexes were precipitated with the anti-GRα antibody (lanes 1–6) or rabbit control serum (lanes 7 and 8) after treatment with DEX. In the *lower two panels*, 10% of cell lysates were run on SDS-PAGE gels, and expressed GRα WT, Q502A mutant, or COUP-TFII was detected with anti-GRα, or -COUP-TFII antibodies, respectively.

pressed COUP-TFII along with WT GRα or GRαQ502A, COUP-TFII was coprecipitated with WT GRα but not with the Q502A mutant receptor (Fig. 3B). Granted that this mutant receptor did not bind COUP-TFII in the GST pull-down assay either, this result indicates that GRα and COUP-TFII form complexes *in vivo* via the surfaces that supported their association observed in the GST pull-down assay (see above).

Functional Interactions between GR and COUP-TFII

We next examined the functional interaction of GRα and COUP-TFII using their respective responsive promoters, the mouse mammary tumor virus (MMTV) promoter that has four GREs and p-416/+32, a short fragment of the rat CYP7A promoter containing two COUP-TFII-responsive elements, in a transient transfection-based reporter assay (Fig. 4). COUP-TFII suppressed DEX-stimulated, GRα-induced transactivation of the MMTV promoter in a dose-dependent fashion in both HeLa and CV-1 cells (Fig. 4A). Overexpression of GRα, on the other hand, enhanced COUP-TFII-stimulated p-416/+32 CYP7A promoter activity in a DEX-dependent fashion in HepG2 cells [Fig. 4B(a)]. Without overexpressing GRα, a weak effect of DEX on COUP-TFII-induced transactivation of CYP7A promoter was observed in HepG2 cells, consistent with their low expression of endogenous GRα and COUP-TFII. In CV-1 cells, which do not have endogenous GRα and GRβ, expression of GRα supported DEX enhancement on COUP-TFII-induced transactivation of the same promoter, whereas GRβ failed to do it [Fig. 4B(b)].

To further explore the interaction between GRα and COUP-TFII, we tested GRα mutants Q501A and Q502A, which are defective in binding COUP-TFII, along with the control mutants E489A and A458T, in the CYP7A reporter assay in CV-1 cells (Fig. 5). As

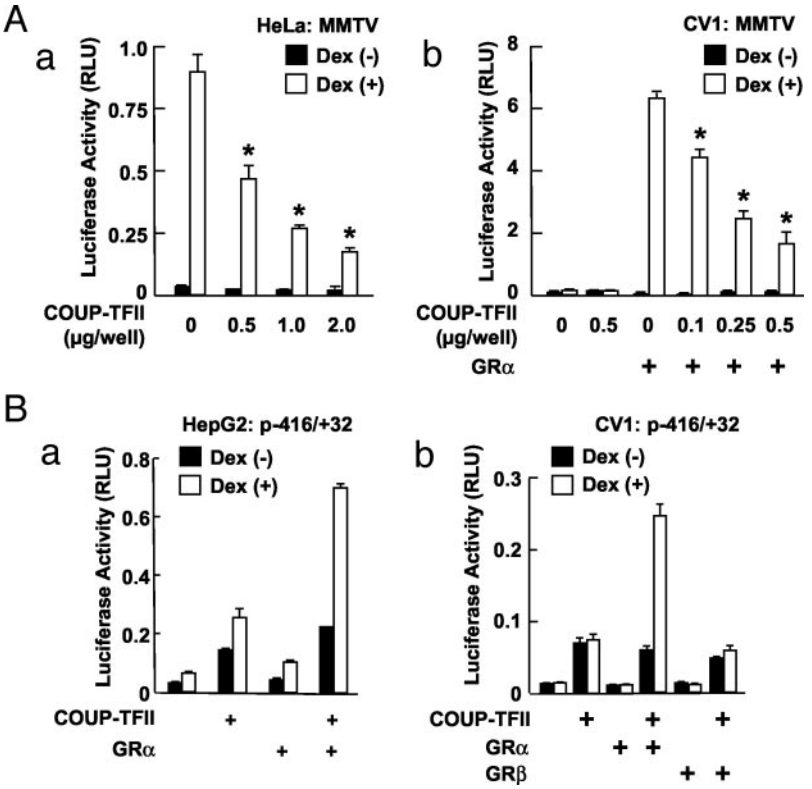

Fig. 4. GRα, But Not GRβ, and COUP-TFII Mutually Affect Each Other's Transactivation Activity on Their Responsive Promoters

A, COUP-TFII suppressed GRα-induced transactivation of the MMTV promoter in HeLa (a) and CV-1 (b) cells. Cells were transfected with increasing amounts of COUP-TFII-expressing plasmid together with pMMTV-Luc and pSV40-β-Gal. pRShGRα was also included in CV-1 cells. *Bars* show the mean ± SE of luciferase activity corrected for β-galactosidase activity. *, $P < 0.01$, compared with baseline. B(a), GRα enhanced COUP-TFII-induced transactivation of the CYP7A promoter in HepG2 cells. Cells were transfected with the COUP-TFII-expressing plasmid and the indicated amounts of GRα-expressing plasmid together with p-416/+32-Luc and pSV40-β-Gal. *Bars* show mean ± SE of luciferase activity corrected for β-galactosidase activity. B(b), GRα is necessary for the enhancement of COUP-TFII-induced transactivation of the CYP7A promoter by DEX, and GRβ did not influence it in CV-1 cells. Cells were transfected with the GRα- or GRβ-expressing plasmid together with the COUP-TFII-expressing plasmid, p-416/+32-Luc, and pSV40-β-Gal. *Bars* show mean ± SE of luciferase activity corrected for β-galactosidase activity.

expected, the former two mutants lost their enhancing effect on COUP-TFII-induced transactivation, whereas the latter two mutants retained it (Fig. 5A). Accordingly, COUP-TFII failed to suppress transactivation activity of GRαQ501A and Q502A on the MMTV promoter (Fig. 5B). All tested GR mutants were expressed in COS7 cells, which derived from the same animal species as CV-1 cells (Fig. 5C). These results strongly indicate that GRα and COUP-TFII influence each other's transcriptional activity, probably through their physical interaction.

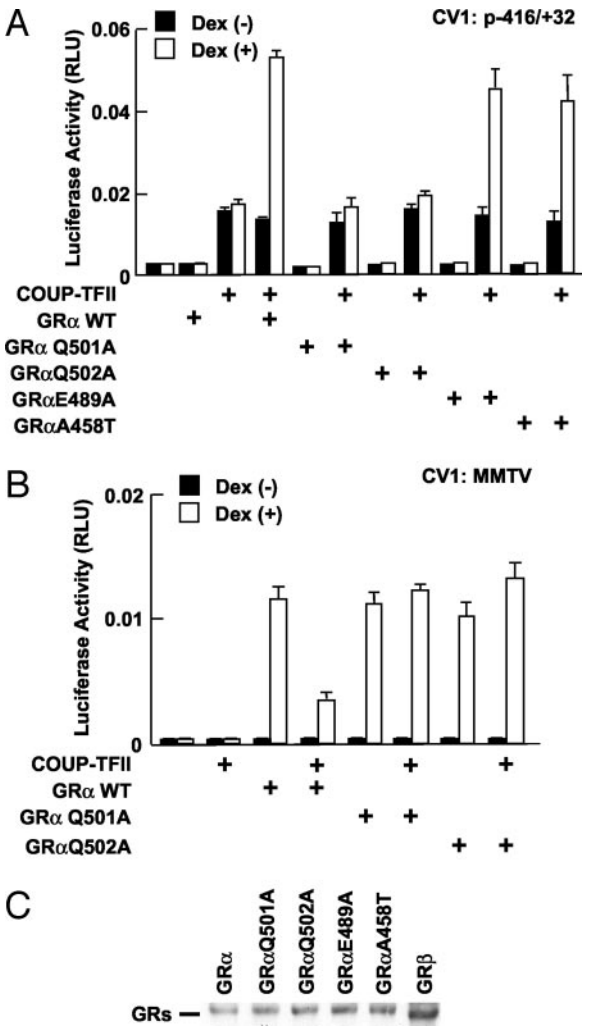

Fig. 5. Physical Interaction between GRα and COUP-TFII Mediates Mutual Effect on Each Other's Transcriptional Activities

A, COUP-TFII-binding-defective GRα mutants Q501A and Q502A lost their enhancing effect on COUP-TFII-induced transactivation of the CYP7A promoter, whereas dimerization-defective GRαA458T preserved it in CV-1 cells. Cells were transfected with indicated GRα mutant-expressing plasmids together with the COUP-TFII-expressing plasmid, p-416/+32-Luc, and pSV40-β-Gal. *Bars* show mean ± SE of luciferase activity corrected for β-galactosidase activity. B, COUP-TFII failed to suppress the transcriptional activity of the COUP-TFII binding-defective GRα mutants Q501A and Q502A on the MMTV promoter in CV-1 cells. Cells were transfected with the indicated GRα mutant-expressing plasmids and/or the COUP-TFII-expressing plasmid together with pMMTV-Luc and pSV40-β-Gal. *Bars* show mean ± SE of luciferase activity corrected for β-galactosidase activity. C, GR mutants are expressed in COS7 cells. COS7 cells were transfected with plasmids expressing indicated GR-related molecules. Cell lysates were run on a 8% SDS-PAGE gel, and GRs were detected by anti-GR antibody that recognizes the immunogenic domain of GR.

GRα Enhances COUP-TFII-Induced Transactivation via Its AF-1 Domain, Whereas COUP-TFII Suppresses GRα in Part by Attracting SMRT via its C-Terminal Portion

We next examined the mechanisms of the interaction between GRα and COUP-TFII, as this affected their transcriptional activities. GRα contains two transactivation domains, AF-1 and AF-2, the activities of which are supported by specific interaction with several coactivators and chromatin modulators. Thus, we examined the contribution of each of these domains on GRα-induced enhancement of COUP-TFII transactivation in HCT116 cells, which express no functional GRα and have low levels of COUP-TFII (data not shown). Coexpression of full-length GRα synergistically enhanced the COUP-TFII-induced transcriptional activity of the CYP7A promoter in a DEX-dependent fashion (Fig. 6A). In contrast, GRα(Δ77–261), which is devoid of the AF-1 transactivation domain, completely lost its enhancing effect, whereas GRα (1–550), which has the AF-1 but not the AF-2 domain, still enhanced COUP-TFII-induced transactivation in a DEX-independent fashion. GRα fragments used in the analysis contain the COUP-TFII-binding domain, and even smaller pieces of this receptor bound COUP-TFII *in vitro* (Fig. 1B). These results suggest that the AF-1 domain of GRα plays an important role in GRα-induced enhancement of COUP-TFII transactivation.

COUP-TF is known to actively suppress the transcriptional activity of several promoters by attracting the corepressor SMRT through the direct binding via its last 35 amino acids (45). This evidence prompted us to examine SMRT on COUP-TFII-induced suppression of GRα transactivation in HCT116 cells (Fig. 6B). Full length COUP-TFII strongly suppressed GRα-induced transactivation of the MMTV promoter in a DEX-dependent fashion. Coexpression of SMRT further strengthened suppressive effect of COUP-TFII on GRα transactivation. In contrast, COUP-TFII (1–380), which is devoid of the SMRT-binding domain, significantly lost the suppressive effect, and SMRT did not effectively cooperate with this mutant COUP-TFII. COUP-TFII (1–380) is very close to COUP-TFII (1–376), which was shown to bind GRα *in vitro* (Fig. 1A). These results indicate that COUP-TFII, at least in part, suppressed the GRα-induced transcriptional activity by attracting SMRT to the promoter region.

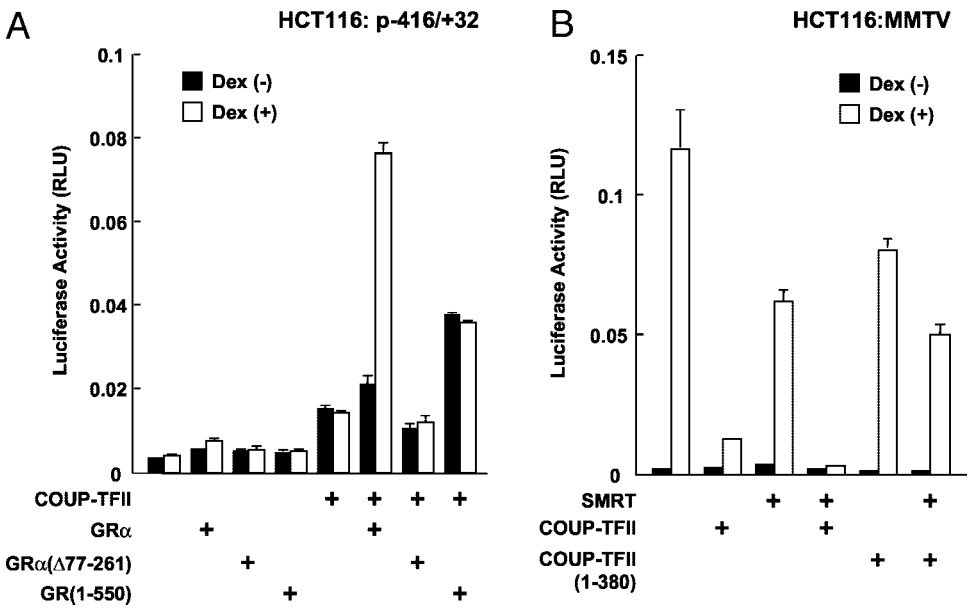

Fig. 6. AF-1 of GRα Is Required for GRα-Induced Enhancement of COUP-TFII Transactivation, Whereas Attraction of SMRT Plays a Role in COUP-TFII-Induced Repression of GRα Transactivation

A, AF-1, but not AF-2, of GRα is required for GRα-induced enhancement of COUP-TFII transactivation in HCT116 cells. Cells were transfected with the indicated wild-type and mutant GRα-expressing plasmids together with the COUP-TFII-expressing plasmid, p-416/+32-Luc, and pSV40-β-Gal. *Bars* show the mean ± SE of luciferase activity corrected for β-galactosidase activity. B, COUP-TFII suppresses GRα-induced transactivation in part through attracting the corepressor SMRT in HCT116 cells. Cells were transfected with the indicated COUP-TFII-expressing plasmids and/or pCMX-SMRT, together with pRShGRα, pMMTV-Luc, and pSV40-β-Gal. *Bars* show the mean ± SE of luciferase activity corrected for β-galactosidase activity.

COUP-TFII Is Necessary for DEX-Induced Stimulation of PEPCK mRNA Expression and Enhancement of PEPCK and CYP7A Promoters' Activity

Previous reports demonstrated that glucocorticoids stimulated the PEPCK promoter and expression of its mRNA through interaction of GRα and COUP-TFII (33, 42). Several reports also indicated that GRα bound to the glucocorticoid response units on the PEPCK promoter, which do not resemble classic GREs but rather contain COUP-TFII-responsive elements (33, 49, 50). Thus, we hypothesized that the physical interaction of GR and COUP-TFII may contribute to the enhancement of this promoter by glucocorticoids. We employed a morpholino oligonucleotide antisense for COUP-TFII to reduce the endogenous expression of COUP-TFII in HepG2 cells. This antisense molecule greatly attenuated DEX-induced stimulation of the endogenous PEPCK mRNA expression in HepG2 cells [Fig. 7A(a)]. Treatment of the cells with the antisense reduced expression of COUP-TFII both at the mRNA and protein levels [Fig. 7, A(b) and B]. In contrast, the antisense treatment did not affect DEX-induced expression of the arginase mRNA, the responsiveness of which is supported indirectly by glucocorticoid-induced induction of the C/EBPβ expression and/or association of C/EBPβ and GRα at the distal enhancer region that contains two C/EBPβ-responsive elements (26, 48) [Fig. 7 A(c)]. Treatment of HepG2 cells with this antisense oligonucleotide also attenuated DEX-induced enhancement of the PEPCK promoter activity as well as the p-416/+32 CYP7A promoter activity (Fig. 7C). Finally, COUP-TFII binding-defective GRαQ502A failed to enhance PEPCK promoter activity in contrast to wild-type GRα in CV-1 cells (Fig. 7D). Taken together, these findings indicate that physical interaction between GRα and COUP-TFII play a crucial role in the stimulation of the PEPCK mRNA expression and promoter activation by glucocorticoids.

DISCUSSION

Both GRα and GRβ physically interacted with COUP-TFII at the DNA-binding domain of the latter via their hinge region *in vitro*, whereas only GRα formed complexes with COUP-TFII on the COUP-TFII-responsive CYP7A promoter in a ligand-dependent manner *in vivo*. The same binding specificity of COUP-TFII to GRα and β was also observed in the regular coimmunoprecipitation assays. GRα may become able to interact with COUP-TFII, which is located constitutively in the nucleus, only after activation by ligand and nuclear translocation *in vivo* (6, 7). GRβ, on the other hand, forms weak complexes with the heat shock proteins and, hence, is readily crossing into the nucleus (51, 52). Regardless of its nuclear localization, however, GRβ was unable to affect COUP-TFII-induced transactivation in our studies.

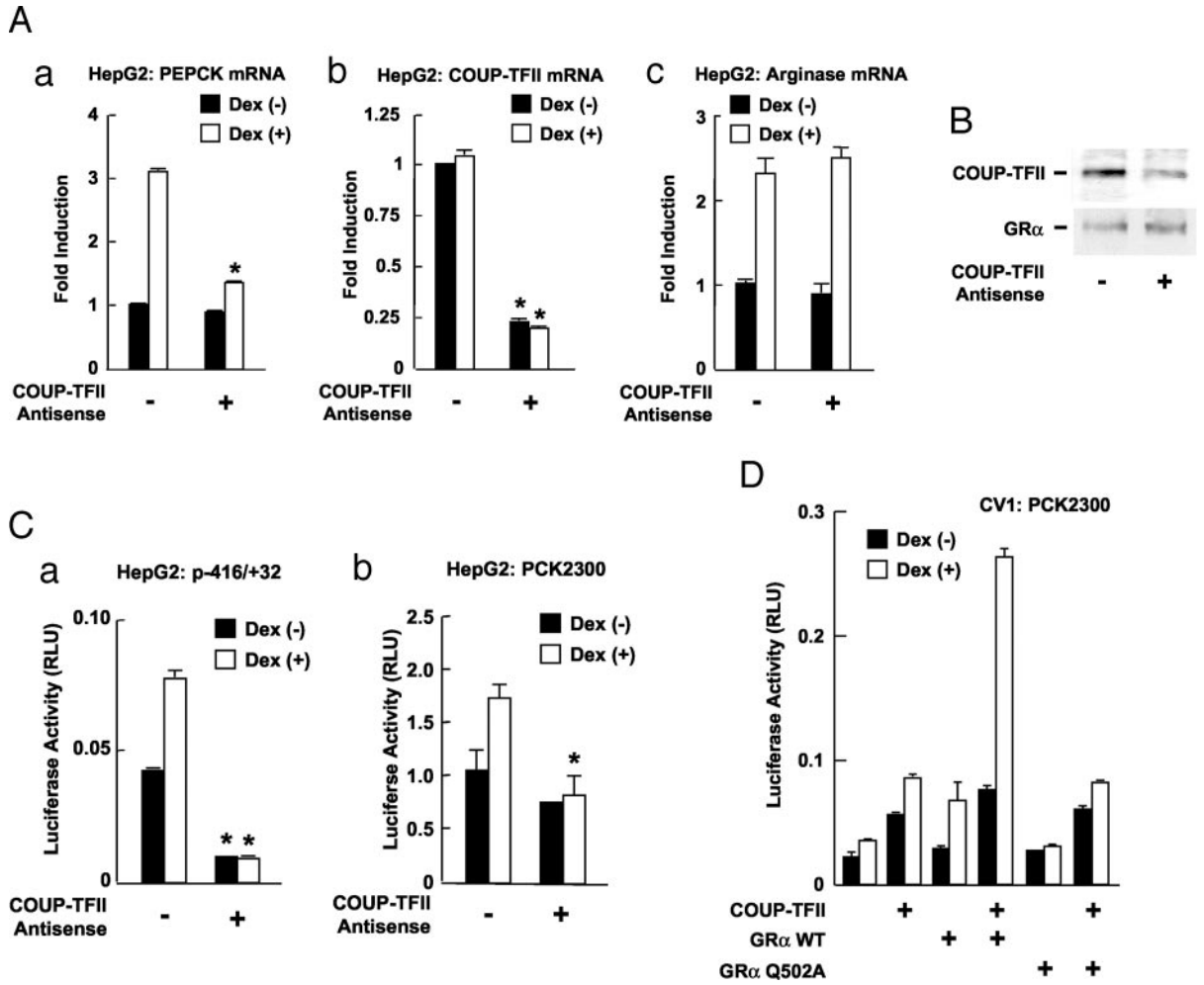

Fig. 7. Endogenous COUP-TFII Is Required for the DEX-Induced Activation of the PEPCK Promoter and Its mRNA Expression

A, COUP-TFII was necessary for the DEX-induced enhancement of PEPCK mRNA expression in HepG2 cells. Cells were treated with the COUP-TFII antisense or the control, followed by the exposure to 10^{-6} M DEX. Relative expression of PEPCK (a) COUP-TFII (b), and arginase (c) mRNAs were determined by real-time PCR. *Bars* show mean ± SE of their fold induction to baseline. *, $P < 0.01$, compared with baseline. B, Morpholino antisense for COUP-TFII suppressed expression of endogenous COUP-TFII in HepG2 cells. Cells were treated with the COUP-TFII antisense or the control antisense, and COUP-TFII was detected in cell lysates in the Western blot using anti-COUP-TFII antibody. As a control, expression of GRα was also examined in the same sample in the Western blot using anti-hGRα antibody. C, COUP-TFII was necessary for the DEX-induced enhancement of CYP7A and PEPCK promoters in HepG2 cells. Cells were treated with the COUP-TFII antisense or the control morpholino oligonucleotide and were transfected with PCK-2300-Luc (a) or p-416/+32-Luc (b), and pSV40-β-Gal, followed by the treatment with 10^{-6} M of DEX. *Bars* show mean ± SE of luciferase activity corrected for β-galactosidase activity. *, $P < 0.01$, compared with baseline. D, Physical interaction of GRα and COUP-TFII is necessary for the DEX-induced enhancement of the PEPCK promoter in CV-1 cells. Cells were transfected with the indicated GRα wild type (WT) or Q502A mutant-expressing plasmids and the COUP-TFII-expressing plasmid together with PCK-2300-Luc, and pSV40-β-Gal, followed by treatment with 10^{-6} M DEX. *Bars* show mean ± SE of luciferase activity corrected for β-galactosidase activity.

In functional reporter assays, COUP-TFII suppressed the transcriptional activity of DEX-stimulated GRα on the MMTV promoter in HeLa, CV-1, and HCT116 cells. Although in earlier studies the other COUP-TF family member, COUP-TFI, was shown to directly bind to the MMTV promoter and to suppress the transcriptional activity of this promoter in a ligand-independent fashion (53), here we showed that COUP-TFII bound to this promoter indirectly, via interaction with GRα, affecting the transcriptional activity of the latter only in the presence of DEX. COUP-TFII was also attracted to the rat TAT promoter, which contains GREs but not COUP-TF-responsive elements. COUP-TFII suppressed GRα transactivation of the MMTV promoter, at least in part, by attracting the corepressor SMRT. The latter binds COUP-TF at its last 35 amino acids and forms complexes with histone deacetylases that tighten the chromatin structure and inhibit access of transcriptional machinery components, such as RNA polymerase II and its ancillary factors (11, 12, 45).

GRα stimulated the transcriptional activity of COUP-TFII on the CYP7A promoter in HepG2, CV-1, and HCT116 cells, also in a ligand-dependent fashion. Because the homodimerization-defective GRα mutant GRαA458T was also able to enhance COUP-TFII-induced transactivation, it is likely that GRα modulates COUP-TFII transactivation in a monomeric form. GRα may function as a DEX-inducible coactivator for COUP-TFII, possibly by attracting cofactors through its transactivation domain AF-1 (11). Because removal of the AF-2 did not abolish GRα-induced enhancement of COUP-TFII transactivation, coactivators such as p300/cAMP response element binding protein-binding protein and p160 proteins, which mainly transduce the transcriptional activity of AF-2, are not likely to mediate the GRα enhancement (11). Rather, molecules that specifically bind and mediate the AF-1 activity may explain GRα-induced enhancement of COUP-TFII transctivation, although the mechanism of AF-1 transactivation is not well elucidated.

We also demonstrated that DEX-bound GRα enhanced the activity of the PEPCK promoter in HepG2 and CV-1 cells. This enhancement could result from the interaction of GRα with a complex promoter element, the glucocorticoid response unit (33, 54). This element contains two GRα-binding sites, GR1 and -2, and three accessory factor-binding sites that support enhancement of this promoter activity by GRα. We demonstrated that treatment with an antisense oligonucleotide against COUP-TFII greatly attenuated DEX-induced stimulation of the endogenous PEPCK mRNA expression and abolished the DEX-induced enhancement of the PEPCK promoter in HepG2 cells. The extent of this effect was similar to that observed on a simple CYP7A promoter, which contains only COUP-TFII-responsive elements. Our COUP-TFII-binding-defective GR mutant lost its ability to enhance PEPCK promoter activity. Because GR1 and 2 do not function as true GREs in the heterologous promoter and two of the accessory factor-binding sites contain COUP-TFII-responsive elements, it is likely that GRα is mainly attracted to the PEPCK promoter through COUP-TFII, and binding of GRα to GR1 and GR2 might play supportive roles in the activation of the PEPCK promoter by glucocorticoids (33, 54).

GRα enhanced the simple COUP-TFII-responsive CYP7A promoter. CYP7A catalyzes the first and rate-limiting step in the conversion of cholesterol to bile acids. The same enzyme may also regulate ketogenesis by suppressing the expression of the mitochondrial 3-hydroxy 3-methylglutaryl coenzyme A synthase (55, 56). COUP-TFII may also influence xenobiotic metabolism by affecting the expression of cytochrome p450 3A, which is the most copious microsomal hemoprotein in cytochrome p450, responsible for catalyzing the oxidative metabolism of clinically employed drugs and environmental chemicals (29, 38). COUP-TFII also activates the promoters of the fatty acid-binding protein, HNF-1 and ornithine transferase (57, 58). COUP-TFII-responsive elements are also found in the promoter regions of several apolipoproteins and insulin (35, 59, 60). Because glucocorticoids affect some of the above-mentioned metabolic pathways, COUP-TFII might participate in such glucocorticoid effects as well.

COUP-TFII also plays important roles in organ and tissue development during embryogenesis, mainly through silencing the transcriptional activity of certain target genes (39, 57). Glucocorticoids, which are produced by embryonic tissues late in organogenesis, may affect such COUP-TFII actions via direct interactions between ligand-activated GRα and COUP-TFII (61). Further investigations are necessary to define this possibility.

MATERIALS AND METHODS

Chemicals

DMEM, McCoy's 5A and Opti-MEM media, fetal bovine serum (FBS), 1 M HEPES buffer solution, antibiotics (penicillin G sodium and streptomycin sulfate), LipofectAMINE 2000, and Lipofectin were purchased from Life Technologies Inc. (Gaithersburg, MD). DEX was purchased from Sigma Chemical Co. (St. Louis, MO). Antisense for human COUP-TFII, encoding 5′-ACGTGCTGACTACCATTGCCATATC-3′, synthesized as morpholino oligonucleotide coupled with ethoxylated polyethilenimine, and the control antisense containing the sense sequence of the above antisense oligonucleotide, were purchased from Genetools (Philomath, OR). Anti-GRα and -GRβ antibodies, and anti-GR antibody, which is raised against a portion located in the immunogenic domain of GR, were purchased from Affinity Bioreagents, Inc. (Golden, CO). Anti-COUP-TFII antibody and rabbit control serum were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Mouse control serum was purchased from Sigma. Protease inhibitors were purchased from Roche Applied Science Co. (Indianapolis, IN). The ChIP assay kit was purchased from Upstate USA, Inc. (Charlottesville, VA).

Plasmids

pLexA-GRα and -GRβ LBDs were constructed by subcloning cDNA fragments corresponding to amino acids 485–777 of GRα and 485–742 of GRβ, respectively, into pLexA (CLONTECH Laboratories, Inc., Palo Alto, CA). p8op-LacZ, which contains 4 LexA-responsive elements upstream of LacZ cDNA, was purchased from CLONTECH. pRShGRα, pRShGRα (1–550), pRShGRβ, and pRSVerbA^{-1} were generous gifts from Dr. R. M. Evans (Salk Institute, La Jolla, CA). pRShGRα(Δ77–261) was constructed by digesting pRShGRα with *Bgl*II and subsequent autoligation. pBK/CMV-GRα and -β were described previously (62). pBK/CMV-GRαA458T, -GRαE489A, -GRαQ501A, and -GRαQ502A, which respectively express the designated mutant GRs, were constructed using PCR-assisted mutagenesis reactions with pBK/CMV-GRα as a template. pCDNA3-COUP-TFII and pCDNA3-COUP-TFII (1–380) were constructed by subcloning the cDNA fragments of full-length or amino acids 1–380 of COUP-TFII from pFL-COUP-TFII into pCDNA3, respectively (Invitrogen, Carlsbad, CA). pFL-COUP-TFII was a generous gift of Dr. M. J. Tsai (Baylor College of Medicine, Houston, TX). pCMX-SMRT, which expresses the full-length SMRT, was a kind gift from Dr. R. M. Evans (Salk Institute). pMMTV-Luc, which has the glucocorticoid-responsive MMTV promoter upstream of the luciferase cDNA, was generously pro-

vided by Dr. G. L. Hager (National Cancer Institute, Bethesda, MD). p-416/+32-Luc, which expresses luciferase under the control of −416 to +32 of the rat CYP7A promoter, containing two COUP-TFII-responsive elements, was kindly provided by Dr. J. Y. L. Chiang (Northeastern Ohio University, Rootstown, OH). pCK-2300-Luc, which expresses luciferase under the control of the PEPCK promoter, was a generous gift of Dr. N. P. Curthoys (Colorado State University, Fort Collins, CO). pSV40-β-Gal, which expresses β-galactosidase under the control of the simian virus 40 promoter, was purchased from Promega Corp. (Madison, WI). pGEX-4T3-GRα (1–777), -GRα (410–777), -GRα (485–777), -GRα (502–777), -GRβ (1–742), -GRβ (410–742), -GRβ (485–742), -GRβ (502–742), -GR (1–410), -GR (1–480), and -GR (410–490) were constructed by subcloning the corresponding DNA fragments of GRα or GRβ into pGEX-4T3 (Amersham Biosciences, Piscataway, NJ). pGEX-4T3-COUP-TFII (1–414), -COUP-TFII (1–376), -COUP-TFII (1–163), -COUP-TFII (163–414), -COUP-TFII (1–75), -COUP-TFII (75–163), and -COUP-TFII (75–414) were constructed by introducing the corresponding cDNA fragments of COUP-TFII into pGEX-4T3.

Yeast Two-Hybrid Screening and Mating Assays

Yeast two-hybrid screening was initially performed using a human Jurkat cell cDNA library with the LexA system and the GRβ LBD, which includes the hinge region of GR and the further C-terminal portion, as a bait. For a mating assay, the RFY206 yeast strain (CLONTECH) was transformed by each of the baits employed in pLexA and p8op-LacZ, whereas EGY48 was transformed with a prey plasmid containing a COUP-TFII cDNA fragment in pB42AD (CLONTECH). Two transformed yeast strains were cultured and mated on appropriate plates and their growth and expression of β-galactosidase were determined.

Cell Cultures and Transient Transfections

Human cervical carcinoma HeLa, African green monkey kidney CV-1 and COS7, rat hepatoma H4IIE, human hepatoma HepG2, and human colon carcinoma HCT116 cells were obtained from the American Type Culture Collection (Manassas, VA). CV-1 and COS7 cells do not express endogenous GRα and COUP-TFII, whereas in HE4IIE and HepG2 cells these molecules are endogenously expressed. HCT cells do not express GRα but have low levels of COUP-TFII, whereas HeLa cells express GRα but not COUP-TFII (data not shown). Cells except HCT116 were grown in DMEM supplemented with 100 U penicillin, 1 μg/ml streptomycin sulfate, 10% FBS, and 25 mM HEPES. HCT116 cells were cultured in McCoy's 5A medium, which receives the same additives. Approximately 24 h before transfection, 1.5–3 $\times$ 10^5 cells were seeded in six-well plates depending on the cell lines. Transfection was performed with LipofectAMINE 2000 or Lipofectin in Opti-MEM (63). The cells were cotransfected with 0.5–1.5 μg/well of pMMTV-Luc, p-416/+32-Luc or pCK-2300-Luc, 0.1–0.5 μg/well of pSV40-β-Gal, 0.1–2.0 μg/well of GR- and COUP-TFII-related plasmids, and/or pCMX-SMRT. Six hours after transfection, Opti-MEM was replaced with regular medium. After 24 h, 10^{-6} M DEX was added. After an additional 16 h, cells were lysed with a reporter lysis buffer (Promega Corp.), and luciferase and β-galactosidase activities were determined using a Monolight 2010 luminometer (BD PharMingen, San Diego, CA), as previously described (64). Luciferase activity was normalized for β-galactosidase activity to correct for transfection efficiency. Molpholino oligonucleotide antisense for the human COUP-TFII or its sense control was introduced into HepG2 cells with ethoxylated polyethylenimine following the manufacturer's recommendations.

GST Pull-Down Assays

In vitro translated and ^{35}S-labeled GRα,β and COUP-TFII were generated with the TNT reticulocyte lysate transcription/translation system (Promega) by using pBK/CMV-GRα, β and pCDNA3-COUP-TFII as templates, respectively. GST-fused GR- and COUP-TFII-related proteins were produced and immobilized on GST beads. They were then mixed in buffer A (50 mM Tris-HCl, pH 8.0; 50 mM NaCl; 0.1% Nonidet P-40; 1 mM EDTA; 10% glycerol; 1 mg/ml BSA) at 4 C for 2 h. The beads were washed four times with buffer A, and proteins were liberated in the SDS-PAGE sample buffer. Samples were loaded and separated on a 10% SDS-PAGE gel. Gels were fixed, dried, and exposed to x-ray films.

ChIP Assays

ChIP assay was performed in H4IIE rat hepatoma cells following the procedure supplied by the manufacturer with minor modifications. H4IIE rat hepatoma cells were seeded in 135-mm plates and were cultured in DMEM containing 10% charcoal/dextran-treated FBS for 24 h. Subsequently, they were exposed to either 10^{-6} M of DEX or vehicle for 16 h. The cells were then fixed, and DNA and bound proteins were cross-linked by treating them with 1% formaldehyde for 10 min, and nuclei were isolated as described previously (65). Amounts of DNA were measured in each sample and nuclei were stored in aliquots at −80 C until assay.

For the ChIP, 100 μg DNA-equivalent nuclei isolated from DEX- or vehicle-treated cells were diluted in 500 μl ChIP dilution buffer [16.7 mM Tris-HCl, pH 8.1; 167 mM NaCl; 0.01% sodium dodecyl sulfate (SDS); 1.1% Triton X-100; 1.2 mM EDTA] containing protease inhibitors and were subsequently sonicated by using a Misonix sonicator (Farmingdale, NY). Samples were then centrifuged in an Eppendorf 5415C Centrifuge (Brinkmann Instruments, Inc., Westbury, NY) at 13,000 rpm for 5 min. The supernatants were collected, and the amounts of DNA were measured as absorbance at 260 nm for the calibration of starting samples. Samples were then incubated overnight with anti-GRα, -GRβor -COUP-TFII antibodies, or control mouse serum at 4 C, and immune complexes were collected by adding Protein A agarose slurry for 1 h at 4 C. After vigorous washing with the ChIP dilution buffer, the complexes were incubated with the elution buffer (0.1 M $NaHCO_3$, 1% SDS), and cross-linked DNA and bound proteins were uncoupled by heating the samples at 65 C for 4 h. DNA was precipitated after adding transfer RNA (25 μg/100 μl) as a carrier.

The promoter region −204 to +20 of the rat CYP7A gene, which contains two COUP-TFII-responsive elements, was amplified from the prepared DNA samples by the Taq DNA polymerase (Applied Biosystems, Foster City, CA) using a primer pair: 5′-CTTCAGCTTATCGAGTATTG-3′ and 5′-CTAGCAAAGCAAGGCTGTC-3′ in GeneAmp PCR, System 9600 (Applied Biosystems). Tandem endogenous GREs of the rat TAT promoter, which are located approximately 2500 bp upstream from its transcription initiation site (47), were amplified by a primer pair: 5′-TCTTCTCAGTGTTCTCTATCAC-3′ and 5′-CAGAAACCGACAGGCGACTACG-3′ (fragment size, 220 bp). The rat arginase promoter fragment (−133-+78) is amplified as a negative control by using a primer pair 5′-CAGGGAAGAGCAACGGGTATTC-3′ and 5′-CTGCTGCTGCTGCTGCTGCATC-3′. Thirty PCR cycles (denaturing, 1 min at 94 C; annealing, 1 min at 50 C; and elongation, 1 min at 72 C) were performed to amplify the DNA. Amplified products were then run on a 2% agarose gel, and visualized DNA bands were photographed.

Coimmunoprecipitation Assays

HepG2 cells or COS7 cells (2 $\times$ 10^6) were plated in 75-cm^2 flasks. COS7 cells were transfected with 7 μg pCDNA3-COUP-TFII and 7 μg pRShGRα WT or Q502A. Cells were

then treated with 10^{-6} M DEX or vehicle for 5 h. Cell lysis and coimmunoprecipitation were carried out by using lysis buffer (50 mM Tris-HCl, pH 7.4; 250 mM NaCl; 0.2% Nonidet P-40; Complete tablets, 1 tablet/50 ml). Proteins were precipitated by antihuman (h)GRα or -hGRβ antibodies, or control serum bound to protein A/G agarose. After blotting on nitrocellulose membranes, coprecipitated COUP-TFII, hGRα, and hGRβ were detected by anti-COUP-TFII, -hGRα, or -hGRβ antibodies. To evaluate endogenous or artificially expressed GRs and COUP-TFII, 10% of cell lysates, which were used in the actual coimmunoprecipitation reaction, were run on SDS-PAGE gels. After blotting on nitrocellulose membranes, the proteins were visualized by using specific antibodies.

The Quantitative Real-Time PCR for the Evaluation of the PEPCK and COUP-TFII mRNA Levels

HepG2 cells (1×10^6) were plated on 100-mm dishes and were treated with morpholino oligonucleotide antisense for human COUP-TFII or its sense control after 24 h. The cells were subsequently exposed to 10^{-6} M DEX or vehicle. After 48 h incubation, the cells were lysed with TRIZOL (Life Technologies, Inc.) and total RNA was isolated according to the instructions of the manufacturer. The reverse transcription reaction was carried out using the random hexamers with TaqMan Reverse Transcription Reagents (Applied Biosystems). To detect mRNA levels of PEPCK, COUP-TFII, arginase, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), primer pairs (PEPCK: forward primer, 5′-CATAAAGGCAAAATCATCATGCA-3′; reverse primer, 5′-TTGCCGAAGTTGTAGCCAAA-3′; COUP-TFII: forward primer, 5′-AGTCGAGCGGCAAGCACTAC-3′; reverse primer, 5′-CACGCTGCGCTTGAAGAAG-3′; arginase: forward primer, 5′-AAAGAACAAGAGTGTGATGTG-3′; reverse primer, 5′-TCTTCCGTTCTTCTTGACTTCTGC-3′; GAPDH: forward primer, 5′-CCACCCATGGCAAATTCC-3′; reverse primer, 5′-TGGGATTTCCATTGATGACAAG-3′) were employed. The real-time PCR reaction, consisting of the heat activation of the Taq polymerase (for 10 min at 95 C) and the subsequent 60 PCR cycles (denaturing, 15 sec at 95 C; annealing/extension, 1 min at 60 C) was performed in quadruplicate using the SYBR Green PCR Master Mix (Applied Biosystems) in an ABI PRIZM 7700 SDS lightcycler (Applied Biosystems). Obtained threshold cycle values of PEPCK and COUP-TFII were normalized for those of GAPDH, and their relative mRNA expressions were demonstrated as fold induction to the baseline. The dissociation curves of used primer pairs showed a single peak, and samples after PCR reactions had a single expected DNA band in an agarose gel analysis (data not shown).

Statistical Analyses

Statistical analysis was carried out by ANOVA, followed by Student's *t* test with Bonferroni correction for multiple comparisons. All transfection experiments were repeated at least three times, and representative results are shown in the figures.

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