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Molecular Mechanisms of COUP-TF-Mediated Transcriptional Repression: Evidence for Transrepression and Active Repression

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COUP-TF, an orphan member of the nuclear receptor superfamily, has been proposed to play a key role in regulating organogenesis, neurogenesis, and cellular differentiation during embryonic development. Since heterodimerization is a common theme within the nuclear receptor superfamily and has been demonstrated to modulate transcriptional properties of heterodimeric partners via allosteric interactions, we have devised a strategy to examine the silencing function of COUP-TF in a heterodimeric context. We find that the intrinsic active repression function of COUP-TF is not affected by heterodimerization. Moreover, COUP-TF can transrepress the ligand-dependent activation of its heterodimeric partners without its own DNA binding site. Using receptor deletion mutants in transfection assays, we show that the region necessary for COUP-TF silencing function is not sufficient for its transrepression activity. Furthermore, our studies indicate that in addition to its active repression function, COUP-TF can repress several different types of activator-dependent transactivation. However, this active repression function of COUP-TF may be differentially regulated by some other activator(s). These studies provide new insights into the molecular mechanism(s) of COUP-TF-mediated repression.

The regulation of gene expression in eukaryotes involves repression as well as activation of transcription (for reviews, see references 8, 31, and 57). A large part of gene regulation, either positive or negative, is governed by sequence-specific transcription factors which bind to *cis*-acting elements located within the promoter regions of responsive genes. Repression of gene expression can be mediated by several different molecular mechanisms (Fig. 1). Perhaps the simplest mechanism involves competition for a common DNA-binding site, whereby a repressor can exclude the binding of an activator by virtue of recognizing the same, overlapping, or adjacent binding sites. This is a passive type of repression. Active repression (silencing) of basal (Fig. 1a) or activated (Fig. 1b) transcription probably occurs by interfering with the formation of a functional preinitiation complex via protein-protein interactions. In such cases, a repressor may also compete with an activator for a limiting common coregulator(s) or general transcription factor(s) and deplete these factors. A second mechanism, called quenching (Fig. 1c) or masking, involves co-occupation of DNA by both the repressor and the activator. However, the repressor function is dominant over the activator function via an interaction which sterically hinders the activation domain of the latter. Often a transcriptional repressor is capable of employing more than one distinct mechanism to inhibit gene expression (31, 51). Many members of the nuclear receptor superfamily have been found to be involved in transcriptional repression by using one or several of these mechanisms (for reviews, see references 39, 51, 61, and 62).

The nuclear receptor superfamily comprises a large group of ligand-dependent transcription factors which control the expression of target genes by binding to their cognate response elements (for reviews, see references 5, 18, 25, and 62). They respond to endocrine, paracrine, autocrine, and possibly intracrine signals to modulate a variety of aspects of development, differentiation, and homeostasis. In addition to the classical receptors, a large number of genes in this superfamily have been cloned through their sequence conservation (for reviews, see references 15, 39, and 46). Since the ligands for this group of receptors have yet to be discovered, they are classified as orphan receptors. One of the most studied of the orphan receptors is COUP-TF. It belongs to the thyroid hormone/retinoic acid (RA) receptor subfamily and has been implicated in neurogenesis, organogenesis, and cell fate determination (for reviews, see references 47 and 50). COUP-TF can form strong homodimers and bind to a wide spectrum of response elements with various arrays of the purine GGTCA core motif (17), allowing COUP-TF to bind to a variety of hormone response elements recognized by other members of the subfamily, including receptors for RA (RAR), 9-*cis* RA (RXR), thyroid hormone (TR), and vitamin D_3; the fatty acid/peroxisome proliferator-activated receptor; and hepatocyte nuclear factor 4. An important consequence of this promiscuous DNA binding is the inhibition of transcriptional activities of TR, RAR, RXR, the vitamin D_3 receptor, the fatty acid/peroxisome proliferator-activated receptor, and hepatocyte nuclear factor 4 on both artificial and native response elements (reference 50 and references therein). In addition, COUP-TF has been found to be capable of actively repressing the basal promoter activity of several target genes (reference 50 and references therein).

Transcriptional repression, mediated by COUP-TF, has been studied in some detail (16, 17, 34, 59). Initial experiments provided evidence that COUP-TF is a response element-dependent repressor and that the putative active repression domain is located within the ligand-binding domain (LBD). COUP-TF acts via several different mechanisms to inhibit target gene transcription, including competition with other nuclear receptors for the occupancy of DNA binding sites, active

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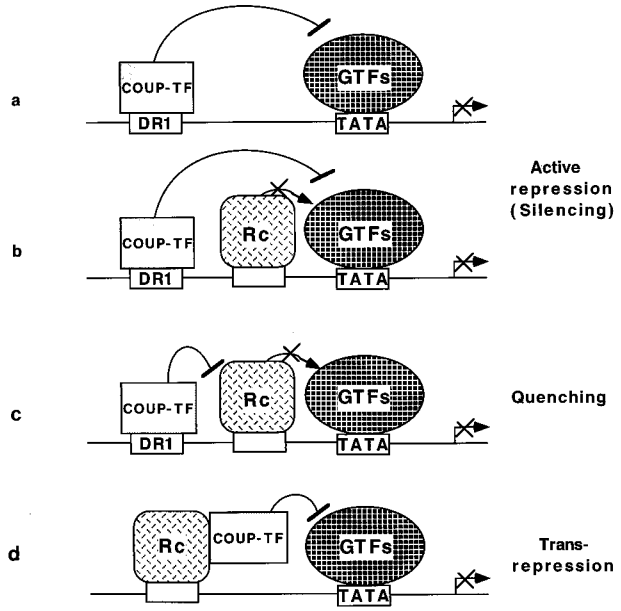

FIG. 1. Mechanisms of transcriptional repression. (a) Active repression of basal transcription; (b) active repression of transactivator-dependent transcription; (c) quenching of transactivator-dependent transcription; (d) transrepression of activated transcription. GTFs, general transcription factors; Rc, activated receptor.

repression of basal level transcription, and titration of the common coregulator, RXR (for a review, see reference 12).

Although the studies cited above help us to understand certain aspects of COUP-TF-mediated repression, a few questions remain unanswered. First, accumulating evidence indicates that heterodimerization is a common paradigm among members of the thyroid hormone/RA receptor subfamily. Detailed analysis has shown that heterodimerization produces novel complexes that bind DNA with altered affinity and thus altered transcriptional properties (reference 21 and references therein). More strikingly, heterodimerization between receptor LBDs can serve as a novel mechanism for allosteric modulation and can confer transcriptional suppression upon a heterodimeric complex (20), as highlighted by the finding that unliganded TR and RAR can act in *trans* to suppress the transcriptional activity of RXR. Such suppression is further modulated by the ligands of TR and RAR (20, 35). Like RXR, COUP-TF can also dimerize with TR and RAR and also with RXR on DNA (6, 10, 16, 34). Experiments performed by Casanova et al. indicate that at least for TR and COUP-TF, heterodimerization can occur in vivo (10) via their LBDs. However, it is not clear whether such a heterodimerization will modulate the active repression function of COUP-TF. Second, although COUP-TF has been shown to actively repress basal promoter activity, the mechanism(s) of this inhibition is largely unclear. The ability to reconstitute nuclear receptor-dependent active repression in vitro with biochemically defined components has enabled searches for partners that interact with these specific repressors (2, 19, 58). Since COUP-TF has been shown to interact with TFIIB in vitro (29, 63), it is proposed that COUP-TF can freeze TFIIB in an inactive conformation via protein-protein interaction and block basal transcription (2, 19). On the other hand, TFIIB, TFIID, and other general transcription factors have been implicated as targets in several different types of activator-dependent transcription. Furthermore, different factors are required to mediate the effects of different transcriptional activators (for a review, see reference 57). Thus, it is important to examine if COUP-TF can also repress activator-dependent transcription and to determine whether COUP-TF has the ability to distinguish between different transcription factors and their mechanisms of transactivation.

In this report, we present data showing that heterodimerization between the LBDs of RXR, RAR, or TR and COUP-TF does not abolish the active repression function of COUP-TF, either in the presence or in the absence of their cognate ligands. By using Gal4-receptor chimeras in transfection assays, we demonstrate that COUP-TF can inhibit transcription in a response element- and DNA-binding domain (DBD)-independent manner once tethered to a promoter via dimerization. This DBD-independent repression is called transrepression (Fig. 1d). We also show, using COUP-TF deletion mutants, that the region required for COUP-TF active repression is not sufficient for transrepression. Furthermore, our studies indicate that in addition to its active repression function, COUP-TF can also repress transactivator-dependent transcription. However, its ability to antagonize transactivator-dependent transcription is dependent on the type of transactivator used. These studies provide new insights into the nature of COUP-TF-mediated repression of target genes and have implications for the physiological roles of COUP-TFs in vivo.

MATERIALS AND METHODS

Cell culture and transfection. L cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U of penicillin per ml, and 100 mg of streptomycin per ml. Cells were plated at 1.3 million/100-mm-diameter dish 24 h prior to transfection. Usually, 15 mg of total DNA including 5 mg of reporter and 0.1 to 10 mg of expression vector was used per 100-mm-diameter dish. A total of 10^{-7} M T3, 10^{-7} M 9-*cis* RA, or 10^{-6} M all-*trans* RA was added after glycerol shock. Transfections were carried out as described previously (38). Cells were harvested for the chloramphenicol acetyltransferase (CAT) assay after incubation for 38 to 44 h with or without the indicated ligand(s). The CAT activities were determined by either thin-layer chromatography (23) or phase extraction (55) assay. All transfections were performed in at least three independent experiments with duplicated samples.

Oligonucleotides and plasmids. Sequences of the oligonucleotides used are as follows: MT696, 5′ ACGTCGACTCAGCCGAGTAC 3′; MT697, 5′ ATTCTA GACTAGGGGGTTTTACCTACCAAAC 3′; MT699, 5′ ATTCTAGACTAG AGCTGCTCGATGACGGAGGA 3′; MT750, 5′ ATTCTAGACTAATGAGA GTTTCGATGGGGGT 3′; MT751, 5′ ATTCTAGACTAAGTAACATATCGC GGATGAG 3′; DR1H1, 5′ TGTCTTAGAGGTCAAAGGTCAAAT 3′; and DR2H2, 5′ GACAATTTGACCTTTGACCTCTAA 3′.

Expression plasmids for full-length human COUP-TFI (pRSV-hCOUP-TFI), human RARα (pRSV-hRARα), human TRβ (pRSV-hTRβ) (17), and mouse RXRβ (pRSV-mRXRβ) (37) have been previously described. Vectors expressing Gal4-mouse RXRβ (pABGal147-mRXRβ) (38), Gal4-VP16 (pABGal94-VP16) (66), Gal4 DBD (pABGal94 or pABGal147), Gal4-human TRβ (pAB Gal94-hTRβ) (4), Gal4-human RARα (pABGal147-hRARα) (3), and Gal4–human COUP-TFI (pABGal147-hCOUP-TFI) (17) have also been described previously. The expression plasmids for mutants of Gal4–COUP-TF chimeras and COUP-TF were constructed in two steps. First, the nucleotide sequences of human COUP-TFI corresponding to amino acids 314 to 408 (MT696 and MT751), 314 to 403 (MT696 and MT750), 314 to 398 (MT696 and MT697), and 314 to 388 (MT696 and MT699) were generated by PCR, and the *Sal*I-*Xba*I fragments were then subcloned into *Sal*I-*Xba*I sites of pT7bSal-tCOUP-TF1 (17). To create the expression vectors pABGal4-COUP-TFD15, pABGal4-COUP-TFD25, and pABGal4-COUP-TFD35, *Sal*I-*Bam*HI fragments of the corresponding pT7bSal constructs were subcloned into *Sal*I-*Bam*HI sites of pAB Gal147-hCOUP-TFI. Likewise, the expression plasmids pRSV-COUP-TFD15, pRSV-COUP-TFD20, pRSV-COUP-TFD25, and pRSV-COUP-TFD35 were generated by inserting the *Sal*I-*Bam*HI fragments of the cognate pT7bSal constructs into pRSV-hCOUP-TFI. To construct the expression plasmids for Gal4-region II (RII) fusion protein, the *Kas*I-*Hin*dIII fragment containing amino acids 768 to 881 was isolated from YEPGal4 (17) and inserted into the *Xma*I-*Hin*dIII site of pABGal94. The expression plasmids Gal4-ftzQ, Gal4-ZenST, and Gal4-CTF1P were generated by isolating the *Xho*I-*Bgl*II, *Xho*I-*Eco*RV, and *Xho*I-*Bgl*II fragments from the cognate pACT-Gal4 plasmids (26) and inserted into the *Xho*I-*Bam*HI, *Xho*I-*Eco*47III, and *Xho*I-*Bam*HI sites of pABGal94, respectively. The junctions of all expression plasmids were verified by sequencing. The constructs 17mer tkCAT(DH/N), (17mer)x2 tkCAT(DH/N), DR5 tkCAT, and (DR1)x2 tkCAT were described previously (16, 17, 40). To generate (DR1)x3

(17mer)x2 tkCAT and (17mer)x2 tkCAT (DR1)x4, concatenated DR1 response elements (DR1H1-DR1H2) were subcloned into *Hin*dIII and *Sma*I sites, respectively, of (17mer)x2 tkCAT(DH/N). If necessary, overhangs were blunt ended with Klenow enzyme.

RESULTS

COUP-TF represses nuclear receptor-mediated transcription in *trans*. Since the in vivo heterodimerization between COUP-TF and other members of the subfamily, such as TR, can take place without stabilization by DNA response elements (10), we examined the active repression function of COUP-TF in the context of heterodimer formation. To avoid the complication due to the intrinsic active repression function of the TR and RAR LBDs (3), we utilized Gal4-RXR as the heterodimeric partner for COUP-TF. The Gal4-RXR chimera binds to its response element (17-mer) as a dimer through a dimerization domain in the Gal4 DBD (9), thus eliminating the problem of competition for DNA-binding sites by COUP-TF.

The expression vectors for Gal4-RXR and COUP-TF were cotransfected into L cells together with a CAT reporter containing a single Gal4-binding site upstream of the *tk* promoter. As expected, since COUP-TF does not bind to this reporter construct by itself, it has little effect on the basal promoter activity when cotransfected with the Gal4 DBD alone (Fig. 2A; compare lanes 1 and 2 with lanes 3 to 6). There is only a one- to twofold repression. However, a clear dose-dependent repression was observed after cotransfection with Gal4-RXR (Fig. 2A; compare lanes 7 and 8 [5- and 20-fold, respectively] with lanes 9 to 12). This result suggests that COUP-TF heterodimerizes with the RXR LBD of Gal4-RXR in vivo without stabilization from the DNA response element, and such an interaction appears not to interfere with the active repression function of COUP-TF; i.e., COUP-TF active repression is dominant in a heterodimeric context. The observed repression was not due to the squelching of general transcription factors by COUP-TF but rather was due to the active repression function, since COUP-TF had little effect on the basal promoter activity when cotransfected with the Gal4 DBD alone. In addition, the observed repression was not due to the inhibition of Gal4-RXR binding to its response element by forming a non-DNA-binding COUP-TF/Gal4-RXR heterodimer. If this were the case, we would expect the RXR activity in the presence of COUP-TF to be the same as that in the absence of a Gal4-RXR expression vector. Since COUP-TF represses the reporter activity below the basal activity (Fig. 2A; compare lanes 9 to 12 with lanes 3 to 6), it must inhibit the reporter gene by being tethered to the RXR LBD bound to the Gal4 response element. The tethering of COUP-TF and repression of transactivation exemplify a novel mechanism of repression which we term transrepression. Our results are consistent with previous observations that nuclear receptors, such as TR and RAR, remain tethered to the Gal4-RXR chimeras in vitro and in cells without stabilization by DNA binding (20, 32, 45, 49).

To investigate whether tethered COUP-TF can also transrepress the activation of various Gal4-receptor fusions in response to their specific ligands, COUP-TF was cotransfected with the Gal4-receptor chimeras. As shown in Fig. 2B, COUP-TF can suppress ligand-dependent transactivation of Gal4-TR (lanes 3 to 6) (14-fold), Gal4-RAR (lanes 7 to 10) (11-fold), and Gal4-RXR (lanes 11 to 14) (7-fold). Thus, ligand binding does not abolish the interaction between COUP-TF and the Gal-receptor chimeras. COUP-TF tethered to the TR, RAR, or RXR LBD is sufficient for the transrepression function. In addition, our results suggest that COUP-TF is able to inhibit activator-mediated transcription in addition to basal activity.

To further substantiate that the observed inhibition is mediated via receptor LBDs, we created a Gal4–COUP-TF chimera by fusing the COUP-TF LBD to the Gal4 DBD. Cotransfection experiments were then performed with Gal4–COUP-TF and wild-type RAR and RXR together with their target constructs. As shown in Fig. 2C, Gal4–COUP-TF was able to antagonize RAR- and RXR-mediated activation of CAT reporters containing RAR (DR5) and RXR (DR1) response elements, respectively. These data confirm that a receptor can be tethered to DNA in the absence of its cognate response element via LBD-LBD interactions with other nuclear receptors. In our case, the tethered COUP-TF LBD can repress the wild-type nuclear receptor activation in *trans*. Since nuclear receptors work as homo- or heterodimers, it is not clear whether Gal4–COUP-TF interacts with nuclear receptors in either their monomer or homo- or heterodimer forms.

Definition of the sequences required for the active repression and transrepression functions of COUP-TF. Taken together, our results indicate that tethered COUP-TF can act in *trans* to suppress basal and activated transcription. Since COUP-TF can also confer direct active repression of basal promoter transcription upon binding to its response element, we sought to examine if the active repression function of COUP-TF explains its transrepression activity. As shown in Fig. 3A, two sets of corresponding COUP-TF mutants were used to provide experimental evidence as to whether the region of COUP-TF transrepression function overlaps that of its active repression function. Various fragments of the COUP-TF C terminus were fused to the Gal4 DBD to generate various expression vectors for C-terminal COUP-TF deletion mutants. The Gal4 DBD contains dimerization and nuclear localization functions, thus eliminating interpretation problems with COUP-TF mutations that affect these functions. The Gal4–COUP-TF deletion mutants were transfected into L cells together with the 17mer tkCAT reporter. As demonstrated previously (16), fusion of the complete C terminus of COUP-TF to the Gal4 DBD results in a potent repressor (Fig. 3B). A 15-amino-acid deletion from the C terminus (Gal4–COUP-TFΔ15) had little effect on Gal4–COUP-TF activity, whereas a 25-amino-acid (Gal4–COUP-TFΔ25) or a 35-amino-acid (Gal4–COUP-TFΔ35) deletion largely impaired its active repression function (Fig. 3B; compare lanes 3 to 6 with lanes 7 to 10). Therefore, we suggest that the C-terminal border of the major active repression function of COUP-TF is located between amino acids 398 and 408. Our results are consistent with those of previous studies in which removal of a similar region in other receptors, i.e., TR and RAR, destroyed the active repression function of these receptors (3).

Using the C-terminal deletion mutants of COUP-TF (Fig. 3A), we then tested the transrepression function of COUP-TF in the heterodimeric context (as in Fig. 2). Interestingly, a 15-amino-acid deletion (COUP-TFΔ15) almost completely abolished the transrepression activity of COUP-TF (Fig. 3C; compare lanes 9 and 10 with lanes 11 and 12). No transrepression activity was observed even when fourfold more COUP-TFΔ15 DNA was transfected (data not shown). This observation was intriguing since the putative heterodimerization domain, which includes nine heptad repeats and the predicted 10 helical structures of the dimerization interface identified in the RXR LBD crystal structure (7), is intact in this mutant. Therefore, sequences other than those important for dimerization are required for transrepression. It is possible, however, that the deletion diminished or destabilized the heterodimerization or interaction between COUP-TF and Gal4-receptor

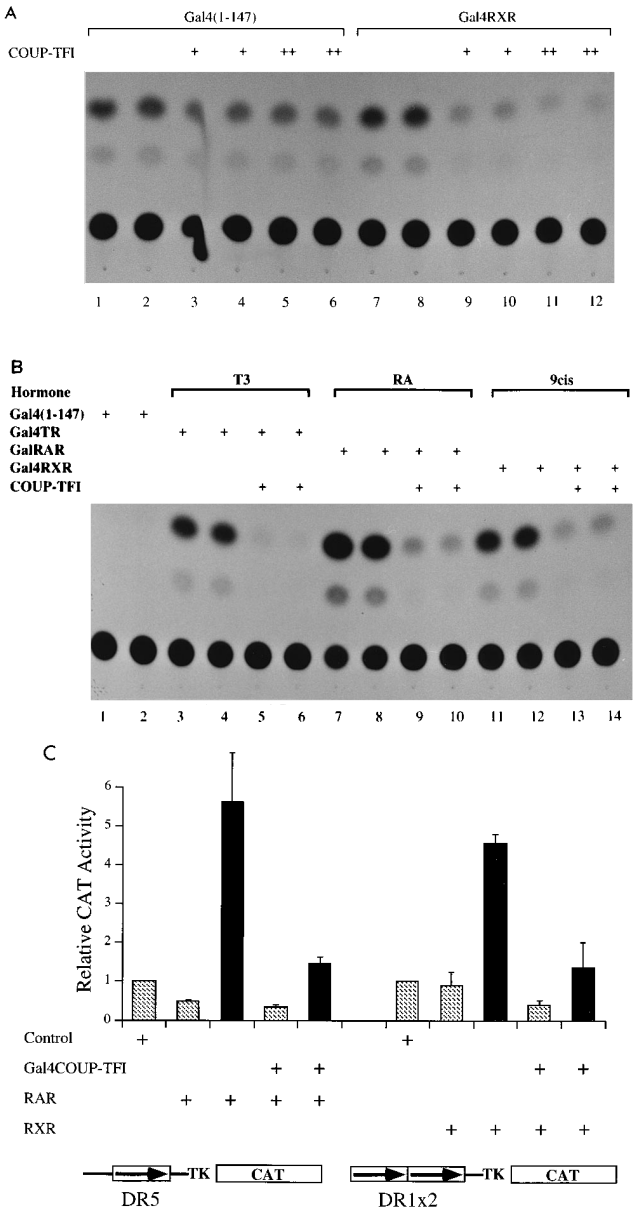

FIG. 2. COUP-TF can transrepress basal transcription as well as ligand-dependent transactivation by members of the thyroid hormone/RA subfamily. (A) Down-regulation of the basal promoter activity by COUP-TF is mediated via interaction with Gal4-RXR. Transfections were performed in L cells with a total of 15 μg of DNA including 5 μg of 17mer tkCAT, 1 μg of pRSVGal4-mRXRβ (or pABGal4), and indicated amounts of pRSVhCOUP-TFI (2 μg for each +). The amount and type of DNA added to each transfection were balanced with empty expression vectors. After glycerol shock, the cells were incubated for 38 to 44 h. Cell extracts were subsequently prepared and assayed for protein concentration and CAT activity. (B) Analysis of COUP-TF transrepression function via Gal4-TR, -RAR, and -RXR chimeras in the presence of their cognate ligands (10^{-7} M T3 in lanes 3 to 6, 10^{-6} M all-*trans* RA in lanes 7 to 10, and 10^{-7} M 9-*cis* RA in lanes 11 to 14). Conditions were as for panel A except that 0.2 μg of pABGal4-hTRβ or pABGal4-hRARα, 1 μg of pRSVGal4-mRXRβ, and 2 μg of pRSVhCOUP-TFI were used as indicated. Hormones or carriers were added after glycerol shock as indicated. (C) Gal4–COUP-TFI antagonizes RAR- or RXR-mediated activation via transrepression. Transfection assays were performed as for panels A and B. Expression vectors for human RARα (1 μg), mouse RXRβ (2 μg), and/or Gal4–human COUP-TFI (6 μg) were cotransfected into L cells with 5 μg of the indicated reporter [DR5 tkCAT or (DR1)x2 tkCAT]. Relative CAT activities were normalized to the control (empty pRSV expression vector plus pABGal4). The hatched bars and solid bars represent the absence and presence of hormones (10^{-6} M all-*trans* RA in the left panel and 10^{-7} M 9-*cis* RA in the right panel), respectively. Error bars indicate standard deviations of three independent transfections done in duplicate.

chimeras, thus resulting in the loss of transrepression. Therefore, these findings suggest that the active repression domain of COUP-TF is not sufficient for transrepression.

COUP-TF represses activator-dependent transactivation. The results presented above revealed that COUP-TF not only has the ability to actively repress basal promoter activity but also has the ability to inhibit nuclear receptor-activated transcription. To substantiate that COUP-TF can actively suppress activator-dependent transcription, we investigated the ability of COUP-TF to repress transactivation by different classes of transcription factors. First, the transactivator Gal4-RII or Gal4-CTF1P was cotransfected with COUP-TF into L cells together with a CAT reporter containing three copies of the COUP-TF response element (DR1) and two copies of the Gal4-activator binding site (17-mer) upstream of the *tk* promoter. Gal4-RII contains the N-terminal 94-amino-acid DBD of Gal4 and the C-terminal 113-amino-acid activation domain (RII) of the yeast transcription factor GAL4. The RII region, which consists of an acidic activation domain, interacts with TBP and the putative coactivators SUG1 and ADA2 (44). Gal4-CTF1P is a fusion protein of the Gal4 DBD and the proline-rich activation domain (amino acids 399 to 499) of CTF1 (CCAAT-box-binding transcription factor 1). It has been demonstrated that the Gal4-CTF1P interacts directly with TFIIB and facilitates TFIIB recruitment during preinitiation complex assembly in both human and yeast systems, thus resulting in transcriptional activation (33). As expected, COUP-TF was able to repress both Gal4-RII- and Gal4-CTF1P-dependent transcription in a dose-dependent manner (Fig. 4A). In fact, COUP-TF-mediated repression can abolish nearly all activator-dependent transactivation (Fig. 4A; compare lanes 1 and 2, and 7 and 8, or 13 and 14).

Next, similar cotransfection experiments were performed with Gal4-ftzQ and Gal4-ZenST. Gal4-ftzQ consists of the Gal4 DBD fused to the last 86 amino acids of the *Drosophila* homeodomain protein Fushi tarazu (ftz), a region shown previously to function as a powerful activation domain. This Gln-rich region has been demonstrated to directly interact with a potential zinc finger structure at the N terminus of TFIIB in vitro, and such an interaction is important for transactivation in vivo (14). Gal4-ZenST consists of the Ser/Thr-rich activation domain of the *Drosophila* zen1 protein and the Gal4 DBD. It has yet to be determined which component(s) of the transcriptional machinery interacts with ZenST. Again, COUP-TF was able to nullify Gal4-ftzQ- and Gal4-ZenST-mediated transactivation when cotransfected into cells (Fig. 4B). Taken together, these results indicate that COUP-TF can indeed function as an active repressor and that it is likely to inhibit transcription of a wide spectrum of genes regulated by different activators.

An alternative explanation for these observations could be that once COUP-TF and an activator both occupy the DNA, COUP-TF masks (quenches) the activation domain of these activators (Fig. 1c). To investigate such a possibility, we reconstructed a CAT reporter with the COUP-TF-binding sites inserted downstream of the CAT gene (Fig. 4C). The rationale is that a quenching factor mediates short-distance (less than 100 bp) repression and its function is distance sensitive (24, 42). Once the binding sites for the activator and the quenching factor are moved further away, the repression activity is greatly diminished. For an active repressor, however, the location of its DNA-binding site will have little effect on its activity. In cotransfection experiments with different types of reporter genes, similar levels of repression on Gal4-RII dependent transactivation were observed (Fig. 4C). These data suggest

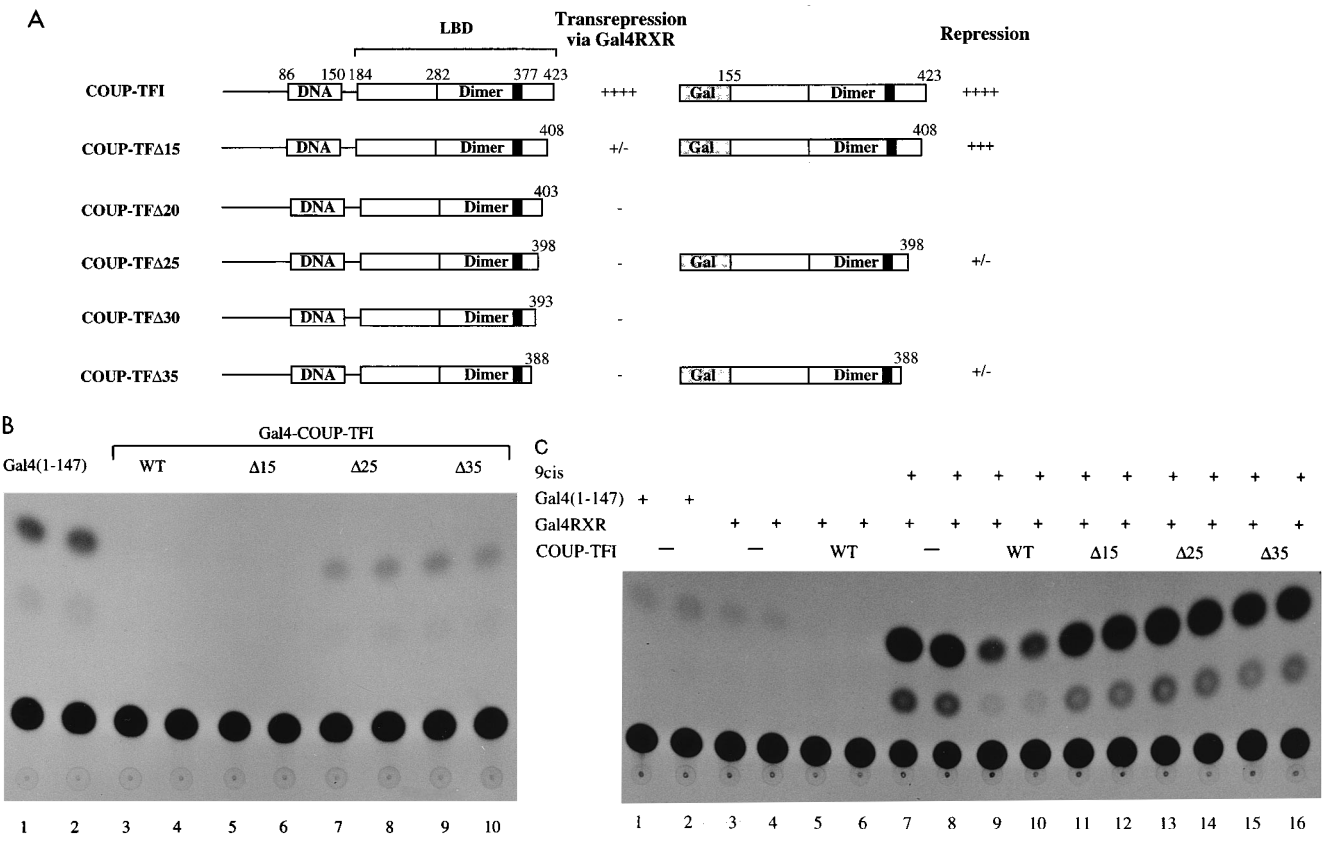

FIG. 3. COUP-TF active repression domain is not adequate for transrepression. (A) Schematic representation of various deletion mutants of COUP-TF and Gal4–COUP-TF fusions. With reference to other nuclear hormone receptors, the sequences from positions 1 to 85, 86 to 149, 150 to 183, and 184 to 423 are designated domains A/B, C, D, and E/F, respectively. Plus signs indicate that the protein retained the transrepression function (left panel) and the active repression function (right panel). The shaded region indicates the ninth heptad repeat ending at amino acid 377. The names of the expression plasmids were derived from those of the deletion mutants. (B) The silencing function of the COUP-TF LBD was greatly diminished when 25 or 35 amino acids but not 15 amino acids were deleted from the C-terminal end. The reporter 17mer tkCAT (5 μg) was cotransfected into L cells with wild-type (WT) Gal4–COUP-TF or various deletion mutants (4 μg of each). (C) The transrepression function of COUP-TF was abolished with a 15-amino-acid deletion from its C terminus. Cotransfection assays were performed under conditions similar to those described for Fig. 2 except that 2 μg of each indicated COUP-TF mutant was used.

that the observed repression is not due to quenching but rather is due to the active repression function of COUP-TF.

Repression of COUP-TF is differentially regulated. It is interesting that COUP-TF can inhibit transactivation by different types of activators, including acidic, proline-rich, Ser/Thr-rich, and glutamine-rich activators. Next, we examined whether COUP-TF can also repress Gal4-VP16-dependent transactivation. Gal4-VP16 contains the Gal4 DBD and the C-terminal acidic transactivation domain of the herpes simplex virus VP16 protein, the strongest activator known to date (60). Unlike the activators described above, VP16 can interact with multiple members of the general transcriptional machinery, including TFIIB, TATA-binding protein (TBP), TFIIH, and TAF40 (see Discussion). Cotransfection experiments were then performed with Gal4-VP16 and COUP-TF. As shown in Fig. 5, although 100-fold more COUP-TF expression vector was transfected, COUP-TF could not repress Gal4-VP16-dependent activation regardless of the location of COUP-TF-binding sites. These findings were in sharp contrast with what is shown in Fig. 4. Thus, the repression function of COUP-TF is unlikely to be universal and is dependent on the context of different transactivators.

DISCUSSION

The C-terminal LBD of nuclear receptors is a complex multiple functional module containing ligand-binding, dimerization, transcriptional repression, and activation functions. The LBD of COUP-TF, an orphan receptor whose ligand has yet to be identified, has been demonstrated to be involved only in dimerization and inhibition of basal promoter activity. The data described above indicate that the COUP-TF LBD can also function as a transrepressor as well as an active repressor. It is therefore conceivable that a major physiological role of COUP-TF is to down-regulate target gene transcription.

Heterodimeric interaction between COUP-TF and TR, RAR, and RXR facilitates COUP-TF-mediated transrepression. Several recent studies using gel mobility shift and cotransfection assays have demonstrated that COUP-TF can form heterodimers with TR, RAR, and RXR both in vitro and in vivo (6, 10, 16, 34). These studies suggest that although the observed heterodimer formation between COUP-TF and other receptors is rather weak in vitro, the in vivo interaction may be more significant (10). Our results confirm this conclusion and demonstrate that such an interaction can be mediated through the LBDs alone (Fig. 2). Our data show that in transfected

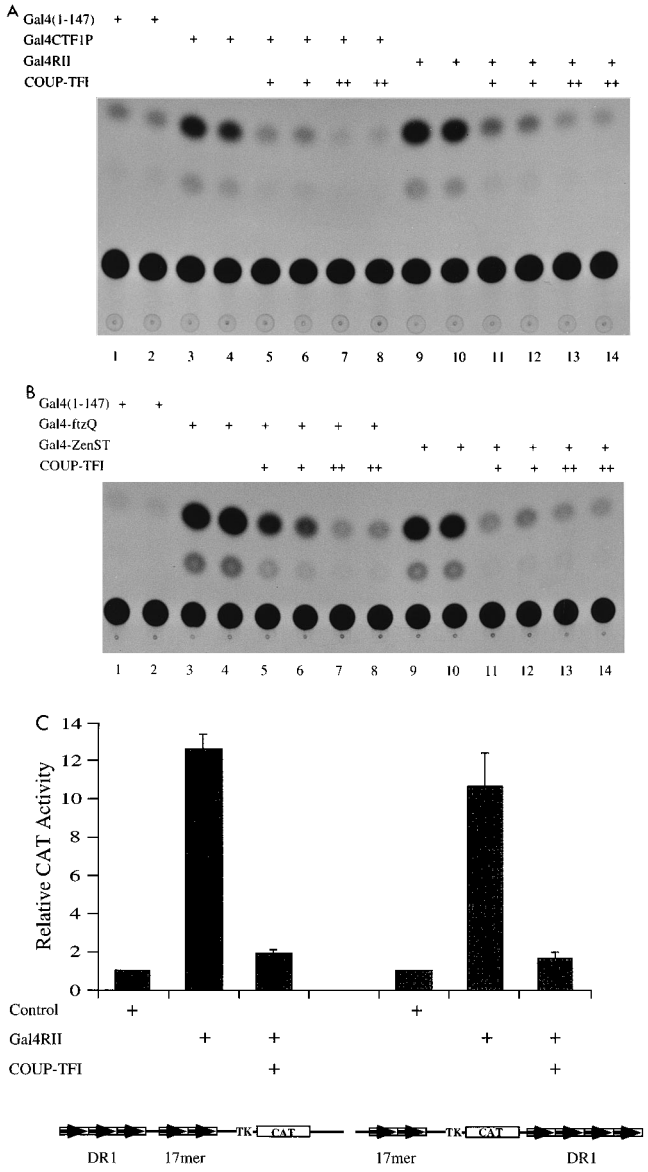

FIG. 4. COUP-TF can down-regulate activator-dependent transcription. (A) Analysis of the active repression of COUP-TF on acidic and proline-rich transactivator-mediated transactivation. The reporter (DR1)x3 (17mer)x2 tkCAT (5 μg) was cotransfected with pRSVGal4-RII (4 μg) or pRSVGal4-CTF1P (4 μg) and/or 2 μg of pRSVhCOUP-TFI. (B) Analysis of the active repression of COUP-TF on glutamine-rich and Ser/Thr-rich activator-dependent transactivation. Conditions were as for panel A except that 1 μg of Gal4-ftzQ and 1 μg of Gal4-ZenST were used. (C) The active repression function of COUP-TF is not affected by the position of the COUP-TF response element. Cotransfection was done as described for panel A except that both (DR1)x3 (17mer)x2 tkCAT and (17mer)x2 tkCAT (DR1)x4 reporters were tested. CAT activities were normalized to the control (empty pRSV expression vector plus pABGal4). Error bars indicate standard deviations of three independent transfections done in duplicate.

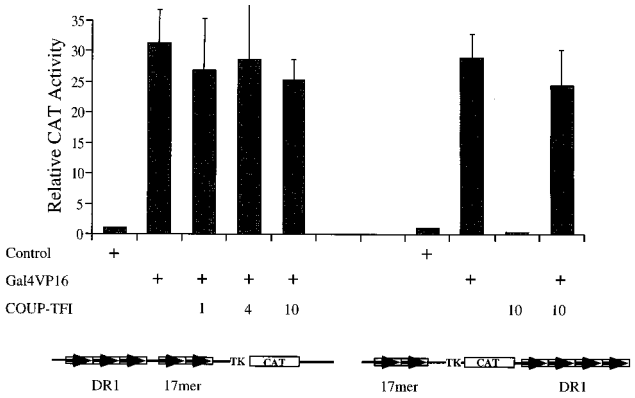

FIG. 5. COUP-TF cannot repress Gal4-VP16-mediated transactivation. Gal4-VP16 (0.1 μg) and indicated amounts (in micrograms) of COUP-TF were cotransfected with 5 μg of either (DR1)x3 (17mer)x2 tkCAT or (17mer)x2 tkCAT (DR1)x4 reporter into L cells. CAT activities were normalized to the control (empty pRSV expression vector plus pABGal4). Error bars indicate standard deviations of three independent transfections done in duplicate.

cells, heterodimeric interaction occurs between the DNA-bound Gal4-receptor chimeras and COUP-TF, for which there is no binding site on the promoter of the reporter gene. Reciprocally, similar heterodimeric interactions can occur between DNA-bound wild-type TR, RAR, or RXR and Gal4–COUP-TF(LBD) in solution. As suggested by Fig. 2A, once a Gal4-receptor chimera and COUP-TF form heterodimers, the heterodimeric complex remains bound to DNA and can subsequently inhibit the basal promoter activity. This is a novel mechanism of repression which we term transrepression. The previously identified dimerization interfaces (7), which are present within the LBDs of RXR, RAR, TR, and COUP-TF, are most likely responsible for these heterodimeric interactions. Furthermore, these heterodimeric interactions can efficiently take place in the absence of ligand and are clearly not affected by the presence of all-*trans* RA, 9-*cis* RA, and T3 when bound to their cognate receptors.

Since the dimerization domain and the active repression function of COUP-TF overlap within the LBD, such a structural arrangement may enable dimerization to serve as an allosteric modulator of active repression. Our data indicate that this may not be the case for COUP-TF, since its active repression function is apparently not abolished when it is recruited to DNA via heterodimerization with unliganded Gal4-RXR (Fig. 2A). In addition, we demonstrated that COUP-TF can clearly transrepress the ligand-dependent activation of Gal4-receptor chimeras once tethered to these proteins (Fig. 2B). This transrepression function is located in the C terminus and independent of the N-terminal region of COUP-TF (Fig. 2C).

Although the details of the mechanism(s) of the COUP-TF transrepression function are unknown, it is possible that once COUP-TF heterodimerizes with TR, RAR, and RXR, it can either suppress the activation functions of these receptors or diminish their ligand-binding abilities. Such mechanisms have been proposed to explain the transrepression functions of other nuclear receptors, including glucocorticoid receptors and RAR (reviewed in reference 48). This hypothesis is also reminiscent of recent studies with RXR-TR or RXR-RAR heterodimers, in which heterodimeric interactions between RXR and TR or RAR inhibit the transactivation and ligand-binding functions of RXR (20, 35, 49). Alternatively, since COUP-TF is a potent repressor and can repress transactivator-mediated transcription (Fig. 4), it is possible that once COUP-TF has been recruited to DNA, via tethering to other receptors, its repression domain can interact with the general transcriptional machinery, either directly or through other cofactors, and down-regulate reporter gene transcription. Thus, any mutation that impairs the heterodimerization function of COUP-TF would also diminish the transrepression function of COUP-TF. This could explain why the regions required for the COUP-TF

active repression and transrepression functions are not superimposable.

Repression mechanism. The active repression functions of nuclear hormone receptors such as TR, RAR, COUP-TF, and ecdysone receptor have been demonstrated by several groups (for reviews, see references 50, 52, 61, and 62). These receptors, once bound to their response elements, can repress the basal activities of different minimal promoters and activate them in the presence of ligands. Although the detailed mechanism is largely unknown, in vitro studies suggest that the active repression functions of these receptors may involve interaction(s) with a corepressor to inhibit the formation of the preinitiation complex assembly (4, 13, 28, 36). Regardless of the exact target of active repression, it is interesting to examine whether these receptors are capable of inhibiting transactivator-dependent transcription, since in many cases TFIIB is also a target for transactivation. We addressed this issue by cotransfecting COUP-TF and several different transactivators into cells.

COUP-TF can function as an active repressor in a dose-dependent manner to inhibit transactivation mediated by acidic (Gal4-RII), glutamine-rich (Gal4-ftzQ), proline-rich (Gal4-CTF1P), and Ser/Thr-rich (Gal4-ZenST) transactivators (Fig. 4A and B). Once again, the inhibition is not due to simple squelching, since similar experiments performed with reporter genes lacking COUP-TF-binding sites showed little COUP-TF-mediated repression (data not shown). We also demonstrate that the active repression function of COUP-TF is position independent, since the COUP-TF-binding sites can be located either upstream of the *tk* promoter or downstream of the CAT reporter gene without affecting active repression. These experiments provide evidence that COUP-TF may not function by a quenching mechanism (Fig. 1c) which involves interactions with certain specific transactivators and short distance repression (24, 42). Moreover, since these different transactivators interact with different components of the general transcriptional machinery, it is unlikely that COUP-TF achieves the repression by preventing all of these transactivators from interacting with their cognate targets. It is more likely that COUP-TF itself interacts with one or few components in the transcriptional machinery (i.e., TFIIB) and freezes the preinitiation complex in an inactive form, thus inhibiting transactivator-dependent activation.

A number of DNA-binding proteins that function as transcriptional repressors have been identified, and several of these may have features in common with COUP-TF. One example is the *Drosophila eve*-encoded protein. Like COUP-TF, eve can repress transcription from a minimal basal promoter containing eve-binding sites, as well as repress transactivator-dependent activation (26, 64). However, the detailed molecular mechanism of eve-mediated repression seems to be different from that of COUP-TF. Unlike eve, COUP-TF lacks any apparent alanine-plus-proline-rich repression domains which seem to be a common motif involved in the repression functions of eve, Krüppel, and msx-1 (11, 26, 41). Also, unlike eve, which can repress promoter transcription in the absence of its binding site, COUP-TF needs to be associated with DNA, either by direct binding (16, 17) or tethered through other nuclear receptors to exert its function. In addition, eve has been demonstrated to interact with TBP and repress transcription via squelching of TBP and/or blocking of the DNA binding of TBP (1, 64). These findings indicate that, like transactivators, repressors can function in diverse ways involving interactions with distinct general transcription factors. The difference in their repression mechanisms could explain why eve can repress Gal4-VP16-mediated transactivation (26) whereas COUP-TF has little effect (Fig. 5).

It is intriguing that Gal4-VP16 can overcome the repression function of COUP-TF. As demonstrated previously, VP16 (Vmw65) by itself does not bind to a target gene directly but is recruited to DNA by associating with the cellular protein Oct-1 (65). VP16 confers rather strong interactions with multiple components in the general transcriptional machinery, including TFIIB (43), TBP (56), TFIIH (67), and TAF40 (22). The interactions with all these general transcription factors may account for the strong VP16-dependent transactivation (57). We speculate that such a wide range of interactions with the transcriptional machinery could offer a potential for VP16 to bypass the inhibitory mechanism(s) mediated by COUP-TF. Alternatively, the interaction between VP16 and these general factors is so strong that it prevents COUP-TF from interrupting. This is highlighted by the fact that once VP16 interacts with TFIIB, it induces a specific conformational change within TFIIB (53). It is feasible that TFIIB with such an active conformation is therefore not capable of interacting with COUP-TF. Although VP16 and ftzQ are both acidic activators and can both interact with TFIIB, it has been demonstrated that they contact different regions of TFIIB, i.e., the C-terminal repeats and the N-terminal zinc finger-like structure, respectively (14, 54). These different interactions may therefore result in distinct conformational changes in TFIIB, which in turn responds differently to COUP-TF-mediated repression. Likewise, the type of interaction between VP16 and TBP is also different from that between RII and TBP (27, 30) and may also induce a different TBP configuration. This difference could explain why RII-TBP interaction is sufficient for GAL4-dependent activation whereas VP16-TBP is not adequate for VP16 transactivation (30, 44) and could also explain the distinct responses of VP16 and RII to COUP-TF-mediated repression. In summary, our data suggest that COUP-TF can repress a rather wide range of transactivator-dependent transcription mechanisms, but in certain cases, such repression can be differentially regulated, depending on the promoter context.

Implication for COUP-TF function in vivo. COUP-TF has been found to be widely expressed during embryogenesis in a spatially and temporally regulated manner (see reviews in references 47 and 50). It is initially expressed in the three germ layers and is subsequently restricted in some of their derivatives. This expression pattern suggests that COUP-TF plays a very important role in embryonic development (50). Our findings reveal a possible molecular mechanism of how COUP-TF regulates developmental processes through gene expression. In addition to its ability to compete for hormone response elements with many receptors and to actively repress basal promoter activity, we found that COUP-TF can also function as a transrepressor and an active repressor of transactivated expression. This ability is potentially important since many new response elements identified for the thyroid hormone/RA receptor subfamily seem to have configurations (i.e., half sites, inverted repeats, or everted repeats) distinct from the common pattern of direct repeats, and it is likely that COUP-TF cannot bind to all of these response elements and exert DNA-dependent repression. However, via transrepression, COUP-TF can be tethered to receptors and recruited to DNA to perform its function. Thus, transrepression clearly brings another level of complexity to the role of COUP-TF during development and differentiation. This complexity provides a potential for fine regulation of target gene expression in response to various differentiation or development signals, since the overall magnitude of induction of gene expression will be determined by the levels of both transactivators and repressors. Hence, the

ability to actively repress transactivator-dependent expression enables COUP-TF to participate in the fine-tuning of a broad range of target genes. The importance of COUP-TF in development is suggested by our preliminary studies on the homozygous null mutations of the COUP-TFI and COUP-TFII genes; in both cases, the null mutation was lethal (49a). Analysis of these null mice may shed more light on the physiological role of COUP-TF during development.

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