Multiple Mechanisms of Chicken Ovalbumin Upstream Promoter
Transcription Factor-dependent Repression of Transactivation by the
Vitamin D, Thyroid Hormone, and Retinoic Acid Receptors*

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The chicken ovalbumin upstream promoter transcription factor (COUP-TF) is a member of the steroid/thyroid hormone receptor superfamily about which little is known of its functional role in the cell. However, it is able to repress hormonal induction of target genes by vitamin D₃ receptor (VDR), thyroid hormone receptor (TR), and retinoic acid receptor (RAR). We have shown previously that COUP-TF can bind a wide variety of A/GGGTCA repeats. This promiscuous recognition of response elements correlates with the ability of COUP-TF I to repress other receptors that bind to A/GGGTCA repeats with different spacings between the half-sites. Here we show that repression of transactivation by these receptors is a general phenomenon for the COUP-TF subfamily, as inhibition is also observed with COUP-TF II. This repression is also dose-dependent on COUP-TF. Inhibition of VDR, TR, and RAR activities also occurs through natural physiological response elements found in the osteocalcin, myosin heavy chain, and βRAR promoters, respectively. In search of the mechanisms of repression by COUP-TF we show that it does not involve the formation of detectable functionally inactive heterodimers between COUP-TF and VDR, TR, and RAR. Instead, we show that the mechanism of repression could occur at three different levels: (a) active silencing of transcription and dual competition for; (b) occupancy of DNA binding sites; and (c), heterodimer formation with retinoid X receptor, the coregulator of VDR, TR, and RAR. The silencing activity was localized to the putative ligand binding domain of COUP-TF. We postulate that COUP-TF may play a master role in regulating transactivation by VDR, TR, and RAR.

Tissue-specific and developmental expression of eucaryotic genes is a complex process which is regulated in large part by cis-acting elements located in the promoter and flanking sequences. These response elements are specific DNA sequences that are recognized and bound by sequence-specific transcription factors. They function to juxtapose the factors at different levels: (a) active silencing of transcription and dual competition for; (b) occupancy of DNA binding sites; and (c), heterodimer formation with retinoid X receptor, the coregulator of VDR, TR, and RAR. The silencing activity was localized to the putative ligand binding domain of COUP-TF. We postulate that COUP-TF may play a master role in regulating transactivation by VDR, TR, and RAR.

One of the best characterized families of transcription factors is the steroid/thyroid nuclear hormone receptor superfamily (which includes the receptors for the sex hormones, retinoids, thyroid hormones, vitamin D₃, and ecdysone) (3). These nuclear receptors regulate the expression of hormone-responsive genes (4). However, in addition to these extensively studied receptors many other genes have been cloned and designated as members of this superfamily on the basis of containing a characteristic double zinc finger DNA binding domain and homology within the ligand binding domain (5,6). Except for retinoid X receptor (RXR), no ligands for these transcription factors have yet been identified, and they have thus been grouped as orphan members of the steroid/thyroid hormone receptor superfamily.

COUP-TF is an orphan member of this superfamily, which, along with the highly homologous COUP-TF II (also called apoAI regulatory protein, ARP-1) form a small but distinct subfamily within the superfamily (7-9). This transcription factor was initially characterized by binding to the chicken ovalbumin upstream promoter (COUP) element (−70 to −90) (10, 11). Dissection of the fine structure of this element revealed that the core sequence of this element is a imperfect A/GGGTCA repeat separated by one nucleotide (12, 13). Analysis of COUP-TF binding sites in other promoters revealed that purified COUP-TFs could bind to many different A/GGGTCA repeats (14, and references therein). Further examination of COUP-TF DNA binding revealed that a single species of recombinant COUP-TF could indeed bind to many A/GGGTCA repeats with different spacings and orientations by structural adaptation (14).

Others have shown that the vitamin D₃, thyroid hormone, and retinoic acid receptors (VDR, TR, and RAR, respectively) also bind to A/GGGTCA direct repeats. Umesono et al. (15) and Naar et al. (16) uncovered the key to the functional discrimination between A/GGGTCA repeats and proposed the 3-4-5 rule. VDR, TR, and RAR specifically activate re-

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The abbreviations used are: RXR, retinoid X receptor; COUP-TF, chicken ovalbumin upstream promoter transcription factor; VDR, vitamin D₃ receptor; TR, thyroid hormone receptor; RAR, retinoic acid receptor; RARα, retinoic acid response element; TK, thymidine kinase; CAT, chloramphenicol acetyltransferase; EMSA, electrophoretic mobility shift assay; VDRE, vitamin D₃ response element; TRE, thyroid hormone response element.

1 The abbreviations used are: RXR, retinoid X receptor; COUP-TF, chicken ovalbumin upstream promoter transcription factor; VDR, vitamin D₃ receptor; TR, thyroid hormone receptor; RAR, retinoic acid receptor; RARα, retinoic acid response element; TK, thymidine kinase; CAT, chloramphenicol acetyltransferase; EMSA, electrophoretic mobility shift assay; VDRE, vitamin D₃ response element; TRE, thyroid hormone response element.
sponse elements containing A/GGGTCA direct repeats with three-, four-, and five-nucleotide spacings, respectively. This rule was extended to cover the direct repeat spacing of one nucleotide when it was shown that RXR specifically activates transcription in a hormone-dependent manner through such an element (17-19). This simplification of the response element discrimination by this group of receptors was recently complicated when the genes encoding the coregulators or auxiliary factors of VDR, TR, and RAR were cloned and identified as RXRs (20-25). RXRs form heterodimers with VDR, TR, and RAR which retain their 3-4-5 pattern of response element recognition and transcriptional cooperation in DNA binding, through RXR, leads to a corresponding enhancement of transcriptional activity that is responsive to the cognate hormones of VDR, TR, and RAR. RXR-receptor heterodimers have recently been shown to be the physiologically active receptor forms in vivo (26, 27).

We have reported previously that inhibition of transactivation by VDR, TR, and RAR appears to correlate with promiscuous binding by COUP-TF I to different spacings of A/GGGTCA direct repeats (14). Recently, Kliewer et al. (28) also demonstrated that COUP-TF I can inhibit transactivation by RXRo. The ability of COUP-TF I to inhibit transcriptional activation by so many receptors with varied A/GGGTCA repeat spacings is intriguing and important because it has significant implications for the metabolic, tissue-specific, differentiation and developmental processes controlled by this subgroup of nuclear hormone receptors. Thus, it is necessary to examine transcriptional repression by COUP-TF more extensively and to elucidate the mechanisms of transcriptional repression. We report here that the repression observed with COUP-TF I also extends to other COUP-TF subfamily members and to natural response elements for the vitamin D3, thyroid hormone, and retinoic acid receptors. We show that inhibition by COUP-TF can occur at three different levels: by competition for DNA binding site occupancy; the enhancement of transcriptional activity that is responsive to the ligand for each receptor was added (Ts, 100 nM; RA, 1 pM; 1,25-dihydroxyvitamin D3, 100 nM). Cell extracts were normalized to the total amount of protein, as determined by the Bradford (59) assay. Chloramphenicol acetyltransferase (CAT) activities were quantitated by determining the radioactivity in each spot by scintillation counting and were expressed as relative CAT activities.

**RESULTS**

**Inhibition of Transactivation by VDR, TR, and RAR Is Characteristic of the COUP-TF Subfamily and Acts through Natural Response Elements**—COUP-TF I (7) and COUP-TF II (8, 9) represent a small but distinct subfamily within the nuclear hormone receptor superfamily. We have shown previously that COUP-TF I can inhibit transactivation by VDR, TR, and RAR (14). To determine whether COUP-TF II also had the ability to inhibit transcriptional activation by VDR, TR, and RAR we cotransfected the COUP-TF II expression vector pMA (4 μg) (9) into CV1 cells with 1 μg of expression vector for each of the receptors and 5 μg of their cognate

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**EXPERIMENTAL PROCEDURES**

**Plasmids and Oligonucleotides**—The oligonucleotides for DR3 and DR5 were synthesized as described previously (14). Two complementary oligonucleotides based on the βRARE (29) were synthesized.

5′-gatccAAAGGGTCAACGGAAACTTCCGAGCTA3′
5′-gatctATGGGCTAAGAAGTTGGGTAC3′

NUCLEOTIDES 1 and 2

βRARE TKCAT was generated by inserting a single copy of the βRARE oligonucleotide into the BamHI-BglII sites of the CAT reporter TKCAT (17) (30). Two complementary oligonucleotides based on DRI (17) were synthesized.

5′-gatcTTAGGGGTCATAAGTGGGA3′
5′-gatctTCCATTGACCTTCCCAGGCCTA3′

NUCLEOTIDES 3 and 4

Two complementary oligonucleotides based on DR4 (17) were synthesized.

5′-gatccTACGGGTCATAAGTGGGA3′
5′-gatctTCCATTGACCTTCCCAGGCCTA3′

NUCLEOTIDES 5 and 6

17-mer X3 TKCAT contained three copies of the GAL4 binding site (31). pRSAGL-COUP was synthesized by subcloning the XmnI-XhoI fragment, encoding the putative ligand binding domain of COUP-TF I, (amino acids 156-423) into the Smal-SalI sites of the expression vector pABGAL (32), which contains the DNA binding and dimerization domains, and the nuclear localization signal of GAL4 (amino acids 1-147) (31, 32). The truncated form of COUP-TF I (pT78-cTUCP-TF I), with amino acids 1-52 deleted, was generated by inserting the AvaI-BglII fragment of the COUP-TF I (pT78-cTUCP-TF I (14) into the SalI site of the pT78S/SalI-Stu vector (33). 5′ overhangs were filled in with Klenow enzyme where necessary.

**In Vitro Transcription/Translation**—For in vitro transcription the plasmids pT78-hRARα (34), pCOUP-TF I, pT78-mRXRα (35), and pT78-cTUCP-TF I were linearized with EcoRI, and pEA101 (hTRβ1) (36) and pEA1001 (hTRβ1) (37), were linearized with HindIII and SalI, respectively. RNA was synthesized in vitro using SP6 RNA polymerase for pCOUP-TF I, T7 RNA polymerase for pEA101, pT78-hRARα, pT78-cTUCP-TF I, and pT78-mRXRα, and T3 RNA polymerase for rTRβ1 according to the manufacturer’s instructions (Promega). Various amounts of in vitro synthesized RNAs were translated in rabbit reticulocyte lysates according to the instructions of Promega. ZnCl2 was added to a final concentration of 0.5 μM. For cotranslation of COUP-TF I RNA and rTRβ1, hRARα, or mRXRα RNAs three different ratios were tested to ensure comparable translation of both factors. Normally a 1:10 ratio of COUP-TF I to TR, 1:5 ratio of COUP-TF I to RAR, or 1:7 ratio of COUP-TF I to RXR RNAs produced satisfactory yields. The products of in vitro translation were analyzed by electrophoretic mobility shift assays or by immunoprecipitation.

**Electrophoretic Mobility Shift Assays (EMSA)**—The oligonucleotides DRI, DR3, DR4, and DR5 were end labeled with [α-32P]UTP, and DNA oligonucleotides were 2-3 × 106 cpm/ml. Each labeled DNA probe was incubated with 1-4 μl of in vitro translated receptors as described previously (14, 38). For antibody up-shift analysis, 1 μl of a 1:5 dilution of the COUP-TF antiserum was preincubated with the reactions for 5 min at room temperature prior to the addition of the probes.

**Competition Assays and Inhibition of Transcription**—Protein A-Sepharose CL-4B was resuspended in suspension buffer TEGN (10 mM Tris. Cl, pH 7.4, 1 mM EDTA, 10% (v/v) glycerol, and 0.1% (v/v) Nonidet P-40). 200-μl aliquots of a 50% (v/v) suspension (approximately 20 mg) of protein A-Sepharose CL-4B were incubated at 4 °C for 1 h with 20 μl of anti-COUP-TF serum (7). The resin was then washed twice each with suspension buffer and then transcription buffer (20 mM Hepes, pH 7.9, 20% glycerol, 0.1 mM KCl, 0.2 mM EDTA, 5 mM MgCl2, 2 mM dithiothreitol). 15-μl aliquots of 100 μl (v/v) suspension (approximately 20 μg) of protein A-Sepharose CL-4B were incubated at 4 °C for 1 h with 20 μl of anti-COUP-TF serum (7). The resin was then washed twice each with suspension buffer and then transcription buffer (20 mM Hepes, pH 7.9, 20% glycerol, 0.1 mM KCl, 0.2 mM EDTA, 5 mM MgCl2, 2 mM dithiothreitol). A 30-μl aliquot of 2 × SDS loading buffer (0.125 M Tris-Cl, pH 6.8, 6% SDS, 10% β-mercaptoethanol, 20% glycerol, 0.025% bromphenol blue) was added, and after boiling for 5 min the supernatant was immediately loaded on an SDS-polyacrylamide (10%) gel (39). The gel was treated sequentially with 40% methanol, 10% acetic acid, and Enhance (Du Pont-New England Nuclear) and deionized H2O prior to drying and fluorography.

**Cell Culture and Transient Transfections**—Transient transfection of 1 × 106 monkey kidney CV1 cells was performed by a modified Polybrene method as described previously (14, 40). Where indicated the ligand for each receptor was added (Ts, 100 nM; RA, 1 μM; 1,25-dihydroxyvitamin D3, 100 nM). Cell extracts were normalized to the total amount of protein, as determined by the Bradford (59) assay (Bio-Rad). Chloramphenicol acetyltransferase (CAT) activities were quantitated by determining the radioactivity in each spot by scintillation counting and were expressed as relative CAT activities.
Mechanism of COUP-TF-dependent Repression of Gene Expression

Upon addition of hormone, induction of transcriptional activity by VDR, TR, and RAR was observed, (8-, 6-, and 6-fold, respectively) (Fig. 1A, compare columns 1 and 2, 4 and 5, and 7 and 8, respectively). Cotransfection of the COUP-TF II expression vector inhibited VDR, TR, and RAR induction of CAT activity by 5-, 3-, and 5-fold, respectively (compare columns 2 and 3, 5 and 6, and 8 and 9, respectively). The observed repression was not because of nonspecific effects, such as promoter competition between the COUP-TF II expression vector and the receptor expression vectors, as equal amounts of empty expression vector containing no COUP-TF or receptor sequences were added to the transfections to which COUP-TF was not added. In all subsequent transfection reactions to which COUP-TF was not added the amount of DNA was equalized in a similar fashion. Thus, repression of transactivation, by VDR, TR, and RAR, is a characteristic of the COUP-TF subfamily as it is exhibited by both COUP-TF I and II.

To determine whether the inhibition of transcriptional activity by COUP-TFs was dose-dependent, a constant amount (1 μg) of the RA receptor expression vector (pRShRARα) was cotransfected with increasing amounts of COUP-TF I expression vector pRShCOUP-TF I (Fig. 1B). The addition of hormone led to a 5-fold increase in CAT activity (Fig. 1B, compare columns 1 and 2) that was progressively repressed by increasing amounts of pRShCOUP-TF I (columns 3–8). An equal amount (1 μg) of COUP-TF I expression vector reduced the transcriptional activity to basal TK promoter levels (Fig. 1B, compare columns 1 and 6). Further increasing the amount of pRShCOUP-TF I to a 4-fold excess above RA receptor DNA, inhibited the CAT activity 12-fold below induced levels and 2-fold below basal activity (compare columns 1 and 2 with 8). Thus, inhibition of RAR-dependent transcriptional activity is dependent on the amount of COUP-TF expression vector cotransfected.

The reporters used in these experiments contained perfect A/GGTC repeats with spacings of three, four, and five nucleotides, which are responsive to VDR, TR, RAR, respectively (14). However, natural response elements for these receptors are quite degenerate. Thus, to determine whether the inhibition of transcriptional activation by these receptors also occurs on natural physiological response elements, we used TKCAT reporters which contained oligonucleotides corresponding to the sequences of natural response elements. The VDRE, TRE, and RARE sequences are from the osteocalcin (41), myosin heavy chain (42), and βRAR (29) promoters, respectively. We observed classical hormone-dependent induction through all of these elements; the inducibility by VDR, TR, and RAR was approximately 5.5-, 8-, and 7-fold, respectively (Fig. 2, compare columns 1, and 2, 4 and 5, and 7 and 8, respectively). Hormone-dependent activation by VDR, TR, and RAR was inhibited (10-, 45-, and 7-fold, respectively) by cotransfection of the COUP-TF I expression vector (Fig. 2, compare columns 2 and 3, 5 and 6, and 8 and 9).

**Fig. 1.** COUP-TF-dependent down-regulation of target gene induction by VDR, TR, and RAR. Panel A, COUP-TF II-dependent down-regulation of hormonal induction of target gene (pAV-hVDR), pRShTRβ (57), and pRshRARα (58) was cotransfected into CV1 cells with 5 μg of cognate reporters (DR3, DR4, and DR5 TKCAT, respectively) in the presence or absence of 4 μg of pMA. The amount of DNA added to each transfection was equalized by adding empty expression vector. CAT activities were determined relative to the activity of each receptor in the presence of hormone but in the absence of COUP-TF II. Panel B, dose-dependent repression by COUP-TF I of RA induction of reporter activity through RA. Increasing amounts (125 ng to 4 μg) of pRShCOUP-TF I were cotransfected with a constant amount of pRShRARα (1 μg) and the reporter DR5 TKCAT (5 μg) into CV1 cells. The amount of DNA added to each transfection was kept constant by compensating with empty RSV expression vector containing no COUP-TF or receptor sequences. The percentage of CAT activity was determined relative to the activity of RA in the presence of RA but in the absence of COUP-TF I. These bar graphs represent the average of duplicate transfections which are representative of four transfections. D3, vitamin D3; T3, thyroid hormone; and RA, retinoic acid.

**Fig. 2.** COUP-TF-dependent repression of reporters containing natural response elements for VDR, TR, and RAR. 1 μg of the receptor expression vectors (pAV-hVDR, pRShTRβ, or pRShRARα) and their cognate reporters (5 μg of VDRE TKCAT, myosin heavy chain TRE (MHC) TKCAT, or βRAR TKCAT, respectively) were cotransfected into CV1 cells in the presence or absence of pRShCOUP-TF I (2 μg) and/or hormone. CAT activities were determined relative to the activity of each receptor in the presence of hormone but in the absence of COUP-TF I. The bar graph is the average of duplicate transfections and is representative of four transfections. D3, vitamin D3; T3, thyroid hormone; and RA, retinoic acid.
Mechanism of COUP-TF-dependent Repression of Gene Expression

9, respectively). Thus, COUP-TF can inhibit transcriptional activation by these receptors through physiological response elements.

Analysis of the Mechanisms by Which COUP-TFs Repress Transcription

by VDR, TR, and RAR—The observed COUP-TF-dependent antagonism of transactivation by VDR, TR, and RAR could be mediated by a number of possible mechanisms (Fig. 3). Mechanism A postulates that COUP-TF can form a functionally inactive heterodimer with VDR, TR, and RAR. Mechanism B suggests that COUP-TF may indirectly inhibit transactivation by these receptors by forming inactive heterodimers with the coregulator RXR. Mechanism C proposes that COUP-TF competes for binding to the response elements for these receptors. Mechanism D raises the question of whether, in addition to these other potential mechanisms, COUP-TF also possesses an active transcriptional silencing function. Thus, we set out to investigate the mechanisms of transcriptional repression by COUP-TF.

COUP-TF Does Not Heterodimerize with TR or RAR—

Several members of this superfamily, such as TR and RAR, form heterodimers with each other (43). It is possible that COUP-TF could act as a negative coregulator of VDR, TR, and RAR by forming transcriptionally nonfunctional heterodimers with these receptors (Fig. 3, mechanism A). To repress hormone-dependent transactivation by these receptors the resultant heterodimers would have to be functionally inactive. The heterodimers might or might not be capable of binding DNA. We analyzed in vitro cotranslated COUP-TF I and TR by EMSA to test for the formation of DNA-binding heterodimers on the DR4 element (Fig. 4A). If heterodimers are formed between COUP-TF and TR we would expect to observe an extra complex of intermediate mobility compared with the homodimer complexes of COUP-TF I and TRβ.

A. Formation of Heterodimers:

RC + COUP-TF → RC COUP-TF

B. Competition for RXR:

RC RXR → RC RXR COUP-TF

C. Competition for the Binding Site:

RC RXR SRE → RC RXR SRE COUP-TF

D. Silencing of transcription:

SRE → COUP-TF

Single retarded complexes, corresponding to homodimers, were observed for COUP-TF I (which we have previously shown only binds DNA as a dimer (14)) and TRβ when they were individually translated (Fig. 4A, lanes 1 and 2). Upon cotranslation of the two species, no third heterodimer complex of intermediate electrophoretic mobility was detected; only homodimers of COUP-TF and TR were observed (Fig. 4A). The slight decrease in the amount of the COUP-TF homodimer complex was a result of competition between the two in vitro expression vectors; that is, the TRβ expression vector translates more efficiently than the COUP-TF vector. Thus, COUP-TF and TR do not form DNA-binding heterodimers. Similarly, no DNA-binding heterodimers were detected between COUP-TF and RARα (data not shown). Thus, COUP-TF does not repress the transcriptional activity of this subgroup of steroid/thyroid hormone receptors directly by forming transcriptionally inactive DNA-binding heterodimers.

To detect the formation of non-DNA-binding heterodimers, we employed communoprecipitation using antibodies raised against COUP-TFs which can recognize COUP-TF dimers (7, 14). In vitro transcribed RNAs encoding RARα or TRβ were either individually translated in vitro in rabbit reticulocyte lysates or cotranslated with in vitro transcribed RNA encoding COUP-TF I. The resultant lysates and a control lysate programmed with only COUP-TF I RNA were immunoprecipitated with antisera raised against COUP-TFs and then analyzed by denaturing polyacrylamide gel electrophoresis (Fig. 4, B and C). Individually translated COUP-TF I (Fig. 4B, lane 5), but not RARα (lane 1) was immunoprecipitated by the COUP-TF antisera (compare lanes 2 and 6) (translated COUP-TF yields two bands). Upon cotranslation of COUP-TF and RARα (lane 3) only COUP-TF I was immunoprecipitated, showing that COUP-TF and RARα do not form non-DNA-binding heterodimers. Similar results were observed with TRβ (Fig. 4C). Individually translated TRβ was not immunoprecipitated by the addition of COUP-TF antisera (lanes 1 and 2). When TRβ and COUP-TF I RNA were cotranslated only COUP-TF I was immunoprecipitated (compare lanes 3–5). In vitro translated TRβ and RARα are capable of forming heterodimers with RXR, indicating they are functional in this sense (data not shown). Thus, COUP-TFs do not inhibit transactivation by these receptors by forming functionally inactive DNA-binding or non-DNA-binding heterodimers.

COUP-TF May Antagonize Transcriptional Activation by VDR, TR, and RAR Indirectly by Heterodimerization with the Coregulator RXR—Having ruled out the formation of direct heterodimers between COUP-TF and TR and RAR as the mode of inhibition of transcriptional activity, we turned our attention to another possible mechanism involving COUP-TF heterodimerization with RXR, the coregulator of VDR, TR, and RAR (Fig. 3, mechanism B). RXRs are required for maximal hormone-dependent transcriptional activity of VDR, TR, and RAR. They function as coregulators that form heterodimers with higher DNA binding affinities than the homodimers (20–27). COUP-TF may indirectly inhibit the transcriptional activity of these receptors by forming heterodimers with RXR. If so, they may sequester the coregulators in a functionally inactive complex. The loss of functionally available RXRs could decrease the DNA binding affinity of VDR, TR, and RAR, thereby reducing their transcriptional activities. To determine if COUP-TFs can form heterodimers with RXR we analyzed the products of cotranslation of COUP-TF I and RXRβ by gel mobility shift assay (Fig. 5). In vitro translated RXRβ and COUP-TF I formed DNA-protein com-

FIG. 3. Models for the mechanism of COUP-TF-dependent inhibition of receptor transactivation activation of gene expression. A, formation of heterodimers between the receptors (RC, VDR, TR, and RAR) and COUP-TF. B, competition for RXR. Formation of heterodimers between RXR and COUP-TF leading to competition between COUP-TF and VDR, TR, and RAR for their coregulators. C, competition for occupancy of DNA binding sites. D, active silencing of the transcriptional machinery by COUP-TF.
plexes with similar electrophoretic mobilities (data not shown). Thus an N-terminally truncated COUP-TF I lacking 54 amino acids (pT7P-tCOUP-TF I) was used, as the complex it forms migrates with a distinct mobility compared with RXRβ (compare lanes 2 and 4). Cotranslation of tCOUP-TF I and mRXRβ RNAs yielded a complex with an intermediate mobility corresponding to a tCOUP-TF I-mRXRβ heterodimer. The COUP-TF homodimer and intermediate heterodimer complexes were up-shifted upon addition of COUP-TF antisem, whereas the RXR homodimer complex was not (data not shown). This proves that the complex of intermediate mobility contains COUP-TF. Thus, COUP-TFs can form DNA-binding heterodimers with RXR and sequester them from the other receptors in a nonfunctional complex, suggesting that this is part of the mechanism to inhibit the transcriptional activity of these receptors indirectly.

COUP-TF Can Directly Inhibit VDR-, TR-, and RAR-dependent Gene Expression by Competition for Occupancy of DNA Binding Sites—In addition to the above indirect mechanism of inhibition, COUP-TF could also repress the transcriptional activity of these receptors by binding to their response elements and competing for occupancy (Fig. 3, mechanism C). We have shown previously that COUP-TF can bind to many A/GGGTCA repeats, arranged both as palindromes and direct repeats with spacings of various lengths. Thus, it is entirely conceivable that COUP-TF can bind to the A/GGGTCA direct repeat response elements of these receptors. To analyze this possibility we end labeled double-stranded oligonucleotides corresponding to DR3, DR4, and DR5 and examined the binding of in vitro translated COUP-TF I by EMSA (Fig. 6A). COUP-TF I binds to the VDRE (DR3, lane 1), the TRE (DR4, lane 2), and the RARE (DR5, lane 3), although with slightly different affinities. To confirm the identity of the factor in the DNA-protein complexes, COUP-TF-specific antisem was added. The antisem retarded the electrophoretic mobility of all three complexes, showing that they contain COUP-TF (lanes 4–6). This agrees with the EMSA in Fig. 4A, which shows binding of both COUP-TF I and TRβ to the DR4 probe. Thus, COUP-TF can bind to the response elements for the VDR, TR, and RAR and therefore can compete for occupancy of the DNA sites to inhibit hormonal induction of transcription. We have also detected the binding of endogenous COUP-TFs, in crude COS cell nuclear extracts to DR3, DR4, and DR5 probes (data not shown), which indicates that the binding of COUP-TFs to these response elements should be physiologically relevant, as it occurs at physiological levels of COUP-TF.

A corollary to this prediction is that transcriptional inhibition by COUP-TF can be overcome by increasing the amount of cotransfected receptor. To test this hypothesis a minimal amount of COUP-TF I expression vector (50 ng) was cotransfected into CV1 cells to inhibit the 10-fold RA induction of DR5 TRCAT by RAR. Increasing amounts of the RARα expression vector were then cotransfected; however, the total amount of DNA in each transfection reaction was kept constant by compensating with empty RSV expression vector (Fig. 6B). COUP-TF repressed retinoic acid induction by 2-fold (compare columns 2 and 3). Increasing the amount of RAR cotransfected to 500 ng restored RA induction levels to those observed in the absence of COUP-TF (column 7). Increasing amounts of pRSrRARα overcame the transcriptional inhibition by COUP-TF and restored transcriptional levels to those observed with RARα on its own. Thus, the transcriptional repression observed with COUP-TF can be competed by increasing the level of the receptor in the cell.

COUP-TF Possesses an Active Transcriptional Silencing
Mechanism of COUP-TF-dependent Repression of Gene Expression

A Probe (DR1)

Antiserum

- - - + + + +

RXR

RXR/COUP-TF

COUP-TF

Mechanism of COUP-TF-dependent Repression of Gene Expression

A Probe (DR1)

Antiserum

- - - + + + +

RXR

RXR/COUP-TF

COUP-TF

Function Located within the C-terminal Putative Ligand Binding Domain—An interesting aspect of COUP-TF-dependent repression of RARα transactivation is that in addition to inhibiting hormone-dependent activation, increasing levels of COUP-TF I can lower reporter expression to below basal levels (Fig. 1B). This leads to the possibility outlined in Fig. 3 (mechanism D) that COUP-TF is an active silencer of transcription. To determine whether the observed repression of DR5 TKCAT basal level transcription was general for all response elements to which COUP-TF binds, we analyzed the effect of cotransfection of COUP-TF I expression vector on basal level transcription from a number of TK reporters (Fig. 7A). Cotransfection of pRShCOUP-TF I into CV1 cells significantly repressed the level of basal transcription from DR3, DR4, and DR5 TKCAT, while having little effect on the level of expression from progesterone response element TKCAT, 17-mer TKCAT, and TKCAT (the parent vector). Thus, the repression of basal level transcription was response elementspecific and a functional consequence of COUP-TF binding and was not caused by the squelching of general transcription factors such as TFIIB, as we have shown a direct interaction between COUP-TF I and TFIIIB (44). However, we cannot entirely rule out squelching as contributing to at least some of the repression we observe.

It has been shown that TRα, TRβ, and RARα possess an active transcriptional silencing domain within their ligand binding domains which is transferable to the heterologous DNA binding domain of GAL4 and can repress basal transcription (32, 45). To determine whether the inhibition of basal transcriptional activity by COUP-TF was caused by competition with endogenous transcription factors which bind to these response elements or by the presence of an active transcriptional silencing domain, we made a GAL-COUP chimera. As the silencing function of TR and RAR was located in the C terminus, the putative ligand binding domain of COUP-TF I (amino acids 156-423) was fused C-terminal to the GAL4 DNA binding domain (GAL4-DBD) (amino acids 1-147) to examine its effect on basal level expression from 17-mer ×3 TKCAT. The 17-mer sequence is a response element to which the yeast transcription factor GAL4 binds to activate transcription (31). Mammalian cells do not possess
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DNA response elements have been classified as simple or complex depending on whether they bind single or multiple factors. Complex cis-elements are generally composed of overlapping response elements which often bind factors from different transcription factor families. Many of the classical steroid response elements can be classified as simple response elements. However, further research has shown that even these simple response elements have a more complex molecular biology than previously realized. One of the earliest examples of this was a simple cis-element that was responsive to both the progesterone and glucocorticoid receptors (46). Subsequently it was shown that the TRE palindromic site was also responsive to retinooids in addition to thyroid hormones (47). With the elucidation of the 1-3-4-5 rule, the underlying mechanism of cis-element recognition was determined for a series of receptors, RXR, VDR, TR, and RAR, respectively, which bind to A/GGGTCA direct repeats (15, 16, 28). In addition, it was shown that the coregulator RXR formed heterodimers with VDR, TR, and RAR, leading to increased DNA binding and transactivation. Thus, simple A/GGGTCA direct repeats are involved in complex molecular interactions of several members of the steroid/thyroid hormone receptor superfamily.

This scenario was further complicated when we showed that COUP-TF I could inhibit transcriptional activation by VDR, TR, and RAR through such direct repeats (14). Transcriptional inhibition of this subgroup of receptors is characteristic of the COUP-TF subfamily of factors as we show that COUP-TF II can also repress the induction of gene expression by these receptors (Fig. 1A). The repression is dose-dependent (Fig. 1B) and evident with physiological relevant response elements present in the osteocalcin, myosin heavy chain, and βRAR promoters (Fig. 2). In contrast to our report, Kliewer et al. (28) have reported that COUP-TF does not repress transcriptional induction through the βRAR element. However, we detect binding of COUP-TF to this element in vitro with lower affinity than to DRI (data not shown), and the observed differences in repression may be caused by the amount of COUP-TF expression vector used, as we used 4-fold more in our experiments. Consistent with our results it was recently shown that COUP-TF I and ARP-1 (COUP-TF II) can repress transcriptional activation by RXRa and HNF4 through their cognate response elements in the apolipoprotein C III and apolipoprotein Al promoters (48, 49). These elements contain A/GGGTCA direct repeats with one-nucleotide spacing, which is the cis-element to which COUP-TF binds with highest affinity (14). Thus, the negative regulatory function of COUP-TF is intrinsic to the COUP-TF subfamily and appears general for all of the response elements to which they bind.

There are a number of possible mechanisms outlined in Fig. 3 whereby COUP-TF could inhibit the activities of VDR, TR, and RAR. We showed that neither DNA binding nor non-DNA-binding heterodimers were formed between COUP-TF and TR or RAR (Fig. 4). This indicates that COUP-TF does not act as a negative coregulator to repress transactivation by these receptors directly by forming nonfunctional heterodimers. Another common feature in the molecular biology of VDR, TR, and RAR (in addition to binding to A/GGGTCA direct repeats) is their requirement for a positive coregulator (RXR) for high affinity DNA binding and maximal transcriptional activity (20-27). If COUP-TF could form a heterodimer with RXR it would sequester the coregulator of VDR, TR, and RAR in inactive complexes and inhibit their transcriptional activity indirectly. We have clearly demonstrated that
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COUP-TF and RXR\(\alpha\) do form DNA-binding heterodimers (Fig. 5). In agreement with our data, Kliewer et al. (28) also reported heterodimer formation between COUP-TF and RXR\(\alpha\). The intermediate mobility complex in Fig. 5 has to be a result of direct protein-protein interactions between COUP-TF and RXR\(\alpha\) on the DNA, as COUP-TF cannot bind to GGTCA half-sites, nor does it bind DNA as a monomer (14). Thus, RXR and COUP-TF are not just binding to adjacent A/GGGTCA half-sites independently. Instead, part of the transcriptional repression of VDR, TR, and RAR by COUP-TF may be because of the formation of heterodimers between COUP-TF and the coregulator RXR.

We have shown previously that COUP-TF displays relatively high binding affinities for other A/GGGTCA direct repeats in addition to DR1 (14). We showed here (Fig. 6A) that COUP-TF can bind to A/GGGTCA direct repeats with three-, four-, and five-nucleotide spacings which correspond to a VDRE, TRE, and RARE, respectively. Such promiscuous DNA binding allows COUP-TF to compete directly with VDR, TR, and RAR for occupancy of their cognate binding sites (Fig. 6). Thus, COUP-TF could competitively antagonize the transactivation of VDR, TR, and RAR by two different mechanisms: (a) competition for formation of inactive heterodimers with the coregulator RXR; and (b) direct competition for occupancy of DNA binding sites. However, the ability of COUP-TF to repress hormonal induction of reporter gene activity to levels below basal expression indicated that the observed repression could not be caused by these mechanisms alone. Rather, COUP-TF appears to function in a manner similar to that observed for unliganded TR and RAR (50, 52, 53). Both of these receptors have been shown to possess an active and transferable transcriptional silencing function within their C-terminal ligand binding domains. Transfer of the putative ligand binding domain of COUP-TF to the GAL4 DBD confirmed that indeed it possesses an active silencing function within its C-terminal domain (Fig. 7B). This active transcriptional silencing function compounds the inhibition contributed by competitive binding. Taken together, these results show that COUP-TF acts on three different levels to inhibit functional activities of VDR, TR, and RAR: active silencing of transcription, competition for the occupancy of DNA binding sites, and titration of the coregulator RXR.

Although the COUP element was initially characterized as a positive element, and COUP-TFs were cloned on the basis of their ability to bind to these response elements, we and others have shown recently that members of the COUP-TF subfamily possess a strong negative transcriptional function (14, 28, 48, 49). Similar inhibitory transcriptional functions have been detected in TR and RAR. However, upon addition of their cognate hormones, the negative transcriptional functions of the latter are converted to a positive transcriptional activity (50–52). By analogy, the negative transcriptional activities displayed by COUP-TFs may be the transcriptional response in the absence of the putative ligand. The binding of ligand could subsequently convert COUP-TF to a positive transcription factor. However, the ligand-COUP-TF complex may or may not produce a positive response on a restricted subset of GGTCA repeats to which COUP-TF is not bound to hormone-dependent transactivation by TR is only seen on a subset of response elements to which it binds (42).

At present there is only circumstantial evidence for a ligand. In addition to the high degree of amino acid conservation in the putative ligand binding domain of COUP-TF between COUP-TF homologs in Drosophila and man (53), a positive transcriptional response can be elicited from a COUP-TF promoter-gene DNA binding domain chimera upon addition of activators such as dopamine, okadaic acid, and 8-bromo-cAMP (64). These compounds mimic the action of hormone with other receptors in an indirect ligand-independent pathway (55). This suggests COUP-TF has the capability for positive transcriptional activity in cells and may be activated by as yet unidentified ligand. Alternatively it may be activated in a ligand-independent mechanism or not at all.

We showed that transcriptional antagonism of RAR activity by COUP-TF could be overcome by cotransfecting increasing amounts of RAR expression vector (Fig. 6A), indicating that competitive binding to identical response elements by members of the steroid receptor superfamily could be a common molecular mechanism controlling the expression of steroid responsive genes, allowing cross-talk between different hormonal and regulatory circuits. Thus, both the level of expression of the receptor and COUP-TF will determine the overall magnitude of induction of gene expression in an inverse concentration-dependent manner, similar to the relationship observed between COUP-TFs and hepatic nuclear factor 4 (HNF4) (48). COUP-TF is expressed ubiquitously, but the level of expression varies from cell type to cell type and tissue to tissue. Differential expression of COUP-TFs, potentially producing different cell-specific regulatory patterns, may impact on metabolic, differentiation, and developmental processes controlled by the thyroid hormones, retinoic acid, and vitamin Ds. We speculate that COUP-TF may play a master role in regulating transactivation by VDR, TR, and RAR. In addition, such a well conserved biological function as transcriptional silencing observed with TR, RAR, and COUP-TF may have an important role to play in these hormonal networks.

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