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Chicken Ovalbumin Upstream Promoter Transcription Factor (COUP-TF) Dimers Bind to Different GGTCA Response Elements, Allowing COUP-TF To Repress Hormonal Induction of the Vitamin D_3, Thyroid Hormone, and Retinoic Acid Receptors

AUSTIN J. COONEY, SOPHIA Y. TSAI, BERT W. O'MALLEY, AND MING-JER TSAI*

Department of Cell Biology, Baylor College of Medicine, Houston, Texas 77030

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Alignment of natural chicken ovalbumin upstream promoter transcription factor (COUP-TF) response elements shows that, in addition to the predominant direct repeat of the GGTCA motif with a 2-bp spacing, there are other functional COUP elements with variations in the GGTCA orientation and spacing. We systematically analyzed the binding of in vitro-synthesized COUP-TFs and showed that COUP-TF is capable of binding to oligonucleotides containing both direct repeats and palindromes and with different spacings of the GGTCA repeats. Subsequently, we analyzed four possible mechanisms proposed to explain how COUP-TF could bind to these spatial variations of the GGTCA repeat. We demonstrated that the functional DNA-binding form of COUP-TF is a dimer which requires two GGTCA half-sites to bind DNA. We demonstrated that the COUP-TF dimer undergoes a remarkable structural adaptation to accommodate binding to these spatial variants of the GGTCA repeats. A functional consequence of the promiscuous DNA binding of COUP-TF is its ability to down-regulate hormonal induction of target gene expression by other members of the steroid-thyroid hormone receptor superfamily such as the vitamin D_3, thyroid hormone, and retinoic acid receptors. Our data indicate that COUP-TF may have an important role in hormonal regulation of gene expression by these receptors.

Many members of the steroid-thyroid hormone receptor superfamily, including the retinoic acid, thyroid hormone, and 1,25 (dihydroxy) vitamin D_3 receptors (RAR, TR, and VDR, respectively) play important roles in the control of vertebrate differentiation, development, and cellular homeostasis. These receptors mediate control of such processes by ligand-dependent regulation of gene transcription. Extensive studies have shown that the members of this superfamily activate and/or repress gene transcription through direct binding to discrete *cis*-acting elements termed hormone response elements. Specific DNA binding is mediated by a distinct structural-functional DNA-binding domain, containing two zinc fingers, which is conserved among all family members (7). Characterization of response elements for the estrogen receptor (ER), the thyroid hormone receptor (TR), the glucocorticoid receptor (GR), and the progesterone receptor (PR) showed them to be closely related palindromic motifs (9, 10, 20, 31). In fact, the last two share identical response elements. The core consensus estrogen response element half-site is the GGTCA pentamer and the glucocorticoid-progesterone response element (GRE-PRE) core consensus half-site is GAACA, with a 3-bp spacing between the half-sites of each response element (31). The palindromic nature of these elements suggested that the receptors bind to their cognate response elements as dimers, which was subsequently shown to be true (14, 35).

The chicken ovalbumin upstream promoter transcription factors (COUP-TFs) have been shown to be orphan members of the steroid-thyroid hormone receptor superfamily (15, 40, 41). The *Drosophila* homolog of COUP-TF, Seven up, plays a critical role in the differentiation of photoreceptor cells (22). COUP-TFs were initially characterized in chick oviduct and HeLa extracts, where they bind as dimers to the COUP element of the ovalbumin gene to activate transcription (1, 28, 42). There are two classes of COUP-TFs defined by their molecular size: low-molecular-weight COUP-TFs are in the 43,000 to 48,000 range and high-molecular-weight COUP-TFs range from 66,000 to 74,000 (40, 41). Genes for two species of low-molecular-weight COUP-TF have been cloned: human COUP-TF I (41) is identical to the orphan receptor hear3 (21), and COUP-TF II is identical to ARP-1, which has been shown to be important in the regulation of the apolipoprotein AI gene (15, 43). The two COUP-TF genes are closely related, with an overall amino acid identity of 87%. The DNA-binding domains and the putative ligand-binding domains have 98 and 97% amino acid identity, respectively (40). Sequence analysis showed they contained zinc fingers characteristic of members of the steroid-thyroid receptor superfamily.

Three amino acids at the C-terminal base of the first zinc finger (known as the P box) are important for recognition of the half-site nucleotide sequence. Based on the amino acid sequence within the P box, the steroid-thyroid receptor superfamily was divided into two groups (37). The GR group, which includes glucocorticoid, mineralocorticoid, androgen, and progesterone receptors, has glycine and serine residues as the first two amino acids of the P box and recognizes the GRE-PRE. Other receptors which have glutamic acid and glycine at the first two positions of the P box are assigned to the ER-TR subfamily, and they recognize GGTCA repeats. On the basis of their P-box sequence,

* Corresponding author.

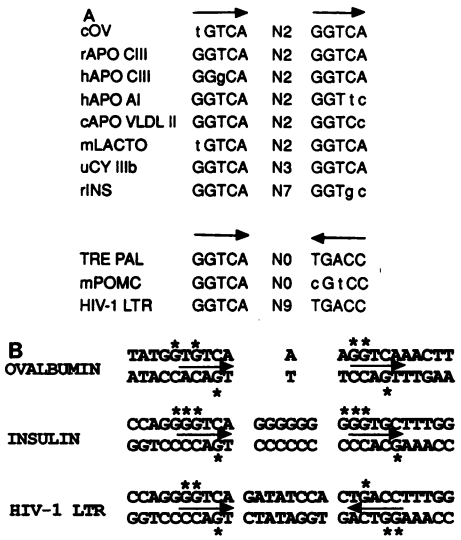

FIG. 1. Summary of natural COUP-TF-binding sites. (A) Alignment of COUP-TF response elements with respect to the orientation and spacing of the GGTCA pentamer motif. The arrow indicates the orientation of the motif. cOV, chicken ovalbumin (28, 42); rAPO CIII, rat apolipoprotein CIII (27); hAPO cIII, human apolipoprotein CIII (11, 15); hAPO AI, human apolipoprotein AI (15); cAPO VLDL II, chicken very low density apolipoprotein II (44, 45); mLACTO, mouse lactoferrin (18); uCY IIIb, sea urchin actin IIIb (7a); rINS, rat insulin II (12, 13); TRE PAL, TRE palindrome (15); mPOMC, mouse pro-opiomelanocortin (5, 6); HIV-1 LTR, human immunodeficiency virus type 1 long terminal repeat (3, 25). N denotes the direct-repeat spacing, and N' denotes the palindromic spacing. (B) Summary of the methylation interference data for binding of COUP-TF to the ovalbumin, insulin, and HIV-1 LTR sequences. The asterisks indicate important guanine contact points, and the arrows highlight the GGTCA repeats.

COUP-TFs are classified as members of the ER-TR subfamily and were predicted to bind to a GGTCA repeat. Indeed, the COUP element is an imperfect direct repeat of the GGTCA motif separated by a 2-bp spacer (26, 36).

In addition to the ovalbumin gene, COUP-TFs have been shown to bind to and activate important regulatory elements in the promoters of a number of other genes, e.g., rat and human apolipoprotein CIII (11, 15, 27), human apolipoprotein AI (15), chicken apolipoprotein VLDL II (44, 45), and mouse lactoferrin (18) (Fig. 1). These elements all contain a GGTCA direct repeat with a 2-bp spacing. In addition to these elements, it was also shown that COUP-TFs can bind to and activate functional elements containing GGTCA repeats whose orientation and spacing are markedly different from those described above. COUP-TFs bind to the RIPE-1 element and activate the rat insulin II promoter, which contains an imperfect GGTCA direct repeat with a 7-bp spacing (12, 13); the sea urchin actin IIIb gene, which contains a direct repeat with a 3-bp spacing (7a); the human immunodeficiency virus type 1 long terminal repeat negative response element, which contains a palindromic element with a 9-bp spacing (3, 25); and the mouse pro-opiomelanocortin promoter, which contains an imperfect palindrome with no spacer (5, 6). COUP-TF also has been shown to bind to the thyroid hormone response element (TRE) palindrome, which also has no spacer (15). This shows the wide functional importance of this diverse collection of response elements whose common denominator is COUP-TF. Comparison of the various COUP-binding sites (Fig. 1A) shows that their common feature lies in the primary sequence, which consists of either perfect or imperfect GGTCA repeats. However, there are dramatic differences in the spatial organization of these repeats. The elements vary both in the number of nucleotides separating the half-sites and in their orientation. Methylation interference analysis of some of these elements showed that COUP-TF makes specific contacts with both GGTCA half-sites (Fig. 1B). Thus, COUP-TF appears to display a promiscuous ability to recognize and bind to GGTCA repeats.

The ability of COUP-TF to bind to diverse GGTCA repeat orientations and spacings is intriguing, especially since COUP-TF has been shown to exist as a stable dimer in solution (28, 42). It is important to understand the mechanism which underlies this promiscuous DNA binding and its relevance to transcriptional activation or repression. Understanding the mechanism by which a single transcription factor can recognize different response elements is relevant to understanding differential regulation of gene expression by tissue-specific and developmentally regulated factors. Thus, we set out to analyze the effect of orientation and spacing of the GGTCA repeats on COUP-TF binding and transcriptional function. The results presented in this report show that COUP-TFs are promiscuous for binding to different arrangements of the GGTCA repeats; they can bind to both direct repeats and palindromes with different spacings. We also elucidated the mechanism by which COUP-TF can bind to these variant DNA elements, and we examined the functional consequences of these variations by transient transfection studies. We demonstrate that COUP-TF can down-regulate hormonal induction of target genes by TR, RAR, and VDR.

MATERIALS AND METHODS

Constructs for in vitro RNA synthesis. pCOUP-TF I was constructed from a full-length COUP-TF I cDNA (465 to 2010) which had been directionally cloned into the *Sma*I and *Eco*RI sites of pGEM7Zf(+) (Promega). A full-length COUP-TF II cDNA was subcloned into the *Eco*RI site of pGEM7Zf(+) to form pCOUP-TF II. The correct orientation was determined by restriction analysis. A truncated form of COUP-TF I (tCOUP-TF I) with amino acids 1 to 52 deleted was subcloned from pCOUP-TF I. pCOUP-TF I was cut with *Xma*I, which cleaves the cDNA at nucleotide 643, and *Aat*II, which cleaves within the multiple cloning cassette of pGEM7Zf(+) 3' of the cDNA. This 1.3-kb fragment was directionally cloned into the *Nco*I and *Aat*II sites of pGEM5Zf(+) (Promega). The *Nco*I and *Xma*I ends were filled in with Klenow fragment and religated. This construct produced a 41-kDa tCOUP-TF I from the ATG supplied by the *Nco*I site. tCOUP-TF I was used to form heterodimers with full-length COUP-TF I and COUP-TF II. The use of the truncated molecule permits differentiation between retarded complexes arising from homodimers and heterodimers in EMSA.

Synthetic oligonucleotides. Oligonucleotides were synthesized on an Applied Biosystems 391 DNA synthesizer and purified through polyacrylamide gels as previously described (27). Two series of complementary oligonucleotides were synthesized and annealed. One series was based on a direct repeat of the GGTCA motif, the other on a palindromic repeat of the GGTCA motif. The spacing between the repeats in both series was varied by 0, 2, 5, 7, 9, and 12 nucleotides. The oligonucleotides were 29 nucleotides long;

to maintain identical lengths between each type, additional nucleotides were added 5′ and 3′ of the GGTCA motifs to compensate for spacer variations. The *Bam*HI site at the 5′ end and a *Bgl*II site at the 3′ end were added for cloning convenience. The oligonucleotide sequences are as follows:

N0 5′-gatccCTTAGGGGTCAGGTCAAATGGACa
5′-gatctGTCCATTTGACCTGACCCCTAAGg

N2 5′-gatccTTAGGGGTCAAAGGTCAAATGGAa
5′-gatctTCCATTTGACCTTTGACCCCTAAg

N5 5′-gatccTAGGGGTCAAATAAGGTCAAATGa
5′-gatctCATTTGACCTTATTTGACCCCTAg

N7 5′-gatccAGGGGTCAAATTCAAGGTCAAATa
5′-gatctATTTGACCTTGAATTTGACCCCTg

N9 5′-gatccGGGGTCAAATATCCAAGGTCAAAa
5′-gatctTTTGACCTTGGATATTTGACCCCg

N12 5′-gatccGGGGTCAAATATAGTCCAAGGTCAAa
5′-gatctTTGACCTTGGACTATATTTGACCCCg

N′0 5′-gatccCTTAGGGGTCATGACCTTTGGACa
5′-gatctGTCCAAAGGTCATGACCCCTAAGg

N′2 5′-gatccTTAGGGGTCAGCTGACCTTTGGAa
5′-gatctTCCAAAGGTCAGCTGACCCCTAAg

N′5 5′-gatccTAGGGGTCAGATACTGACCTTTGa
5′-gatctCAAAGGTCAGTATCTGACCCCTAg

N′7 5′-gatccAGGGGTCAGATTCACTGACCTTTa
5′-gatctAAAGGTCAGTGAATCTGACCCCTg

N′9 5′-gatccGGGGTCAGATATCCACTGACCTTa
5′-gatctAAGGTCAGTGGATATCTGACCCCg

N′12 5′-gatccGGGGTCAGATATAGTCCACTGACCTa
5′-gatctAGGTCAGTGGACTATATCTGACCCCg

The half-site oligonucleotide contains part of the ovalbumin promoter (−20 to −56), which contains a GGTCA half-site. The sequence is as follows.

Half-site
5′-cctgtGGGTGGGTCACAATTCAGACTATATATTCCCCAGagct
5′-CTGGGGAATATATAGTCTGAATTGTGACCCACCC

The oligonucleotides spanning the COUP element, −70 to −92, of the ovalbumin promoter (42) and oligonucleotides based on the PRE of the tyrosine aminotransferase gene (31) were also generated. The sequences of the oligonucleotides inserted into the TKCAT reporter to construct DR3 and DR5 are as follows:

DR3 5′-gatccGATTCAGGTCAAGGTAGGTCAGCACa
5′-gatctGTGCTGACCTCCTTGACCTGAATCg

DR5 5′-gatccGATTCAGGTCACCAGGAGGTCAGCa
5′-gatctGCTGACCTCCTGGTGACCTGAATCg

In vitro transcription and translation. For in vitro transcription, the plasmids pCOUP-TF I, pCOUP-TF II, and tCOUP-TF I were linearized with the enzymes *Eco*RI, *Cla*I, and *Aat*II, respectively. RNA was synthesized in vitro, using Sp6 RNA polymerase for COUP-TF I and tCOUP-TF I and T7 RNA polymerase for pCOUP-TF II, according to the manufacturer's instructions (Promega). After synthesis, the RNA was capped with GTP, *S*-adenosylmethionine, and guanylnyl transferase (Bethesda Research Laboratories), according to Promega's protocol. Five hundred nanograms of in vitro-synthesized RNA was translated in vitro in a rabbit reticulocyte lysate (Promega) with [^{35}S]methionine (1,120 Ci/mmol; ICN Biomedicals) or unlabeled methionine. After synthesis, $ZnCl_2$ was added to a final concentration of 0.5 mM. The in vitro-translated products were then analyzed directly either by electrophoresis in sodium dodecyl sulfate (SDS)-10% polyacrylamide gels or by electrophoretic mobility shift assays (EMSAs). The SDS-polyacrylamide gels were treated with ENHANCE (Dupont, NEN Research Products) before drying and fluorography.

EMSAs and partial proteolytic digestion. EMSAs were performed as previously described (28) except 2 to 4 μg of poly(dI-dC) · poly(dI-dC) (Pharmacia) was used as a nonspecific competitor instead of *Hin*fI-digested pBR322. For antibody upshift analysis, the antiserum was preincubated with the in vitro translation products at room temperature for 5 min before the addition of the probe. Oligonucleotides used as probes were end labeled and blunt ended with Klenow enzyme and the appropriate radionucleotide (29). For the partial proteolytic digestion, in vitro-translated COUP-TF I was bound to either the direct or palindromic repeat probes in an EMSA reaction for 15 min at room temperature. Then increasing amounts (0 to 5 μg/ml, final concentration) of trypsin, dissolved in H_2O (5 μg/ml, stock solution), were added to the EMSA reactions and incubated at room temperature for an additional 15 min. The reactions were stopped by the addition of leupeptin (50 μg/ml, final concentration) and soybean trypsin inhibitor (50 μg/ml, final concentration) (32). Each reaction mixture was then electrophoresed in a native 5% polyacrylamide gel in 0.5× Tris-borate-EDTA running buffer (1× TBE is 0.04 M Tris-acetate–0.001 M EDTA) at room temperature. The gels were then autoradiographed at −70°C with two Cronex Lightning-Plus intensifying screens. For competition analysis, the quantity of shifted probe was determined by laser densitometry by using an LKB Ultroscan XL densitometer which was used to scan the autoradiographs. To determine the molar excess of competitor that reduced the shifted probe by 50% compared with control lanes, the amount of probe shifted was plotted versus the amount of oligonucleotide used.

Transient transfections and CAT assays. Transcriptional activity was measured by transient transfection of derivatized TKCAT reporters into monkey kidney CV1 cells. Cells (10^6) were transfected by a modification of the Polybrene method (4). Cells were then grown in Dulbecco modified Eagle medium supplemented with Nutridoma (Boehringer Mannheim). The cells were incubated with or without ligand (T_3, 100 nM; retinoic acid [RA], 1 μM; 1,25 (dihydroxy) vitamin D_3, 100 nM). Chloramphenicol acetyltransferase (CAT) activities were determined 40 h after the cells were treated with 25% glycerol. Cell extracts were normalized to the total amount of protein, as determined by the Bradford assay (Bio-Rad), because the COUP-TF expression vector represses expression of β-galactosidase expression vectors. The CAT activities were quantitated by cutting the thin-layer chromatography plates and determining the radioactivity in each spot by scintillation counting. The fold induction was determined relative to the activity of each reporter in the absence of hormone. Samples (5 μg) of reporter constructs TRETKCAT, DR3TKCAT, DR4TKCAT, and DR5TKCAT were cotransfected with 2.5 μg of the expression plasmids pRShCOUP-TF I, pRShTRβ (34), pRShRARα (8), and pAV-hVDR (17). pRShCOUP-TF I was formed by cloning the full-length COUP-TF I cDNA (465 to 2010) downstream of the Rous sarcoma virus promoter in the Rous sarcoma virus eukaryotic expression vector.

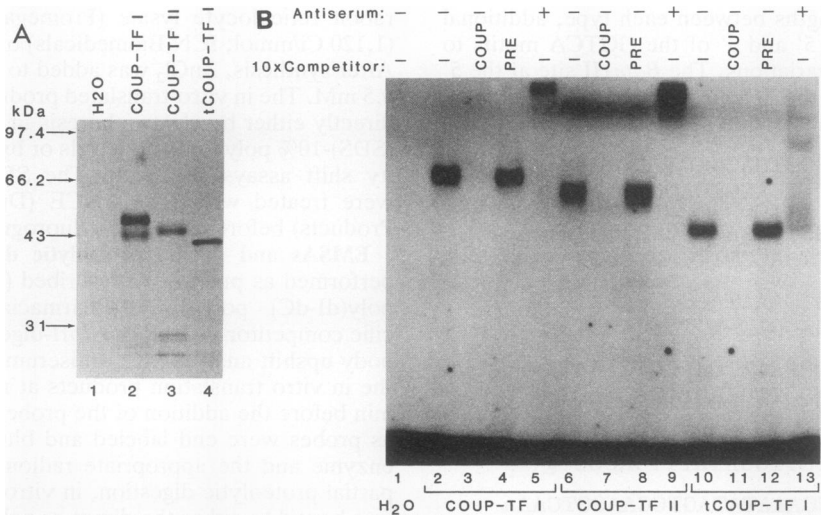

FIG. 2. Analysis of in vitro-synthesized COUP-TFs. (A) Analysis of in vitro-translated COUP-TFs by SDS-10% polyacrylamide gel electrophoresis as described in Materials and Methods. Lanes: 1, mock-translated reaction; 2, COUP-TF I; 3, COUP-TF II; 4, tCOUP-TF I. (B) Analysis of the DNA-binding and antigenic properties of in vitro-translated COUP-TFs by EMSA. The COUP oligonucleotide (Materials and Methods) was end labeled and used as the probe. Protein-DNA complex formation was inhibited with 10-fold molar excesses of unlabeled competitor COUP or PRE oligonucleotides. The in vitro-translated products were preincubated with a 1:5 dilution of the COUP-TF antiserum for 5 min before the addition of probe.

RESULTS

In vitro-synthesized COUP-TFs bind DNA specifically in a COUP element-dependent manner. To characterize the binding of COUP-TFs to spatial variants of the GGTCA repeats and to determine how a stable dimer can bind to structurally distinct elements, we synthesized COUP-TFs in vitro for analysis of their DNA-binding properties. COUP-TF I, COUP-TF II, and tCOUP-TF I (a truncated COUP-TF with the first 52 amino acids deleted) were synthesized in a coupled in vitro transcription-translation system (Fig. 2A). The mock-translated control produced no detectable product (Fig. 2A, lane 1). In vitro translation yielded polypeptides of the expected sizes; however, COUP-TF I was translated as two polypeptide species (Fig. 2A, lane 2). The major species is a polypeptide of the expected molecular weight of 47,000. The minor species probably arises from internal initiation of translation or limited degradation.

The in vitro-synthesized COUP-TFs were analyzed by EMSA (Fig. 2B) to determine whether their DNA-binding properties and antigenic characteristics were similar to those of native COUP-TFs (3, 41). The specificity of DNA binding was assessed by competition of binding to the COUP oligonucleotide probe with excess unlabeled oligonucleotides (Fig. 2B). It was evident that each of the in vitro-synthesized COUP-TFs could bind to DNA specifically in a COUP-dependent fashion, since excess unlabeled COUP oligonucleotides (lanes 3, 7, and 11), but not PRE oligonucleotides (lanes 4, 8, and 12), could eliminate the retarded complexes. In addition, the in vitro-synthesized COUP-TFs were recognized by COUP-TF antiserum, as the mobility of the DNA-protein complexes was decreased upon addition of antiserum (Fig. 2B, lanes 5, 9, and 13). Thus, the in vitro-synthesized COUP-TFs have DNA-binding specificities and antigenic determinants indistinguishable from those of HeLa COUP-TFs (3, 41) and are suitable for our in vitro binding studies.

COUP-TFs can bind to direct and palindromic repeats of GGTCA motifs with variable spacing. The response elements for COUP-TFs have been identified in the promoters of many genes (Fig. 1A). Comparison of these sequences showed that repeats of the GGTCA pentamer are the common motif in these elements. This was confirmed by methylation interference analysis of several of these elements, which showed that the COUP-TF dimer makes contacts with both half-sites (Fig. 1B). From these observations, it appeared that COUP-TF displayed a promiscuous ability to bind to different spatial arrangements of the GGTCA repeat. To determine, in a methodical fashion, the relative binding affinity of COUP-TFs for different orientations and spacings of the GGTCA motif, we synthesized two series of oligonucleotides. One series was based on a direct repeat of the GGTCA core motif, with spacings of 0, 2, 5, 7, 9, and 12 nucleotides. The spacings were defined from the last A of the 5′ GGTCA to the first G of the 3′ GGTCA. The other series was based on a palindromic repeat of the core motif, with spacings similar to the direct repeats, but defined from the last A of the 5′ GGTCA to the first T of the 3′ TGACC (see Materials and Methods). The spacings between the GGTCA repeats were chosen to reflect the observed spacings in natural COUP elements.

To determine the relative binding affinity of COUP-TFs for each of these oligonucleotides, the N2 direct repeat, the predominant element found in vivo, was end labeled and used as a probe in competition analysis. Figure 3A shows a representative competition experiment used to determine the relative binding affinity of COUP-TF I for each of the oligonucleotides. The amounts of each competing unlabeled oligonucleotide were empirically determined to calculate the molar excess of competitor required to reduce the quantity of shifted protein-DNA complex by 50%. The binding affinity of COUP-TFs for each oligonucleotide was determined relative to competition by the N2 direct repeat, which was designated 100%. These experiments were repeated three times for COUP-TF I, COUP-TF II, and the heterodimer of tCOUP-TF I and COUP-TF II. The heterodimer of tCOUP-TF I and COUP-TF II was formed by cotranslation

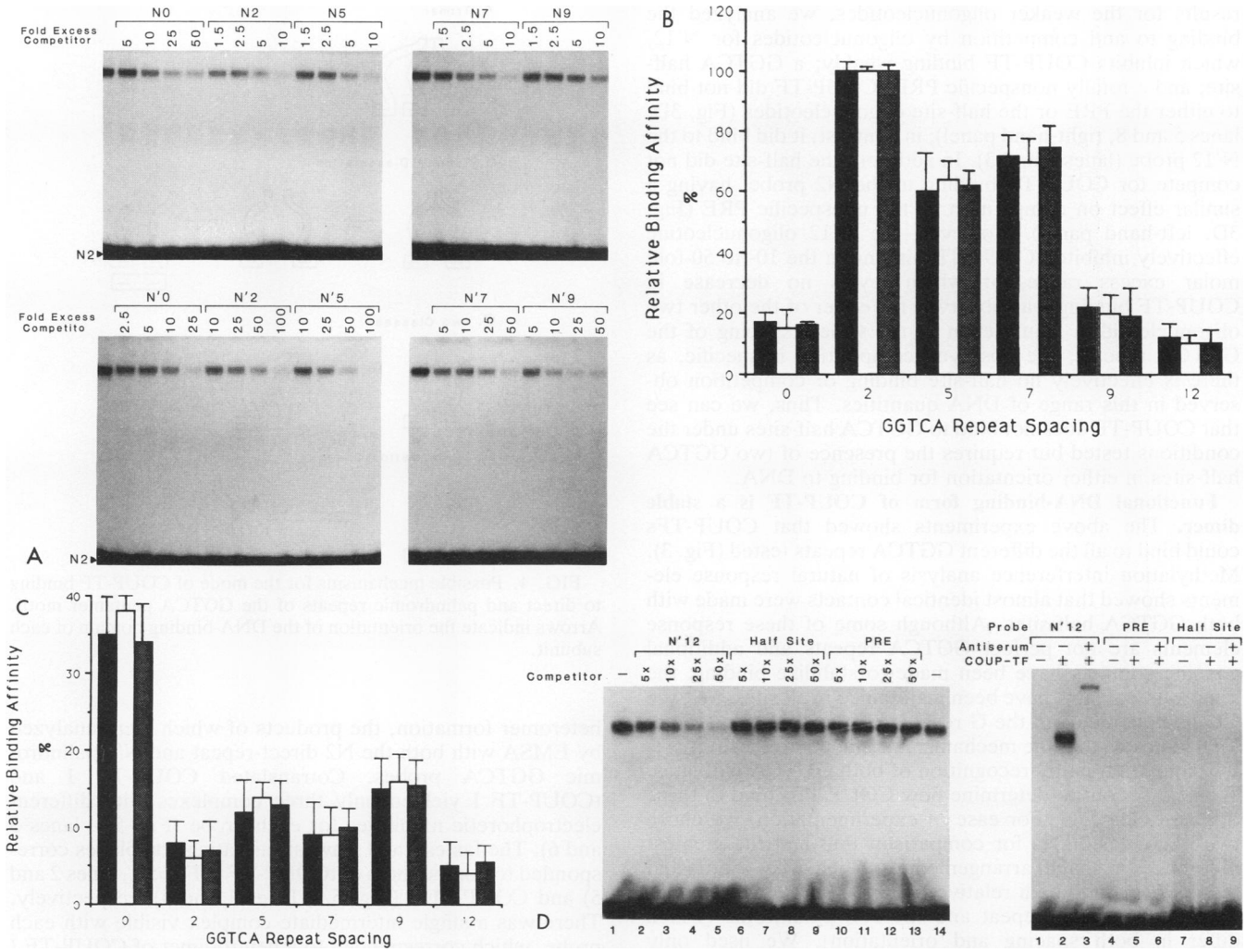

FIG. 3. Analysis of COUP-TF binding to a series of oligonucleotides containing variations in the orientation and spacing of the GGTCA pentamer motif. (A) Competition analysis of COUP-TF I binding to the N2 direct-repeat probe. Binding was inhibited with an increasing molar excess of each of the other oligonucleotides in the EMSA. The upper two panels are competition analysis with the direct-repeat series of oligonucleotides (N0 to N9), and the lower two panels are competition analysis with the palindromic series of oligonucleotides (N'0 to N'9). (B and C) Summary of the relative DNA-binding affinities of COUP-TFs for the direct and palindromic series of oligonucleotides. (B) Bar graph of the relative binding affinities of COUP-TF I, COUP-TF II, and the tCOUP-TF I–COUP-TF II heterodimer for the direct-repeat series of oligonucleotides. (C) Bar graph of the relative binding affinities of COUP-TF I, COUP-TF II, and the tCOUP-TF I–COUP-TF II heterodimer for the palindromic series of oligonucleotides. Relative binding affinity was determined with respect to competition by the N2 direct-repeat oligonucleotide, which was designated 100%. Black, open, and hatched bars represent COUP-TF I, COUP-TF II, and the COUP-TF I–COUP-TFII heterodimer, respectively. The error bars represent standard deviations. (D) Analysis of COUP-TF binding to a GGTCA half-site. Left-hand panel, competition analysis of COUP-TF binding to the N2 probe. COUP-TF binding was incubated with an increasing molar excess of each of the unlabeled oligonucleotides, which includes the N'12, half-site, and PRE oligonucleotides. Right-hand panel, direct EMSA analysis of COUP-TF binding to the labeled N'12, PRE, and half-site oligonucleotides and COUP-TF antiserum upshift of retarded complexes.

of their respective mRNAs, which yielded three easily distinguishable retarded complexes when analyzed by EMSA, corresponding to the two homodimers and the intermediate heterodimer (data not shown). A summary of the results of these experiments is shown in Fig. 3B and C.

The binding of COUP-TFs to the N2 direct-repeat probe was inhibited by all the oligonucleotides containing the different repeat orientations and spacings of the GGTCA motif, yielding relative binding affinities ranging from 5 to 100%. There were no significant differences between the relative binding affinities of COUP-TF I, COUP-TF II, and the COUP-TF I and II heterodimer for each of the spatial variants (Fig. 3B and C). COUP-TFs had the following order of relative binding affinities for the direct-repeat series: N2 > N7 > N5 > N9 > N0 > N12. COUP-TFs had the following order of relative binding affinities for the palindromic series: N'0 > N'9 > N'5 > N'7 > N'2 > N'12. COUP-TFs generally displayed higher relative binding affinities for direct repeats compared with palindromes.

Some of the oligonucleotides, such as N12 and N'12, only inhibited from 5 to 20% of the level of N2 itself. At the limit of the repeat spacing, COUP-TF may essentially bind only to individual half-sites. To determine the contribution of half-site binding by COUP-TF to the observed competition

results for the weaker oligonucleotides, we analyzed the binding to and competition by oligonucleotides for N'12, which inhibits COUP-TF binding weakly; a GGTCA half-site; and a totally nonspecific PRE. COUP-TF did not bind to either the PRE or the half-site oligonucleotides (Fig. 3D, lanes 5 and 8, right-hand panel); in contrast, it did bind to the N'12 probe (lanes 2 and 3). In addition, the half-site did not compete for COUP-TF binding to the N2 probe, having a similar effect on competition as the nonspecific PRE (Fig. 3D, left-hand panel). However, the N'12 oligonucleotide effectively inhibited COUP-TF binding in the 10- to 50-fold molar excess range, at which levels no decrease in COUP-TF binding was observed for either of the other two oligonucleotides. Thus, even at the widest spacing of the GGTCA repeats, the observed competition is specific, as there is effectively no half-site binding or competition observed in this range of DNA quantities. Thus, we can see that COUP-TF does not bind to GGTCA half-sites under the conditions tested but requires the presence of two GGTCA half-sites in either orientation for binding to DNA.

Functional DNA-binding form of COUP-TF is a stable dimer. The above experiments showed that COUP-TFs could bind to all the different GGTCA repeats tested (Fig. 3). Methylation interference analysis of natural response elements showed that almost identical contacts were made with both GGTCA half-sites. Although some of these response elements are not perfect GGTCA repeats and additional guanine contacts have been made to stabilize binding, the important contacts have been maintained in all sites, i.e., the GG dinucleotide and the G residue on the opposing strand. This indicates that the mechanism of binding to these sites is the same, that is, by recognition of both GGTCA half-sites. We next set out to determine how COUP-TFs bind to these different elements. For ease of experimentation, we chose two oligonucleotides for comparison that had the greatest differences in spatial arrangement of the GGTCA motifs and that still retained high relative binding affinities for COUP-TF: the N2 direct repeat and the N'0 palindrome (which differ in both spacing and orientation). We used only COUP-TF I as the model for these experiments, as no significant binding preferences were observed between COUP-TF I, COUP-TF II, or the heterodimer. A number of proposed mechanisms are illustrated in Fig. 4 that would permit binding to opposite orientations of the GGTCA repeat.

HeLa COUP-TFs exist in solution as dimers (28). However, we wanted to rule out the possibility that the functional DNA-binding form of the translated receptor is not a dimer but a trimer. In mechanism A (Fig. 4), COUP-TF forms a DNA-binding trimer, in which the subunits associate via alternate dimerization interfaces, allowing each pair in the trimer to bind only to direct or palindromic repeats. It has been determined previously that the heat shock response element is a complex element consisting of a 5-bp core sequence in multiple copies and orientations. It was also demonstrated that heat shock transcription factor binds to these elements as a trimer, which allows it to bind to tail-to-tail and head-to-head orientations and other combinations of these elements (30). To test this model, trimer formation by COUP-TF should be detectable upon cotranslation of full-length and truncated COUP-TF. Employing EMSA, trimer formation should yield four species with different electrophoretic mobilities, based on the sizes of the possible combinations of monomer subunits: two homotrimers and two intermediate heterotrimers. COUP-TF I and tCOUP-TF I RNAs were cotranslated in vitro to allow

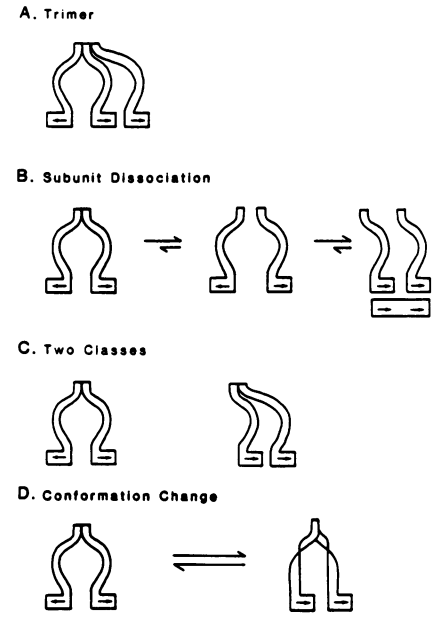

FIG. 4. Possible mechanisms for the mode of COUP-TF binding to direct and palindromic repeats of the GGTCA pentamer motif. Arrows indicate the orientation of the DNA-binding domain of each subunit.

heteromer formation, the products of which were analyzed by EMSA with both the N2 direct-repeat and N'0 palindromic GGTCA probes. Cotranslated COUP-TF I and tCOUP-TF I yielded only three complexes with different electrophoretic mobilities for each probe (Fig. 5A, lanes 3 and 6). The fastest- and slowest-migrating complexes corresponded to homodimers of tCOUP-TF I (Fig. 5A, lanes 2 and 5) and COUP-TF I (Fig. 5A, lanes 1 and 4), respectively. There was a single intermediate complex visible with each probe, which corresponded to a heterodimer of COUP-TF I and tCOUP-TF I. This result shows that COUP-TF I does not bind to DNA as a trimer and corroborates previous

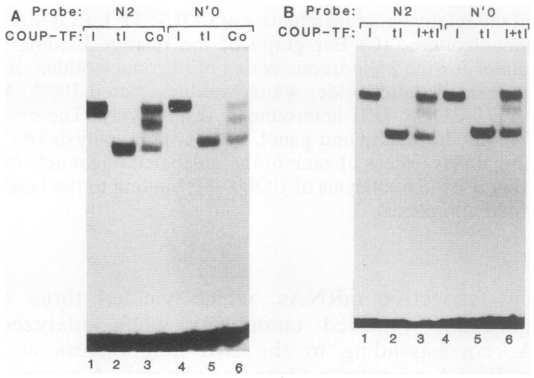

FIG. 5. Analysis of COUP-TF binding to direct and palindromic repeats of the GGTCA motif. (A) EMSA of cotranslated COUP-TF I and tCOUP-TF I binding to the N2 direct-repeat and the N'0 palindromic-repeat oligonucleotides. Lanes: 1 and 4, COUP-TF I; 2 and 5, tCOUP-TF I; 3 and 6, cotranslated COUP-TF I and tCOUP-TF I. (B) EMSA of a mixture of separately translated COUP-TF I and tCOUP-TF I for binding to the N2 direct-repeat and N'0 palindromic-repeat probes. Lanes: 1 and 4, COUP-TF I; 2 and 5, tCOUP-TF I; and 3 and 6, mixture of COUP-TF I and tCOUP-TF I.

biochemical studies showing that COUP-TFs exist as stable dimers in solution (28).

These results, however, do not rule out the possibility outlined in mechanism B (Fig. 4) which proposes that COUP-TF undergoes dissociation and reassociation of the dimer, which would permit COUP-TF to bind as a monomer and dimerize after binding to DNA, and/or associate via two different dimerization domains with different specificities for the orientation of the repeats. In fact, it has been reported that under certain conditions TR can bind to its response element as a monomer (16). To assess this possibility, COUP-TF I and tCOUP-TF I were translated separately and then mixed and immediately analyzed by EMSA with both the N2 direct-repeat and N'0 palindromic probes. If the COUP-TF dimers dissociate prior to binding, then heterodimers should form after mixing, as observed for cotranslation (Fig. 5A). When independently translated COUP-TF I and tCOUP-TF I were mixed, there was only minor heterodimer formation on either probe (Fig. 5B, lanes 3 and 6), compared with the heterodimer formation observed upon cotranslation of these two species (Fig. 5A, lanes 3 and 6). The small amount of heterodimer formed probably derives from subunit exchange; however, the lack of equimolar amounts of heterodimer shows that dissociation of the dimer is not an obligatory requirement for binding to either response element. Therefore, our results indicate that there is no significant dissociation or reassociation of COUP-TF dimers prior to binding to these elements. We conclude that the ability of COUP-TF to recognize and bind to direct or palindromic GGTCA repeats is not dependent on dissociation of the stable dimers prior to DNA binding.

COUP-TF does not exist as two independent DNA-binding species in solution. Results obtained to this point indicate that the functional DNA-binding entity of COUP-TF is a stable dimer which must be capable of binding to different spatial arrangements of the GGTCA repeat. Binding could be achieved by two possible mechanisms: either more than one distinct dimer species exists in solution or there is structural flexibility within a single dimer species. Mechanism C (Fig. 4) proposes that promiscuous DNA recognition could be achieved if COUP-TF exists as two distinct noninterchangeable species of stable dimer, in which the subunits associate via alternate dimerization domains. One species would be capable of binding only to direct repeats, and the other would recognize only palindromic repeats. To explore this possibility, we analyzed COUP-TF binding to both N2 direct and N'0 palindromic probes by cross-competition with homologous and heterologous unlabeled oligonucleotides (Fig. 6). The probes and in vitro-synthesized COUP-TF were preincubated for 5 min to allow formation of the COUP-TF–DNA complex before the addition of unlabeled oligonucleotides. If COUP-TF exists as two noninterchangeable species of dimer, then the complex formed on one probe would not be eliminated when challenged with heterologous unlabeled oligonucleotides corresponding to the other probe. As expected, protein-DNA complexes that were formed with both probes (Fig. 6, lanes 1 and 6) were eliminated when challenged with the homologous unlabeled oligonucleotides (Fig. 6, lanes 2 and 5). In addition, cross-competition with the heterologous unlabeled oligonucleotides also eliminated formation of the retarded complex (Fig. 6, lanes 3 and 4). These results agree with our previous competition analyses (Fig. 3), which also showed palindromes successfully competing for COUP-TF binding to the N2 direct repeat. Cross-competition between direct and palindromic GGTCA repeats indicates that the COUP-TF species bound to each

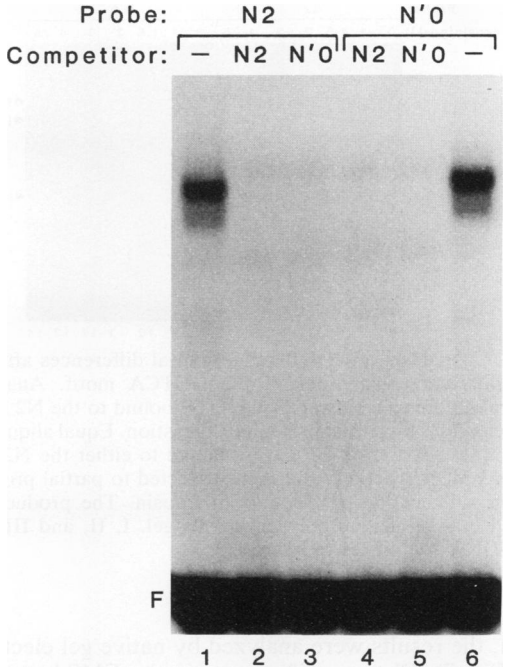

FIG. 6. Analysis of cross-competition of COUP-TF binding to the N2 direct-repeat and N'0 palindromic-repeat probes by EMSA. Tenfold molar excesses of N2 (lanes 2 and 4) or N'0 (lanes 3 and 5) unlabeled oligonucleotides were incubated with in vitro-synthesized COUP-TF I after addition of the probes to the EMSA reactions. Lanes: 1 to 3, N2 probe; 4 to 6, N'0 probe.

probe retains the ability to recognize and bind to both direct and palindromic repeats. This observation was confirmed by EMSA of COUP-TFs affinity purified over a column containing an N2 direct-repeat oligonucleotide. COUP-TFs so purified still retained the ability to bind to the N'0 palindromic probe (data not shown). Thus, COUP-TF exists as a single species of stable dimer that is capable of recognizing and binding to both direct and palindromic repeats of the GGTCA motif or exists as two dimer forms which are interchangeable but without first dissociating into monomers.

Structural adaptation underlies the ability of COUP-TF dimers to bind to direct and palindromic repeats of the GGTCA motif. Having ruled out mechanisms A to C (Fig. 4), we are left with the possibility that the COUP-TF dimer is remarkably flexible and undergoes structural adaptation to accommodate binding to the different GGTCA spatial variants (Fig. 4, mechanism D). The first indication that the quaternary structure of COUP-TFs bound to each of the spatial variants was different was observed when COUP-TF binding to each oligonucleotide was analyzed by EMSA. The mobility of each COUP-TF:DNA complex was slightly different and was probably due to both structural differences between the COUP-TF dimers bound to the different GGTCA repeats and differences in DNA bending exerted by COUP-TF on the various oligonucleotides (data not shown). To examine these potential structural changes more directly, we used limited proteolytic digestion in conjunction with EMSA (32). In vitro-translated COUP-TF I was incubated with the N2 direct-repeat and N'0 palindromic-repeat probes under the conditions used above and then subjected to partial proteolytic digestion with increasing amounts of

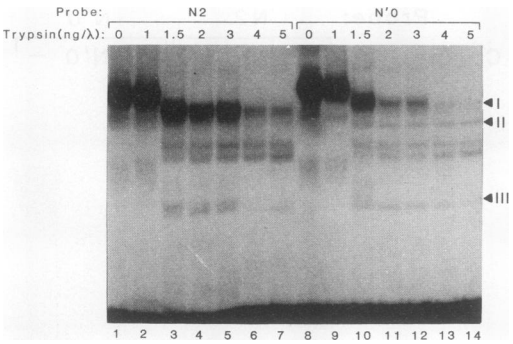

FIG. 7. Analysis of COUP-TF structural differences after binding to different orientations of the GGTCA motif. Analysis of structural differences between COUP-TF bound to the N2 and N'0 oligonucleotides by partial proteolytic digestion. Equal aliquots of in vitro-synthesized COUP-TF I were bound to either the N2 or N'0 probe in EMSA reactions and then subjected to partial proteolytic digestion with increasing amounts of trypsin. The products were analyzed in a 5% native polyacrylamide gel. I, II, and III denote protein-DNA complexes of interest.

trypsin; the results were analyzed by native gel electrophoresis (Fig. 7). Obvious differences in the EMSA pattern of partially digested COUP-TF were observed when bound to the N2 direct repeat (Fig. 7, lanes 1 to 7) compared with the N'0 palindromic repeat (Fig. 7, lanes 8 to 14). COUP-TF I bound to the palindrome is significantly more susceptible to tryptic digestion than that bound to the direct repeat; this is most clearly observed for complex I. There were two additional smaller complexes, labeled II and III, which differ when COUP-TF was bound to the palindromic or direct repeat probes. Complex II is more evident with the N'0 probe, although it may be obscured by the strong complex I band on the N2 probe. However, comparing lanes 6 and 7 with lanes 13 and 14, complex II is more resistant to proteolytic digestion when bound to N'0 than when bound to N2. Complex III appears as a doublet on the N2 probe but only as a singlet on the N'0 palindromic probe. These differences in the proteolytic patterns are striking and are indicative of significant structural differences between the COUP-TF dimers bound to these two probes. These structural differences are consistent with the proposed mechanism by which the COUP-TF dimer binds to the different GGTCA repeats by structural adaptation.

COUP-TF-dependent down-regulation of hormonal induction of target gene expression by VDR, TR, and RAR. Having established that COUP-TF can bind to spatial variants of the GGTCA repeat as a dimer by structural adaptation, the next problem to address is the functional consequence of these variations on the transcriptional activity of COUP-TF. COUP-TF is an orphan receptor for which a postulated ligand has not yet been identified. Thus, we are unable to analyze the effect of GGTCA repeat variations on ligand-induced transactivation, as has been done for TR, RAR, and VDR (23, 39). Since COUP-TF binds to the N'0 palindrome, which is a response element for RAR and TR (38), as an alternative approach, we wanted to determine whether COUP-TF I could affect TR and RAR function on such a reporter. Thus, we cotransfected expression vectors for COUP-TF I and/or TR and RAR with TRETKCAT in the presence or absence of hormone (Fig. 8A and B). When retinoic acid was added to the cells cotransfected with RAR, we observed a hormonally dependent induction of transcription (Fig. 8A, lanes 2 and 3). This hormonal induction of target gene expression by RAR was inhibited by cotransfection of COUP-TF I (Fig. 8A, lanes 3 and 4). Inhibition of hormonally induced transcription was also observed with TR (Fig. 8B). Thyroid hormone stimulated transcription of TRETKCAT 8-fold (Fig. 8B, lanes 2 and 3), and COUP-TF

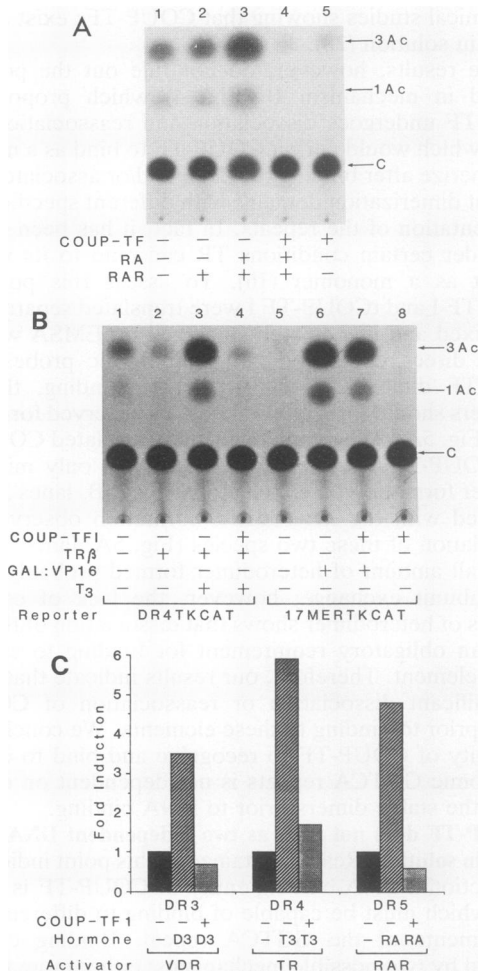

FIG. 8. COUP-TF-dependent down-regulation of hormonal induction of target gene expression by RAR, TR, and VDR. (A) Single copy of the TRE palindrome (N'0) oligonucleotide was cloned 5' of the thymidine kinase promoter (−107 to +54) fused to a CAT reporter (TKCAT) and cotransfected with RAR (pRShRARα) and COUP-TF I (pRShCOUP-TF I) expression vectors into CV1 cells. After hormone treatment for 40 h, the cells were harvested and CAT activities were analyzed. (B) TRETKCAT reporter was cotransfected with TR (pRShTRβ) and COUP-TF (pRShCOUP-TF I) in the presence or absence of hormone (T_3). In a parallel experiment, pABGal:VP16 was cotransfected with its reporter 17-mer TKCAT in the presence or absence of pRShCOUP-TF I. The [^{14}C]chloramphenicol and the 1- and 3-acetylated forms of [^{14}C]chloramphenicol are indicated by C, 1Ac, and 3Ac, respectively. (C) Single copies of the GGTCA direct-repeat oligonucleotides DR3, DR4, and DR5 (N4, N5, and N6, respectively) were cloned 5' of TKCAT and cotransfected with VDR (pAV-hVDR), TR (pRShTRβ), RAR (pRShRARα), and COUP-TF (pRShCOUP-TF I) expression vectors into CV1 cells as above. These results represent the average of duplicate transfections differing by less than 10% and are representative of at least seven experiments with different DNA preparations.

inhibited this induction 15-fold (lanes 3 and 4). To demonstrate that this inhibition of hormonal induction was specific, we analyzed the effect of COUP-TF I on the transcriptional activation of the 17-mer TKCAT reporter by a Gal:VP16 chimer [Gal4 (amino acids 1-147:VP16]. Gal:VP16 also induces transcription of its cognate reporter (Fig. 8B, lanes 5 and 6); however, cotransfection of COUP-TF I only represses this induction twofold. The repression is overrepresented because the COUP-TF I expression vector is cotransfected at a 50-fold excess over that of Gal:VP16, whereas in all other experiments, it is cotransfected in amounts equal to those of the other expression vectors. When lower amounts of the COUP-TF expression vector were used (50 ng), so that an equal amount was coexpressed with the Gal:VP16 expression vector, then no effect of COUP-TF on Gal:VP16 transactivation was observed (data not shown). This result indicates that the repression of thyroid hormone and RA-dependent transcription by COUP-TF is not due to a nonspecific effect such as affecting either the growth of CV1 cells or the expression of a general transcription factor. The small amount of repression of Gal:VP16 activity may be due to squelching of a general transcription factor. Thus, COUP-TF can specifically inhibit hormonally induced transcription directed by palindromic response elements.

Recently, Umesono et al. (39) have shown that the specific response elements for VDR, TR, and RAR are direct repeats of the GGTCA motif with spacings of 3, 4, and 5 bp (equivalent to N4, N5, and N6), referred to as DR3, DR4, and DR5. This raises the possibility that COUP-TF also plays a role in modulating receptor activation of these reporters since COUP-TF can bind with relatively high affinity to direct repeats with this range of spacings (Fig. 3). To analyze this possibility, we cotransfected expression vectors encoding COUP-TF I with VDR, TR, or RAR in the presence or absence of their cognate hormones to determine the effect on transcription from TKCAT reporters containing their respective oligonucleotides. VDR-, TR-, and RAR-dependent transcription was stimulated 3.5-, 6-, and 5-fold, respectively, upon addition of their cognate hormones. This activation was repressed 4.5-, 4-, and 8-fold, respectively, in the presence of COUP-TF (Fig. 8C). Therefore, COUP-TF displays a potent ability to inhibit hormonal induction of target genes by VDR, TR, and RAR.

DISCUSSION

The GGTCA motif has been proposed to be the primordial DNA-binding element for the steroid-thyroid hormone receptor superfamily, as it is the target element of the ecdysone receptor in the *Drosophila* hsp27 promoter (20). Many members of this superfamily, including TR, RAR, VDR, and several orphan receptors, can bind to this core element. The genes encoding these receptors are expressed with an overlapping tissue distribution and developmental pattern, making it unclear how distinct sets of genes are regulated by these receptors. Recent evidence suggests that, in addition to the primary sequence, the orientation and spacing of the GGTCA motifs determine the specificity of hormonal regulation of distinct but overlapping sets of genes (23, 39). Umesono et al. (39) have shown that VDR, TR, and RAR specifically activate transcription of genes containing GGTCA direct repeats with spacings of 3, 4, and 5 bp, respectively (i.e., equivalent to N4, N5, and N6). Thus, the ability of a receptor to recognize, discriminate, and bind to repeat variants of the GGTCA core sequence is critical to its function.

Analysis of natural COUP-TF response elements has shown that COUP-TF can bind to elements with different spacing and orientation of the GGTCA motif repeat (Fig. 1), which is corroborated by our results shown in this report (Fig. 3). COUP-TF requires at least two GGTCA motifs in either relative orientation to bind to DNA, because we cannot detect appreciable binding to the GGTCA half-site. This agrees with our previous mutational analyses of the COUP element, which showed that two intact GGTCA half-sites are required for COUP-TF binding (12), and the methylation interference summary in Fig. 1B, which shows COUP-TF dimers contact both half-sites equally. Thus, COUP-TF must bind the different GGTCA repeats as a complete functional unit, in contrast to the binding of GR to a suboptimal GRE with a 4-bp spacing. GR makes specific contacts with only one half-site and nonspecific contacts with the other half-site to stabilize binding (19).

COUP-TF I, COUP-TF II, and heterodimers of these two species exhibited similar relative binding affinities for all the GGTCA spatial variants tested (Fig. 3). This was not unexpected since the amino acid identity between the DNA-binding domains of these two transcription factors is very high (98%). Thus, any functional differences between these two factors probably lies outside of their ability to recognize and bind to DNA response elements. As expected, the GGTCA spatial variant for which COUP-TF has the greatest relative binding affinity is the direct repeat with a 2-bp spacing, which is the predominant element to which COUP-TF has been shown to bind in vivo (Fig. 1). In addition, we demonstrated that COUP-TF is capable of binding to all the spatial variants of the GGTCA motif tested and displays significant relative binding affinities for direct repeats with 5-, 7-, and 9-bp spacers (60, 75, and 28%, respectively) and to a palindromic repeat with no spacing (34%) (Fig. 3). The functional relevance of elements for which COUP-TF has a 5- to 10-fold-lower binding affinity is, at present, unknown.

Other members of the steroid-thyroid receptor superfamily, TR and RAR, can bind to GGTCA palindromes and direct repeats, which have been shown to be the functional elements for these receptors (23, 39). TR can bind as a monomer or a dimer depending on the conditions (16, 39). COUP-TF, however, exists as a stable dimer in solution (28). This raised the question of how a steroid-thyroid hormone receptor family member, which binds to an element as a dimer, can accommodate the structural alterations required to permit binding to these widely differing elements. A number of mechanisms were proposed. Mechanisms A and B (Fig. 4) depend on the possibility that the functional DNA-binding form is not a dimer; rather, it is either a trimer or a monomer, which may have gone undetected in previous biochemical studies. Both mechanisms A and B require COUP-TF to have two dimerization interfaces. The results showed that the functional COUP-TF DNA-binding entity is neither a trimer nor a monomer but that it is a dimer which does not undergo significant dissociation and reassociation (Fig. 5). These results are consistent with our previous biochemical studies (28, 42). Thus, a mechanism was proposed that would enable COUP-TFs to bind to spatial GGTCA variants as a stable dimer. Mechanism C (Fig. 4) postulates that COUP-TFs exist as two distinct stable non-interchangeable dimers that associate via alternate dimerization interfaces which thereby determine the spatial orientation recognized. Cross-competition analysis clearly showed that COUP-TF bound to either direct or palindromic repeats retained the ability to recognize the other response element

(Fig. 6), proving that COUP-TF does not exist as two separate species of dimer. Instead, COUP-TF must exist as a single species of dimer capable of binding to all the variants of the GGTCA motif tested.

To achieve promiscuous DNA binding within a single species of stable dimer, the conformation and association of COUP-TF dimers must be flexible enough to accommodate the structural changes required. The ability of COUP-TF to bind to different response elements in the chicken ovalbumin and the rat insulin II promoters, which contain different spacings of the GGTCA direct repeats, has been previously studied (12, 13). Purine and phosphate contact points encompassed the GGTCA direct repeats, but were different for the two elements. Computer modeling of this data predicted that COUP-TF would wrap around the COUP element but, in contrast, would be confined to one face of the helix and more extended on the rat insulin RIPE I element (13). These differences in DNA binding suggested significant structural differences between the COUP-TF dimers bound to these two elements, which would be programmed by the helical periodicity of the GGTCA repeats. Analysis of COUP-TF by partial tryptic digestion, in combination with EMSA (Fig. 7), showed that the COUP-TF dimer bound to different GGTCA variants has distinctly different susceptibilities to tryptic digestion, indicative of different dimer structures. Thus, COUP-TF must undergo considerable structural adaptation to accommodate binding to both direct and palindromic repeats with different spacings. We envision a structural change within the COUP-TF dimer that either allows one of the DNA-binding domains to rotate 180 degrees with respect to the other or permits a 90-degree rotation of both DNA-binding domains while maintaining dimerization through a single interface. However, we cannot rule out the possibility that COUP-TF has two closely spaced dimerization interfaces between which the subunits slide without significant dissociation of the dimer.

In contrast to the promiscuous DNA binding displayed by COUP-TF, other members of this superfamily of receptors have a very strict requirement for the appropriate orientation and spacing of the core binding motif. For example, ER, GR, and PR have strong binding affinities only for a palindrome with a 3-bp spacing. These differences may be due to size considerations and the nature of their dimerization domains. COUP-TF is one of the smallest members of this superfamily, having a relatively short amino-terminal domain compared with ER, GR, and PR. This structure could permit promiscuous DNA binding because there might be less steric hindrance from the smaller domain, allowing the freedom required for any structural changes in the flexible hinge region. In addition, it has been shown that COUP-TFs lack a dimerization domain in the DNA-binding domain (15) which has been observed in other receptors (ER [14], GR [19, 35], and PR [2]). The absence of this structure may contribute to the freedom of binding to spatial variants. This dimerization domain may restrict the ability of GR and PR to bind only to palindromes with a 3-bp spacing.

The functional consequence of promiscuous DNA binding is apparent in the ability of COUP-TF to down-regulate hormonal induction of target gene expression. In all cases, we observed hormone-dependent activation by VDR, TR, and RAR of their cognate reporters which was inhibited by cotransfection of COUP-TF I (Fig. 8). The ability of COUP-TF to bind to diverse spatial arrangements of the GGTCA repeats permits it to repress transcriptional activation by this subset of steroid-thyroid hormone receptors on both palindromes and direct repeats. In addition to competitive binding, the mechanism of repression may also involve the formation of inactive heterodimers with the receptors themselves or their coregulator retinoid X receptor (RXR). The observed repression is specific as COUP-TF has little effect on transcriptional activation by Gal:VP16. However, as there is no effective way to quantitate the amounts of COUP-TF expressed in CV1 cells relative to the other receptors and Gal:VP16, we cannot therefore entirely rule out the possibility that squelching is the cause of the repression. RA, thyroid hormone and 1,25 (dihydroxy) vitamin D_3 are hormones that have potent effects on vertebrate development and cellular differentiation and homeostasis. The morphogen RA has dramatic effects on development, as it can establish the anterior-posterior axis in the developing chick limb bud (33). Thyroid hormone is involved in cellular homeostasis, while 1,25 (dihydroxy) vitamin D_3 plays a critical role in skeletal bone remodeling (24). This raises potentially significant implications for the role of COUP-TF in development and differentiation.

One of the functions of COUP-TF may be to modulate the response of target genes to hormones such as RA, thyroid hormone, and 1,25 (dihydroxy) vitamin D_3 by repressing transcriptional activation. Seven up (the *Drosophila* homolog of COUP-TF) plays a critical role in photoreceptor cell differentiation (22). COUP-TFs may have a dual role in development and differentiation. They may regulate specific developmental pathways, such as for the photoreceptor cells, directly through a putative ligand. Or alternatively, as the data presented here suggest, its developmental role may also involve cross-talk between COUP-TF and the developmental pathways controlled by RA, thyroid hormone, and 1,25 (dihydroxy) vitamin D_3; in these cases, COUP-TF may inhibit specific hormonal responses of their cognate receptors, thus altering cell fate. The functional implications raised by these data are presently under investigation, especially the mechanism of the functional repression by COUP-TF of hormonal induction of gene expression by this subgroup of receptors and its role in differentiation and development.

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