

Negative Feedback Control of the Retinoid-Retinoic Acid/Retinoid X Receptor Pathway by the Human TR4 Orphan Receptor, a Member of the Steroid Receptor Superfamily*

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Amino acid sequence analysis indicates that the human TR4 orphan receptor (TR4) is a member of the estrogen/thyroid receptor subfamily of the steroid/thyroid receptor superfamily and recognizes the AGGTCA direct repeat (DR) of the hormone response element. Here we demonstrate using the electrophoretic mobility shift assay that TR4 binds specifically to DR with a spacing of 1 and 5 base pairs (DR1 and DR5), which are the response elements for retinoic acid receptor (RAR) and retinoid X receptor (RXR), respectively. A reporter gene assay using chloramphenicol acetyltransferase demonstrated that TR4 repressed RA-induced transactivation in a TR4 dose-dependent manner. Inhibition of the retinoid signal pathway also occurs through natural response elements found in CRBPII and RAR β genes. Our data suggest that the mechanism of repression may not involve the formation of functionally inactive heterodimers between TR4 and RAR or RXR. Instead, we show that TR4 may compete for hormone response elements with RAR and RXR due to its higher binding affinity. Furthermore, treatment of F9 murine teratocarcinoma (F9) cells with 10^{-6} M all-*trans*-retinoic acid increased TR4 mRNA levels, and this change was accompanied by an increased amount of endogenous TR4 protein that can bind to RXRE in electrophoretic mobility shift assay. Our data therefore strongly suggest that the retinoid signal pathway can be regulated by TR4 in a negative feedback control mechanism, which may restrict retinoic acid signaling to certain elements in a cell-specific fashion.

The steroid/thyroid hormone receptor superfamily is a large group of related transcriptional factors that control cellular differentiation, development, and homeostasis by direct interaction with distinct *cis*-elements in target genes (1). This superfamily includes receptors for steroids, thyroid, vitamin D₃, retinoids, and a large number of orphan receptors whose cognate ligands are still unknown (2). Regardless of whether transcriptional activity is controlled by ligand binding, each of these proteins is able to bind to specific DNA sequences called hormone response elements (HREs)¹ in their target genes. The

sequence-specific DNA binding properties of nuclear receptors are determined by their highly conserved DNA binding domains (DBD). The P box of the DBD formed by three amino acids at the C-terminal base of the first zinc finger is responsible for the recognition of response elements. Based on the sequence within the P box and the generic recognition sequence of the P box for the HRE, the steroid/thyroid hormone receptor superfamily can be divided into the GR and ER/TR subfamilies (3). The GR group, which includes GR, MR, PR, and AR, prefers to bind to the glucocorticoid response element (5'-AGAA-CAnnnTGTCT-3') palindromic consensus sequence. Other receptors that have glutamic acid and glycine at the first two positions of the P box are assigned to the ER/TR subfamily. This subfamily, which includes ER, T₃R, VD₃R, RARs, RXRs, and most of the orphan receptors, recognize the AGGTCA direct repeat or palindromic motif. However, some members prefer to bind to the single half-site of AGGTCA as a monomer, such as the steroidogenic factor 1 (SF-1)(4), TR3 orphan receptor/NGFI-B/nur77 (5), and the thyroid receptor (T₃R) (6).

The human and rat TR4 were originally isolated from human prostate, testis, and hypothalamus cDNA libraries (7). The open reading frame of human TR4 encodes a protein of 615 amino acid residues with a calculated molecular mass of 67.3 kDa. Based on the modular structure and presence of a conserved DBD, which includes two zinc fingers that have a high degree of nucleotide sequence homology (65%) with the TR2 orphan receptor (7–9), TR4 belongs to the subfamily of TR2 orphan receptors within the steroid/thyroid receptor superfamily. Northern blotting and *in situ* hybridization studies reveal that TR4 is widely expressed in the adult rat brain (10), and most intense labelings for TR4 transcripts are detected within the granule cells of the hippocampus and cerebellum. On the basis of the sequence in the P box, TR4 has been speculated to belong to the ER/TR subfamily because of its ability to bind to AGGTCA direct repeats. In our previous studies, we found that TR4 may repress the expression of SV40 major late promoter, which contained an imperfect AGGTCA motif with a spacing of 2 bp between the half-sites (DR2) (11). By contrast, TR4 may also induce transcription of a thyroid receptor-regulated gene with a DR4 motif (12).

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¹ The abbreviations used are: HRE, hormone response element; TR4, TR4 orphan receptor; AR, androgen receptor; ER, estrogen receptor; GR, glu-

corticoid receptor; PR, progesterone receptor; MR, mineralocorticoid receptor; T₃R, thyroid receptor; VD₃R, 1,25-dihydroxyvitamin D₃ receptor; COUP-TF, chicken ovalbumin upstream promoter transcription factor; atRA, all-*trans*-retinoic acid; 9cRA, 9-*cis*-retinoic acid; RARE, retinoic acid receptor response element; RXRE, retinoid X receptor response element; EMSA, electrophoretic mobility shift assay; CAT, chloramphenicol acetyltransferase; CHO cell, Chinese hamster ovary cell; DBD, DNA binding domains; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; IPTG, isopropyl-1-thio- β -D-galactopyranoside.

The retinoid signaling pathway is mediated by retinoid X receptors (RXRs) and retinoic acid receptors (RARs) through interaction with their HREs. This happens by formation of either a RXR-RAR heterodimer, a RXR-RXR homodimer, or heterodimers with other orphan receptors, such as LXR or NGFI-B. The RXR-RAR heterodimer mediates the effects of atRA and 9-*cis*-retinoic acid (9cRA) through interaction with DR5. In this complex, RXR functions as a silent partner that occupies the 5' half-site of DR5-RE (13). Alternatively, RXR may become an active ligand-binding heterodimer partner with LXR or NGFI-B and mediate the response of target genes to 9cRA (14, 15). Finally, RXR can mediate 9cRA action by binding through a DR1 element under the form of an RXR-RXR homodimer (16).

Here we present evidence that TR4 can bind strongly to response elements for RXR (RXRE-DR1) and RAR (RARE-DR5). The consequence of TR4 interaction with DR1-RXRE and DR5-RARE in the promoter context was then determined by using a transfection gene assay. The molecular mechanism for this regulation was further examined by Northern blotting analysis and EMSA. Together these results strongly suggest

that TR4 is a central regulator in the retinoic acid signal transduction pathway.

EXPERIMENTAL PROCEDURES

Construction of Plasmids—The chimera receptor TR4-AR-TR4 was constructed by PCR site-directed mutagenesis to create two restriction enzyme sites, one is in front of DBD (*Bgl*II), the other is right after the DBD (*Xho*I), in both TR4 and AR. The DBD of AR was then removed by cutting pSG5AR with *Bgl*II and *Xho*I and ligated into pCMX-TR4, which had also been digested with these two enzymes.

Production of Monoclonal and Polyclonal Antibodies against Human TR4—To obtain a large amount of TR4 for use as an antigen in raising specific monoclonal antibodies, the *Escherichia coli* pET expression system was used. The expression of TR4 from the pET system was performed according to the manufacturer's instruction (Novagen) with the addition of six consecutive histidine residues at the N-terminal TR4. The lysates were centrifuged at 40,000 rpm for 20 min at 4 °C. The cellular extraction was then either analyzed on SDS-PAGE followed by Coomassie Blue staining or purified by a one-step metal chelating chromatography (Novagen). For the production of monoclonal and polyclonal antibodies, the antigen was prepared by directly cutting from the SDS-PAGE and emulsified with Freund's complete adjuvant.

In Vitro Transcription/Translation of Nuclear Receptors—Four expression vectors, pCMX-TR4, pCMX-4A4, pSG5-RAR α , and pCMX-RXR α , were utilized to produce *in vitro* transcribed and translated proteins in a rabbit reticulocyte-based transcription/translation kit (TNT coupled reticulocyte lysate system; Promega, Madison, WI).

Nuclear Extracts—Nuclear extracts were prepared following the mini-extract procedure (17). In short, the cells, either with or without treatment of RA for 24 h, were harvested and lysed by pushing through a 25-gauge hypodermic needle. The nuclear pellet was resuspended in buffer C (500 mM NaCl, 20 mM Hepes, 25% glycerol, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM dithiothreitol) and incubated on a rotating wheel for 30 min at 4 °C. The nuclear debris was pelleted by centrifugation for 30 min, and supernatant was dialyzed for 2 h against buffer D (20 mM Hepes, 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol). Protein concentration was determined by Bradford reagent (Bio-Rad). Two μ g of nuclear extracts were used in each 20- μ l DNA-protein interaction.

Cell Culture and Transient Transfection—Chinese hamster ovary (CHO) cells were routinely maintained in DMEM with 5% heat-inactivated fetal bovine serum (FBS). CHO cells (3×10^5) were seeded in 6-cm culture dishes 24 h before transfection. The medium was changed to DMEM with 5% charcoal dextran-treated FBS at least 1 h before transfection. The cells were transfected using a modified calcium phosphate precipitation method previously described (18).

Northern Blotting Analysis—F9 (10^6) cells were seeded in DMEM containing 5% charcoal dextran-treated FBS. After 24 h, the cells were treated with 10^{-6} M atRA and harvested at 0.5, 1, 2, 4, 8, 24, 48 h, and even longer to 6 days after atRA treatment. Total RNA from the RA-

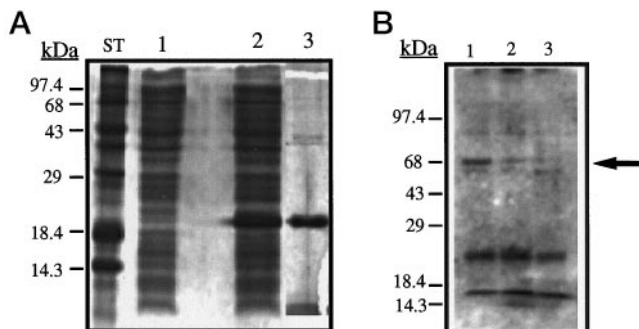
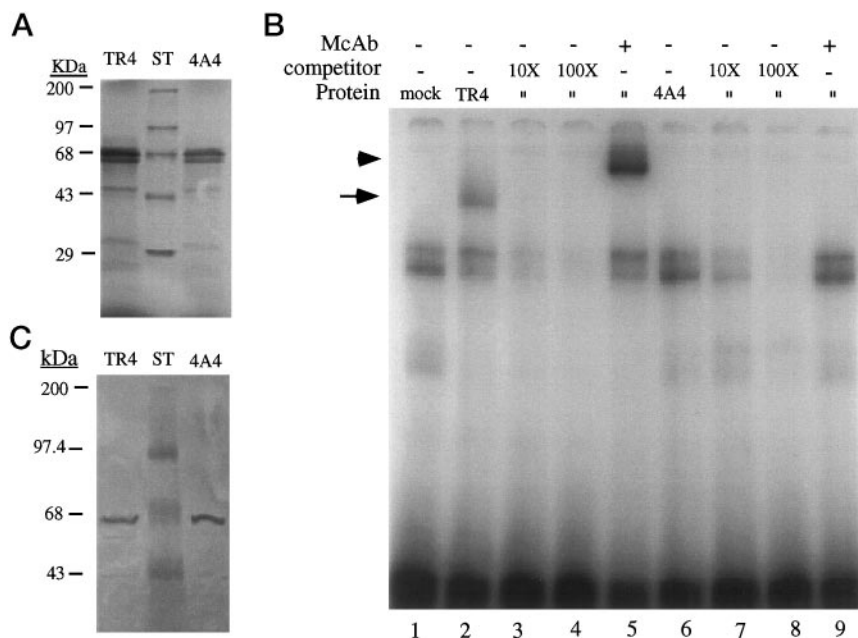


FIG. 1. Production of anti-TR4 monoclonal antibodies. A, pET14b-NTR4 expression plasmid was transformed into BL21(DE3)pLysS host strain bacteria and cultured in NZCYM medium. Analysis of the protein composition before (lane 1) and after (lane 2) IPTG induction in 12.5% SDS-PAGE. The IPTG-induced N'-terminal TR4 protein was purified by one-step metal chelating chromatography (lane 3) and analyzed in 12.5% SDS-PAGE. B, Western blotting analysis of anti-TR4 monoclonal antibody G232-151.4 with the whole cell extracts which were transfected with 4 μ g of pCMX-TR4 (lane 1), pSG5-TR2 (lane 2), and pSG5 (lane 3). A TR4 band with an expected molecular mass (67.3 kDa) was indicated as an arrow.

FIG. 2. Binding of *in vitro* expressed wild-type TR4 and chimera 4A4 to RXRE-CRBPII. A, [³⁵S]methionine incorporated *in vitro* translated TR4 and 4A4 were analyzed in 10% SDS-PAGE. B, ³²P-labeled RXRE-CRBPII oligomer was used in the EMSA with *in vitro* translated TR4 and 4A4. The migration position of the specific binding formed by the DNA-protein complex and the supershifted band formed by adding monoclonal antibody are indicated as an arrow and arrowhead, respectively. C, Western blot analysis of the expression levels of wild-type TR4 and chimera 4A4 proteins following transfection. 4 μ g of pCMX-TR4 or pCMX-4A4 was transfected into CHO cells, and 24 h after transfection cells were lysed and applied into 10% SDS-PAGE (30 μ g/lane). Polyclonal antibody against both TR4 and 4A4 was used as a primary antibody in the Western blot analysis.



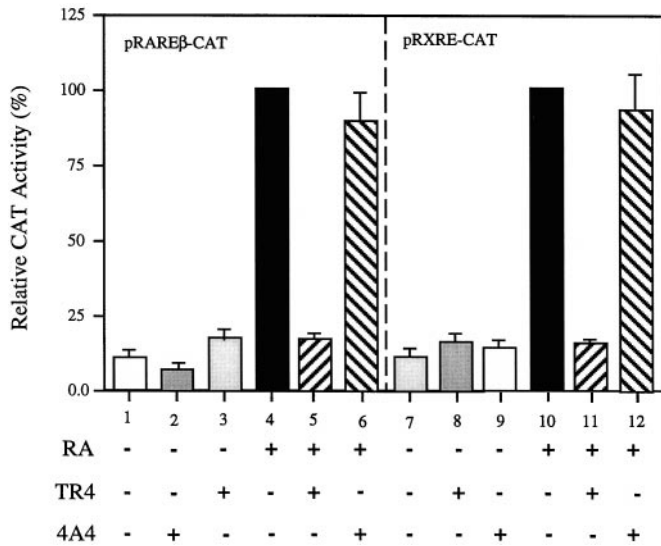


FIG. 3. Suppression of RA-induced pRARE-CAT and pRXRE-CAT reporter activity by TR4. Expression vectors (2 μ g of pSG5-RAR α and pCMX-RXR α) and their cognate reporters (3 μ g of pRARE-CAT or pRXRE-CAT) were co-transfected into CHO cells in the presence (lanes 1, 5, 8, and 11) of pCMX-TR4 or pCMX-TR4/AR/TR4 (lanes 2, 6, 9, and 12) (3 μ g). As indicated, cells were treated with 10^{-6} M tRA. CAT activities were determined and expressed as the relative activity of each reporter in the presence of tRA but in the absence of TR4. Bars represent the mean \pm S.D. of three individual experiments.

treated F9 cells was prepared by the ultracentrifugation method as described previously (19). A probe covering the N-terminal of TR4 was released by the digestion of *Eco*RI and *Aat*II and labeled with [α - 32 P]dCTP by using a random primer DNA labeling system (Life Technologies Inc.).

In Situ Hybridization Analysis—Embryo collection, section preparation, and *in situ* hybridization were performed as described previously (20).

Other Methods—EMSA, DNA-protein binding assay, and Western blot analysis were performed as described previously (21, 22).

RESULTS

Production of Anti-TR4 Monoclonal Antibodies—Using an *E. coli* expression system, we were able to generate large quantities of the N-terminal domain of TR4 to use as an antigen for the production of monoclonal antibodies. The reason we used the N-terminal domain of TR4 as an antigen is that this domain is the least conserved domain compared with other members of steroid/thyroid receptors. As shown in Fig. 1A, the N-terminal domain of TR4 encoded a 21-kDa IPTG-inducible protein (Fig. 1A, lane 1 versus 2). This induced protein, which was tagged with six N-terminal His residues, was further purified by one-step affinity chromatography and analyzed on 12.5% SDS-PAGE. As shown in Fig. 1A, lane 3, a major band of the right size was detected. For the production of the monoclonal antibody, the purified N-terminal of the TR4 peptide was cut from the gel and used for monoclonal antibody production. After screening, at least 40 monoclonal antibodies showed positive in enzyme-linked immunosorbent assay, and most of them were IgG or IgM subtypes. Western blotting analysis demonstrated that monoclonal antibody G232-151.4 can specifically recognize endogenous TR4 in CHO cell extract with a much lower intensity (Fig. 1B, lane 3, co-transfection of the expression vector only) than the cell extract from the co-transfection of TR4 (lane 1). There are no further enhanced TR4 bands that can be detected in CHO cell extract, which was transfected with TR2, indicating that the antibody does not cross-react with TR2 (lane 2).

TR4 but Not Chimera Receptor 4A4 Binds to RXRE-DR1 Specifically—Previously we were able to identify a DNA re-

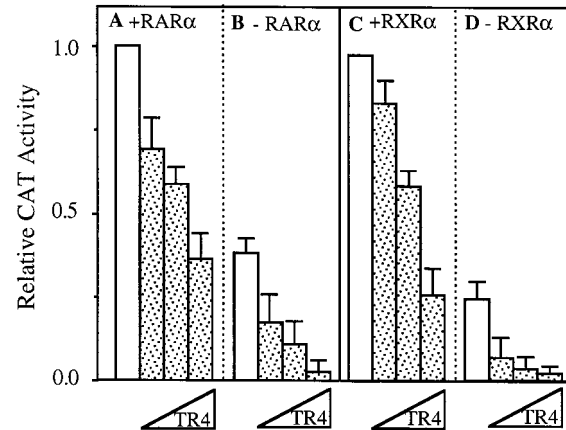


FIG. 4. Dose-dependent suppression of RA-induced pRARE-CAT and pRXRE-CAT reporter activity by TR4. Increasing amounts of pCMX-TR4 (1–3 μ g) were co-transfected with 3 μ g of pRARE-CAT (panels A and B) or pRXRE-CAT (panels C and D) in the presence (panels A and C) or absence (panels B and D) of their cognate expression vectors (2 μ g of pSG5-RAR α and pCMX-RXR α). Cells were treated with 10^{-6} M tRA for 24 h after transfection. The percentage of the CAT activity was determined relative to the CAT activity in the absence of TR4. Bars represent the mean \pm S.D. of three individual experiments.

sponse element for TR4 (TR4RE-SV40) containing an imperfect direct repeat of the AGGTCA consensus motif with a 2-base pair spacing (DR2) in the transcriptional initiation site of the SV40 major late promoter (11). Because the RXRE and RARE in the 5' promoter region of CRBP2 and RAR β were similar to TR4RE-SV40, we were interested in determining if TR4 might also bind specifically to these HREs and if it plays a role in the regulation of the retinoid signal pathway. Wild-type TR4 and chimera 4A4 were *in vitro* translated to produce proteins of the expected molecular mass of 67.3 kDa as shown in Fig. 2A. To characterize the binding specificity for TR4 and 4A4 to RXRE-CRBPII-DR1, EMSA was performed. As shown in Fig. 2B, a specific DNA-protein complex was revealed when 1 μ l of *in vitro* translated TR4 was incubated with 0.1 ng of 32 P end-labeled CRBP2 oligonucleotides (lane 2, arrow) which was different from mock-translated control protein (lane 1). This complex could be eliminated in the presence of a 10- and 100-fold molar excess of unlabeled RXRE-CRBPII oligonucleotides (lanes 3 and 4). Furthermore, the monoclonal anti-TR4 antibody (G232-151.4) could supershift this DNA-protein complex (lane 5, arrowhead). In contrast, there is no specific interaction between the probe and the chimera receptor 4A4 protein (lanes 6–9). Similar results were detected when we replaced the 32 P-CRBPII-DR1 with 32 P-RAR β -DR5 oligonucleotide (data not shown). These data demonstrated that TR4 can specifically bind to and form a single complex with RXRE-CRBPII-DR1, and also the DNA binding domain of TR4 is essential for the DNA binding.

To examine the expression level of both wild type and chimera TR4 proteins after transfection, polyclonal antibody against both proteins was produced and examined. As shown in Fig. 2C, a single band with 67.3 kDa was detected in both TR4 and 4A4 overexpressed CHO cell lysates with an equal expression level. These data clearly demonstrated that either wild type TR4 or chimera receptor 4A4 can be equally transfected into cells. Therefore, it can further support our following transfection experiment.

Suppression of RAR β and CRBP2 Promoter Activities by TR4—Transient transfection in CHO cells of CAT genes driven by promoters with retinoid response elements were then used to study the potential roles of TR4 in regulating RXR/RAR transcriptional activation. tRA (10^{-6} M) induced the expres-

FIG. 5. Suppression of RARE β and CRBP II gene expression by TR4. *Panel A*, the suppression effect of TR4 on CAT gene expression with RAR β promoter with RARE (pRAR β -CAT) or without RARE (p Δ RAR β -CAT) was tested. Reporter plasmids (3 μ g) and pSG5-RAR α (2 μ g) were co-transfected into CHO cells in the presence (lanes 3 and 6) or absence (lanes 1, 2, 4, and 5) of pCMX-TR4. Cells were then treated with 10^{-6} M tRA (lanes 2, 3, 5, and 6). *Panel B*, the suppression of TR4 of CAT containing the CRBP II promoter with RXRE (pCRBP II-CAT) or without RXRE (p Δ RAR β -CAT) was tested. Reporter plasmids (3 μ g) and pCMX-RXR α (2 μ g) were co-transfected into CHO cells in the presence (lanes 3 and 6) or absence (lanes 1, 2, 4, and 5) of pCMX-TR4. Cells were then treated with 10^{-6} M tRA (lanes 2, 3, 5, and 6; panels A and B). The percentage of CAT activity was determined relative to that in the presence of tRA but in the absence of TR4. Bars represent the mean \pm S.D. of three individual experiments.

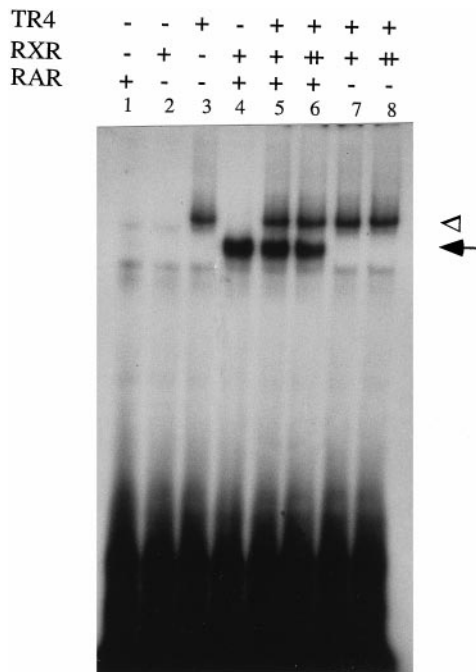
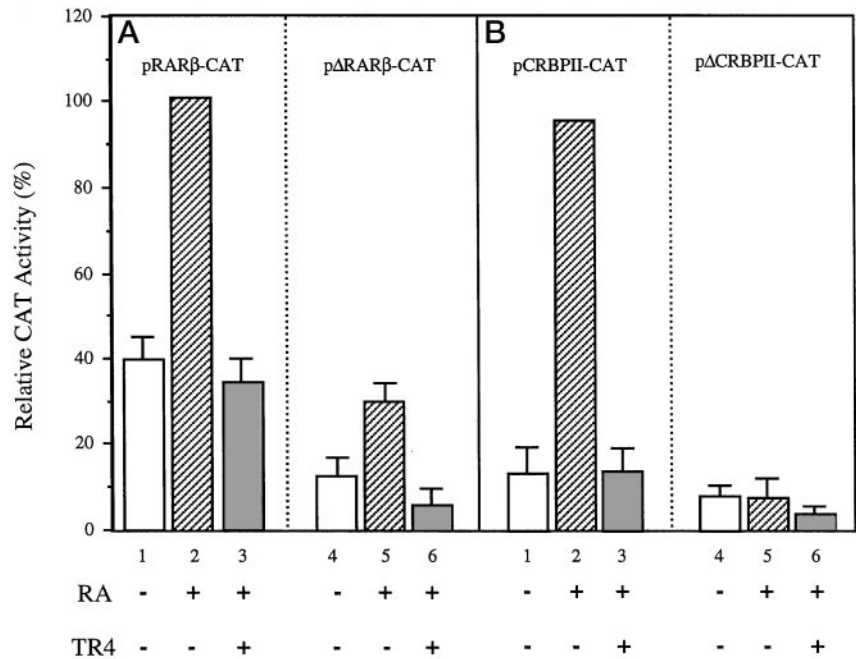


FIG. 6. TR4 does not form a heterodimer with RAR α or RXR α to RARE or RXRE-CRBP II oligonucleotides. *In vitro* translated TR4 (2 μ l), RAR α (2 μ l), and RXR α (1 μ l) were incubated in combinations with 0.1 ng of 32 P-labeled RXRE oligonucleotide and analyzed by an EMSA. The arrow indicates the complex formed by the RAR α /RXR α heterodimer, and the open triangle identifies the complex formed by the TR4.

sion of both pRARE-CAT (a CAT expression vector with insertion of RARE oligonucleotides) and pRXRE-CAT (a CAT expression vector with insertion of RXRE oligonucleotides) nearly 7-fold (Fig. 3, lanes 4 and 10). This induction was repressed to basal levels when the TR4 expression vector (pCMX-TR4) was co-transfected into the CHO cells (Fig. 3, lanes 5 and 11). However, the TR4-mediated repression could not be detected when we replaced TR4 with the chimera receptor TR4-AR-TR4 (4A4), in which the DNA binding domain of TR4 is exchanged with that of androgen receptor (Fig. 3, lanes 6 and 12). The effects of TR4 and 4A4 expression on the basal promoter activ-

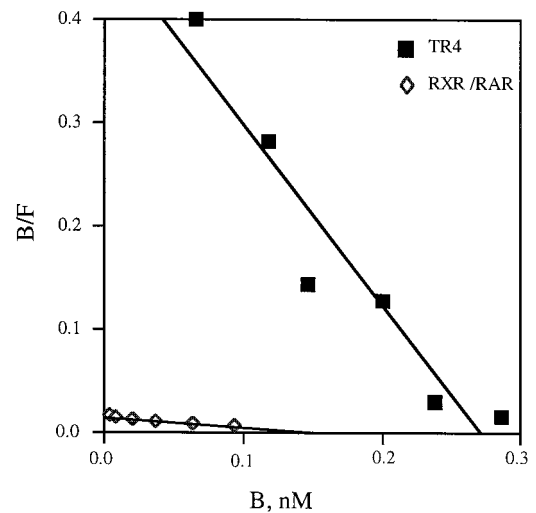


FIG. 7. Binding affinity for the TR4 and RAR α /RXR α to RXRE-CRBP II. Constant amounts of *in vitro* expressed TR4, RAR α , and RXR α were used in the EMSA with various concentrations of 32 P-labeled RXRE-CRBP II oligonucleotide probe. The specific DNA-protein complexes and free probes were detected by autoradiography, excised from the gel, and counted directly in a scintillation counter. The ratio between DNA-protein (nM) and free DNA with respect to DNA-protein was plotted. The dissociation constant (K_d) value was determined from the negative reciprocal of the slope of the line generated from the experimental data.

ity levels were also tested, and there is no significant change (Fig. 3, lanes 1, 2, 7, and 8). Moreover, our data suggested that TR4-mediated suppression of pRARE-CAT and pRXRE-CAT was TR4 dose-dependent both in the presence and absence of exogenous RAR or RXR (by co-transfection with pSG5-RAR α or pCMX-RXR α (Fig. 4).

The RARE (GGTTCAccgaaAGTTCA) and RXRE (AGGTCAcAGGTCAcAGGTCAcAGTTCA) sequences used in the suppression studies were obtained from the promoter regions of RAR β (23) and CRBP II genes (16), respectively. We therefore determined if TR4 could also bind to these elements in the context of their own natural promoters and affect their transcription. DNA fragments containing the promoter regions of these respective genes, with and without RARE or RXRE, were

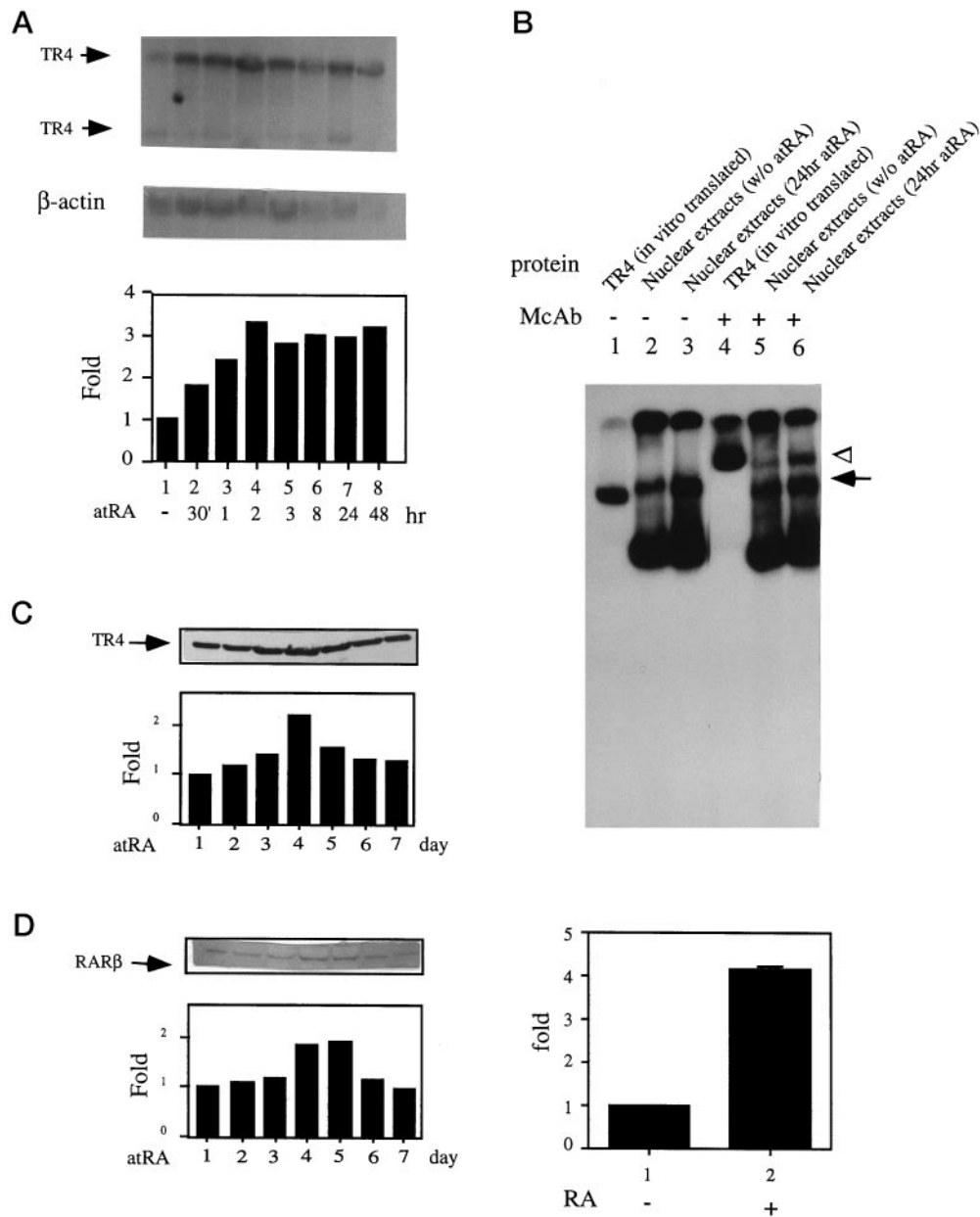


FIG. 8. Feedback control of RA signal pathway by TR4. *A*, total RNA (25 μ g) was isolated from either untreated or RA-treated F9 cells at different times (from 30 min to 48 h). The probe used for the hybridization was the N-terminal domain of TR4 labeled randomly with [α - 32 P]dCTP. The hybridization bands were quantified by PhosphorImager. The positions of β -actin and TR4 transcripts are indicated. *B*, 0.1 ng of 32 P-RXRE-CRBP II probe was incubated with *in vitro* translated TR4 (lanes 1 and 4), the nuclear extracts from the untreated (lanes 2 and 5), or 24-h RA-treated F9 cells (lanes 3 and 6) with (lanes 4, 5, and 6) or without (lanes 1, 2, and 3) adding the 1 μ l of specific anti-TR4 monoclonal antibody (G232-151.4). They were then analyzed in EMSA. The migration position of the specific binding formed by the DNA-protein complex and the supershifted band formed by adding anti-TR4 monoclonal antibody are indicated as an arrow and open triangle. The specific bands were quantified by PhosphorImager. *C* and *D*, 30 μ g of proteins from RA-treated F9 cell lysates (1 to 7 days) was analyzed by the Western blot. The TR4 polyclonal antibody and the RAR β antibody (C-19) were used as primary antibodies. A horseradish peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology) was used as the second antibody, and the color was then developed utilizing the chemiluminescent ECL Western blotting detection system (American Life Science). The results from two independent experiments are summarized.

inserted in front of CAT expression vectors (named as pRAR β -CAT and pCRBP II-CAT versus p Δ RAR β -CAT and p Δ CRBP II-CAT, respectively). atRA (10^{-6} M) induced CAT expression from the pRAR β -CAT and pCRBP II-CAT (approximately 2.5-fold and 8-fold, respectively), but not p Δ RAR β -CAT and p Δ CRBP II-CAT (Fig. 5). Furthermore, this RA-dependent induction was repressed to basal levels when the TR4 expression vector was co-transfected into CHO cells. These results clearly demonstrate that TR4 can repress the retinoid-RAR/RXR signal transduction system.

Interaction between TR4, RAR/RXR, and RARE/RXRE—To explore the direct interaction between TR4 or RAR/RXR upon

binding to the RARE or RXRE, we performed a series of studies using an EMSA. As shown in Fig. 6, *in vitro* translated TR4 binds specifically to RXRE with one clear band shift (lane 3). As expected, no visible band shifts were obtained when *in vitro* translated TR4 was replaced by either *in vitro* translated RAR α (lane 1) or RXR α (lane 2). However, a clear band appeared (lane 4) when both *in vitro* translated RAR α and RXR α were added. This band migrated differently from that of the TR4, which migrated more slowly (lane 3 versus 4). When *in vitro* translated TR4 was added together with *in vitro* translated RAR α /RXR α , two distinct bands could be observed, one for TR4 and another one for RAR α /RXR α . There was clearly no intermedi-

ate band between TR4 and the RAR α /RXR α heterodimer (lane 5), even when the amount of *in vitro* translated RXR α in the assay was increased (lanes 6 and 8). Similarly, no intermediate band could be visualized between TR4 and the RAR α /RXR α heterodimer when RXRE-CRBPII was replaced with RARE-RAR β (data not shown). Together, these data indicate that there is no significant heterodimer formation between TR4 and RAR or RXR. Clearly, these results suggest that TR4 functions as a competitor for RARE/RXRE binding.

RXRE-CRBPII Binds to TR4 with Higher Affinity Than to the RAR/RXR Heterodimer—To explore the affinity between these receptors and HREs, we performed EMSA to determine the dissociation constant by Scatchard plot analysis. Scatchard plot analysis of the DNA-protein complexes in the EMSA demonstrated that the dissociation constants (K_d) for TR4 to RXRE-CRBPII and RAR α /RXR α for RXRE-CRBPII were 0.5 nM and 10.0 nM, respectively (Fig. 7). Therefore, TR4 has an affinity for RXRE-CRBPII that is 20-fold greater than that of the RAR α /RXR α heterodimer. Our data suggest that a simple competition between TR4 and RXR α /RAR α for the same HRE may be the potential mechanism for the TR4-mediated suppression of the retinoid-RAR/RXR pathway.

Negative Feedback Control of the Retinoid Pathway by TR4—To explore the mechanism for TR4-mediated repression of the retinoid signal pathway, we checked to see whether retinoids can regulate TR4 in F9 cells that are sensitive to the RA treatment. To investigate expression of TR4 during differentiation of F9 cells, 25 μ g total RNA from untreated F9 cells, or after treatment with 10^{-6} M atRA at different times, was analyzed by Northern blotting. As shown in Fig. 8A, an inducible band, which corresponds to the size of TR4 mRNA (9 kilobases), was visible in atRA-treated F9 cells. The induction increased gradually from 30 min to 2 h after RA treatment (Fig. 8A, lanes 1–4) and reached a plateau 2 h after treatment (Fig. 8A, lanes 5–8).

Induction of TR4 by the treatment of atRA also occurred at the protein level. As shown in Fig. 8B, the F9 nuclear extracts give a shifted band with 32 P-RXRE-CRBPII that migrated at a similar position as compared with the *in vitro* translated TR4 (lane 1 versus 2). This specific DNA-protein complex was enhanced by culturing F9 cells with atRA for 24 h (lane 3). Furthermore, this enhanced band was partially supershifted by anti-TR4 monoclonal antibody (G232-151.4). Quantitation of the specific band supershifted by anti-TR4 antibody indicated an increase of nearly 4-fold after atRA treatment for 24 h (lane 5 versus 6). These data clearly demonstrate that RA can induce TR4 expression both at RNA and protein levels and therefore may represent a negative feedback system that controls RA-mediated modulation of gene expression. To prove this hypothesis, the effects of TR4 induction on endogenous gene expression after a longer RA treatment was investigated. As shown in Fig. 8C, the expression level of TR4 in protein level was gradually increased after 2 days of 10^{-6} M atRA treatment. However, the expression level declines gradually after 5 days of treatment and continues to decrease to the basal after 7 days of treatment. We also checked the expression of RAR β , a retinoic acid-induced gene. Its protein expression was increased at the beginning of treatment and decreased after 6–7 days of exposure to atRA (Fig. 8D). All these data demonstrated that induction of TR4 expression down-regulated both its own expression and that of RA-induced target gene and that further supports the feedback control mechanism.

TR4 Expression Domains Overlapped with Those of RA Receptors—It is well accepted that retinoic acids affect differentiation in various developmental systems via nuclear RA receptors. To analyze whether the interaction between TR4 and RA

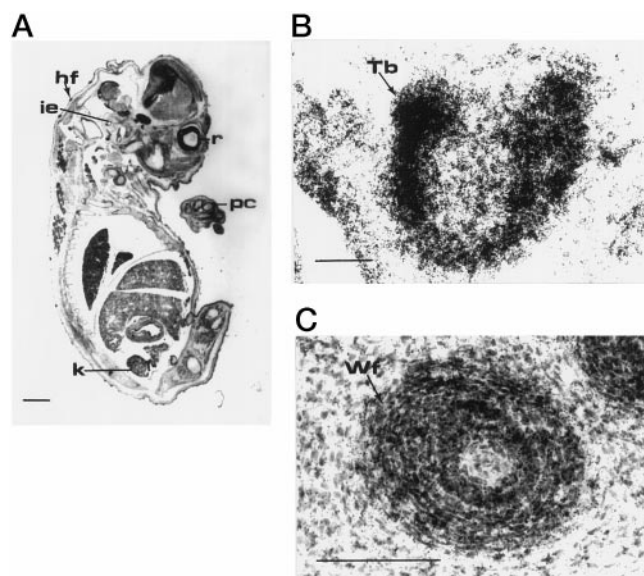


FIG. 9. Localization of TR4 transcripts in vitamin A-target organs during mouse embryogenesis. Sagittal sections of mouse embryos at gestation day 14 (A) and day 16 (B and C) were analyzed by *in situ* hybridization with [35 S]UTP-labeled mouse TR4 antisense riboprobe. Autoradiograms were photographed under light field illumination with (C) or without (A and B) hematoxylin staining. Tissues with strong hybridization signals (dark areas) are labeled. High magnifications (B and C) show intensive TR4 signals in the tooth bud (Tb) and whisker follicle (Wf). Other abbreviations used are: hf, hair follicle; ie, inner ear; k, kidney; pc, perichondrium; r, retina. The bars represent 1 and 0.1 mm of length in panels A and B and C, respectively.

receptors could occur *in vivo*, we examined the TR4 expression pattern by *in situ* hybridization and compared it to that of RA receptors. As shown in Fig. 9, TR4 transcripts were intensively accumulated in the paraventricular brain area, retina, vestibular epithelium, perichondrium, kidney, and hair follicles of an embryo at gestation day 14 (Fig. 9A). The TR4 labeling was also detectable in tooth bud and whisker follicles at gestation day 16 (Fig. 9, B and C). It is worth noting that sense TR4 riboprobe did not detect any specific signal. Thus, this TR4 distribution pattern appears to be specific. This pattern does not directly follow expression patterns reported for any RA receptors.

When comparing the TR4 expression patterns to those of RA receptors, we noticed that TR4 was co-localized with RA receptor members to various extent. For example, RAR γ and TR4 transcripts were clearly seen at embryo day 14 in the perichondrium and the roots of the developing whisker follicles (24). RAR β was co-localized with TR4 in regions where teeth develop as epithelial invaginations into the underlying mesenchyme (25, 26). During the development of sensory epithelia of the eye, inner ear, and olfactory system, the expression of RAR α , RAR γ , and RXR γ was detected together with TR4 (25, 26). Among the RA receptors, only RXR γ transcripts were detected in brain areas that express TR4. Interestingly, the TR4 labeling pattern in kidney is very similar to that of CRBP and highly overlaps with RAR β , suggesting co-expression of these three factors in kidney (25, 26). Our *in situ* data therefore support the idea that modulation of retinoic acid signaling by TR4 could occur in physiological conditions.

DISCUSSION

The data reported here suggest an important biological role of TR4 as a negative feedback regulator for the retinoid signaling pathway. Because the level of RA-induced transactivation can be repressed by co-transfection of TR4, but not the chimera receptor, TR4-AR-TR4 (Fig. 3), the DBD of TR4 is essential for repression. This result also argued against the possibility that

TR4 may compete with RAR or RXR for the co-factors needed for the RA activation. Moreover, our data (Fig. 5) suggested that the repression of RA-induced transactivation by TR4 is RARE- and RXRE-dependent. Together, these results indicate that both the DNA sequence in HRE and the promoter context may contribute to the repression of RA-induced transactivation by TR4.

There are two possible mechanisms to explain how nuclear receptors can repress the RA-induced transactivation pathway. The first one is through protein-protein interaction by formation of heterodimer with RXR. For example, COUP-TF has been demonstrated to repress RA-induced transactivation by forming a heterodimer with RXR (22). However, our results from the EMSA demonstrated that the mechanism of repression by TR4 may be different from that displayed by COUP-TF. Our data showed that both TR4 and RXR/RAR heterodimer can bind to RARE or RXRE probes specifically but failed to show any unique bands formed between TR4 and RXR/RAR when these three receptors were combined in EMSA analysis. This strongly suggests that TR4 may repress RAR/RXR without heterodimerization with either RAR α or RXR α . The second mechanism for repression is competition with these receptors for binding to a common response element. In this regard, we compared the binding affinity between TR4 and RXR/RAR to the RXRE-CRBPII DNA fragment in EMSA. TR4 binds to RXRE-CRBPII with a 20-fold higher affinity than RXR/RAR. The strong correlation observed between effective binding of TR4 to a response element and its repressing effect provides convincing evidence that TR4 represses the retinoid signaling pathway by a simple competition mechanism. Therefore, TR4 can compete with and displace the RXR/RAR heterodimer from the DNA binding site to achieve effective repression. Our data further confirmed that this repression of RA-mediated transactivation is TR4-dose dependent, as increasing levels of transfected TR4 led to a stronger inhibition of RA-mediated transactivation. Such a finding is similar to our previous results for the TR2 orphan receptor, a closely related subclass member (21, 27, 28). Both TR2 and TR4 can potentially function as repressors for the retinoid-RAR/RXR signal transduction pathway by competition with the HRE. A similar example of the dose-dependent regulation of gene expression in this steroid/thyroid superfamily is the repression of *OCT3/4* gene by COUP-TFs. It has been demonstrated that COUP-TFs can repress the *OCT3/4* gene by binding to the RAREoct site with much higher affinity than the RXR/RAR heterodimer (29).

Based on the above conclusions, it is possible that gene regulation can be controlled by different ratios and relative affinities of a gene's inducers and repressors. In F9 cells that differentiated upon treatment of RA, we noted that TR4 mRNA was increased nearly 4-fold by adding RA. This inducibility was also supported by EMSA results. Although many nuclear hormone receptors may bind to RXRE-CRBPII in differentiating F9 cells (Fig. 8B, lanes 2 and 3), TR4 may represent one of the very few proteins that can also be induced during the RA treatment. It may indicate that retinoid pathways involve a very complicated and balanced control between TR4 expression and RA concentration.

Taken together, the repressive effects of TR4 on RA-induced transactivation suggest that TR4 may play a role in a negative

feedback system, which controls RA-mediated modulation of gene expression. Moreover, TR4 may represent a master regulator in the retinoid signal pathway due to the dominant effects it exerts with higher affinity to the HREs and the potential amplification of this repression effect by its increasing expression during RA treatment.

In summary, the discovery of negative feedback control regulation of RA-mediated gene induction by TR4 provides evidence that TR4 is a regulator of cell proliferation and differentiation. Further studies of TR4 in retinoid systems may provide us with clues as to their physiological role in humans and other systems.

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