

TR4 Orphan Receptor Represses the Human Steroid 21-Hydroxylase Gene Expression through the Monomeric AGGTCA Motif

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The human TR4 orphan receptor (TR4) is a member of the nuclear receptor superfamily. It functions as a transcriptional factor which regulates and controls many important physiological functions. It has been documented that TR4 may bind as a homodimer to a DNA response element containing two direct repeats of the AGGTCA consensus motif. Surprisingly, our data reveal that the expression of the human steroid 21-hydroxylase (21-OHase) gene could be repressed by TR4 via the monomeric AGGTCA motif (–228TR4RE) at its 5' flanking region (nucleotide numbers 1431–1444, 5'-GGAAAAGGTCAGG-3'). Electrophoretic mobility shift assay showed specific binding with a dissociation constant of 0.4 nM between TR4 and the monomeric –228TR4RE motif. However, TR4 does not form heterodimers with either retinoid X receptor alpha or SHP (short heterodimer partner) orphan receptor. Additionally, both dual-luciferase and chloramphenicol acetyltransferase assays demonstrated that TR4 can function as a repressor via the –228TR4RE of the 21-OHase gene. In conclusion, our data suggest that TR4 may bind to a monomeric DNA response element and play an important role in the suppression of the 21-OHase gene expression. © 2001 Academic Press

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Abbreviations used: TR4, TR4 orphan receptor; 21-OHase, steroid 21-hydroxylase; SHP, short heterodimer partner orphan receptor; HRE, hormone response element; DR, direct repeat; PPAR, peroxisome proliferator-activated receptor; NGFI-B, nerve growth factor inducible-B; NBRE, NGFI-B response element; CAT, chloramphenicol acetyltransferase; RXR, retinoid X receptor; EMSA, electrophoretic mobility shift assay; RBA, relative binding affinity; CRBP II, cellular retinoic acid binding protein II; SV40, simian virus 40; RARE, retinoic acid response element; VDRE, vitamin D₃ response element; TRE, thyroid hormone response element; ERE, estrogen response element.

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It has been known for more than two decades that steroid and thyroid hormones function through the action of specific receptor proteins (1, 2). Nuclear hormone receptors comprise a huge family of transcriptional factors that regulate complex gene networks in a wide variety of biological processes, such as growth, development, and differentiation (1). Members of this nuclear receptor superfamily include receptors for steroid hormones, thyroid hormones, vitamin A and D derivatives, as well as a large group of orphan receptors whose cognate ligands remain to be identified. Upon binding to their respective hormonal ligands, the nuclear receptors may undergo an activation or transformation step (3). The hormone–receptor complex, serving as a *trans*-regulator, may specifically bind to a *cis*-acting DNA sequence, known as a hormone response element (HRE), and thereafter regulate the transcription of target genes (2).

Orphan receptors are the title used to group receptor-like proteins that have no known ligand or function (4). The human TR4 orphan receptor (TR4) cDNA isolated from prostate and testes libraries shares structural homology with members of the nuclear receptor superfamily (5). TR4, also named as TAK1, is most closely related to the previously identified human TR2 orphan receptor (6, 7). In addition, TR4 was designated as the TR2 β , while the TR2 orphan receptor was referred to as TR2 α (4). A unified nomenclature system further classified TR4 as NR2C2a (8). Recent evidence showed that TR4 may heterodimerize with TR2 and androgen receptor in different signaling pathways, respectively (9, 10). The expression of TR4 transcripts was detected during brain development in rat, and widely in many mouse tissues including the central nervous system and peripheral organs, such as the adrenal cortex (11, 12). These data suggest that TR4 may play a role in a relatively wide variety of biological processes.

Differential recognition of target genes by the nuclear receptors is determined by at least three properties: protein–DNA interactions, protein–protein interactions, and protein environment (2). For the protein–DNA interaction, DNA-binding domains of family members selectively interact with HREs, which are structurally related but functionally distinct. Based on the zinc finger model, the proximal box in the DNA-binding domain of receptor proteins may determine target HRE specificity (13). Consequently, the TR4 orphan receptor containing the EGCKG amino acid sequence in the proximal box of the DNA-binding domain, can be grouped into members of the estrogen receptor subfamily, recognizing direct repeats of the hexameric consensus motif AGGTCA (13). Thus far, we have demonstrated that TR4 may bind to bipartite repeats of many target genes, including a direct repeats spaced by two nucleotides (DR2) of the simian virus 40 major late promoter (14), a DR1 in the fifth intron of the ciliary neurotrophic factor receptor gene (12), DR1 and DR5 of the retinoids target genes (15), a DR4 of the thyroid hormone target genes (16), and a DR3 of the vitamin D₃ target gene (17). Moreover, TR4 has also been shown to bind as a homodimer to DRs of synthetic and natural targets, a DR1 in the promoter of the peroxisome proliferator-activated receptor alpha (PPAR α) target genes, and an imperfect DR0 in the promoter of the human luteinizing hormone receptor (18–20).

Steroid 21-hydroxylase (21-OHase; EC 1.14.99.10) belongs to the cytochrome P450 superfamily, and is one of the key enzymes in biosynthesis of adrenal steroid hormones, leading to the production of cortisol and aldosterone (21). The expression of the 21-OHase gene is restricted to the adrenal cortex, where it is essential for the production of both glucocorticoids and mineralocorticoids. 21-OHase mediates the conversion of progesterone to 11-deoxycortisol. Since cortisol is the primary feedback inhibitor of adrenocorticotropin secretion, 21-OHase deficiency is one of the major causes of increasing levels of adrenocorticotropin causing congenital adrenal hyperplasia. This disorder is thus the most common autosomal recessive defect in humans (22). The human 21-OHase gene has been cloned and characterized (22–24). Subsequently, multiple regulatory elements have been identified from the 5' flanking region of the 21-OHase gene (25, 26). In addition, members of the nuclear receptor superfamily are suspected to regulate the expression of the adrenal steroidogenic enzymes (26). Indeed, it has been demonstrated that the nerve growth factor inducible-B (NGFI-B)/nur77/TR3 orphan receptor may bind to the mouse 21-OHase –65 element, and upregulate this gene expression (27). The mouse 21-OHase –65 element does not exist in the 5' flanking region of the human gene. However, there is a perfect monomeric DNA response element containing the single AGGTCA core motif preceded by an

AT-rich sequence located in the –228 element (nucleotide numbers 1431–1444, GenBank Accession Nos. M12792 and M23280) in the human 21-OHase gene (24).

It has been assumed that members of the nuclear hormone receptors are able to bind to HREs in three different ways, monomeric, homodimeric, and heterodimeric categories (2, 4). Most classical steroid hormone receptors have been characterized to form homodimers or heterodimers with another member of the superfamily. However, only a few members have sufficient affinity to bind to HREs as a monomer. Indeed, NGFI-B was first demonstrated to be a monomeric nuclear receptor which may bind to the NGFI-B response element (NBRE) (28, 29). Two elements, referred to as the A and T boxes, at the C-terminal region of the second zinc finger of the NGFI-B/nur77/TR3 orphan receptor are critical for specific DNA recognition (28–30). Several orphan receptors, for example, ROR/RZR, NGFI-B/nur77/TR3, FTZ-F1, and SF-1/Ad4BP, may also bind as monomers to HREs composed of a single AGGTCA motif preceded by an AT-rich sequence (29). This highlights the importance of non-zinc finger regions of receptor proteins that also contribute to the specificity of DNA binding. Surprisingly, we were able to demonstrate that TR4 may also specifically bind to monomeric DNA response elements. Our data demonstrate that TR4 may function as a repressor in the human steroid 21-hydroxylase gene expression via the –228TR4RE. Thus, the 21-OHase gene could represent a unique target downregulated by TR4.

MATERIALS AND METHODS

Plasmid construction. Plasmid pCMX-TR4 consists of the full-length coding sequence of TR4 cDNA in the pCMX vector under the control of both the human cytomegalovirus and T7 promoters for *in vivo* and *in vitro* expression, respectively (14). Plasmids 100CAT21 and 389CAT21 containing different lengths of the natural promoter regions of the 21-OHase gene in front of the chloramphenicol acetyltransferase (CAT) reporter gene were kindly provided by Dr. Michael R. Waterman as described previously (31). Plasmids pCMX-RXR α and pT7hmSHP were kindly provided by Drs. Ronald M. Evans and Wongi Seol, respectively (14, 32). One copy of the –228TR4RE (nucleotide numbers 1431–1444, 5'-GGAAAAAGGTCAAGG-3') of the human 21-OHase gene (24) was cloned into the *Bgl*III site of the pGL3-promoter vector (Promega), named pGL3-228.

Coupled *in vitro* transcription and translation. Plasmids containing the full-length TR4, retinoid X receptor alpha (RXR α), and short heterodimer partner (SHP) cDNAs were *in vitro* transcribed and translated directly by the TNT system (Promega) as previously described (14). Depending on the purpose of the experiment, the reactions were carried out in the presence or absence of L-[³⁵S]methionine (DuPont NEN) in the transcription-translation mixture. The *in vitro* translated products were then analyzed by electrophoresis in SDS–10% polyacrylamide gel and used as the protein source in electrophoretic mobility shift assay (EMSA).

Electrophoretic mobility shift assay. EMSA was conducted as previously described (14). The double-stranded NBRE oligonucleotides (5'-GATCGAGTTTTAAAAGGTCATGCTCAATTT-3') and the

A	DR1	agcttcAGGTCAg	AGGTCAgagagct	
	DR2	agcttcAGGTCAgg	AGGTCAgagagct	
	DR3	agcttcAGGTCAagg	AGGTCAgagagct	
	DR4	agcttcAGGTCAcagg	AGGTCAgagagct	
	DR5	agcttcAGGTCAccagg	AGGTCAgagagct	
	DR6	agcttcAGGTCAaccagg	AGGTCAgagagct	
	CRBP II	gatcAGGTCA	cAGGTCAc	AGGTCAc AGTTCA
	SV40 +55		gttaAGGTTCgt	AGGTCAtgga
	RARE β	gatcgggtagGGTTCAccgaa		AGTTCActcg
	VDRE		cgattcAGGTCAagg	AGGTCAgcac
	TRE		tcttagGGGTCAaata	AGGTCAaatg
	ERE		tcAGGTCActg	TGACCTga
B	NBRE	gatcagagTTTTAAAAGGTCAgtctcaattt		
	21-OHase -228TR4RE		ggAAAAGGTCAgg	

FIG. 1. Sequence list of bipartite repeats and monomer DNA response elements. (A) The core consensus AGGTCA sequences (capital) of bipartite repeats, including direct repeats spaced by 1 to 6 nucleotides (DR1-6), cellular retinoic acid binding protein II (CRBP II), +55 region of the major late promoter of simian virus 40 (SV40 +55), retinoic acid response element beta (RARE β), vitamin D₃ response element (VDRE), thyroid hormone response element (TRE), and palindromic estrogen response element (ERE) are aligned. (B) The core consensus AGGTCA motifs (capital) of monomeric response elements, including NGFI-B response element (NBRE) and -228 region of the human 21-OHase gene, are aligned.

-228TR4RE of the human 21-OHase gene were ³²P-end-labeled as probes (28, 33). For cold competition reactions, unlabeled double-stranded oligonucleotides were mixed with the labeled probe prior to the addition to the reactions. For antibody supershift analysis, 1 μ l of monoclonal anti-TR4 antibodies (G232-416.3 and G232-85.6) was added into the reactions for 15 min at room temperature prior to loading on a 5% native gel, respectively (34). Radioactive bands were visualized by autoradiography (Fuji RX film) and quantified by PhosphorImager (Molecular Dynamics).

Competitive EMSA. To determine the relative binding affinity (RBA), EMSA was performed as above using the [³²P]NBRE as a probe, except that increasing concentrations of unlabeled annealed competitors were mixed with the probe prior to adding into the reactions. Radiolabeled bands were quantified by PhosphorImager (Molecular Dynamics). The RBA was calculated from the relative ratio between the intensity of the specific band shift and that of the nonspecific band shift. Competitive displacement curves were graphed as the competitor concentration (ng) versus the percentage of the intensity remaining in the specific band with 100% bound, representing the amount in the absence of competitor DNA as previously described (15). The IC₅₀ represents the competitor concentration that inhibits 50% of the RBA (15).

Scatchard analysis. The DNA-protein binding affinity assay between TR4 and either the NBRE or the -228TR4RE of the human 21-OHase gene was carried out as previously described (14) with modifications that will be described elsewhere.

Cell culture, transfection experiments, CAT, and dual-luciferase reporter gene assays. Mouse Y-1 adrenocortical tumor cells, kindly provided by Dr. Bernard P. Schimmer, were cultured in Dulbecco's modified Eagle medium (Life Technologies) supplemented with penicillin (100 units/ml), streptomycin (100 μ g/ml), amphotericin (0.25 μ g/ml), and 15% heat-inactivated (56°C for 30 min) fetal bovine serum (Harlan). Transient transfection of the Y-1 cells plated at an initial density of 3 \times 10⁵/60-mm dish were transfected by the calcium phosphate precipitation method as described previously (14). Using 389CAT21 and 100CAT21 reporter plasmids, a CAT assay was conducted as described previously (14). To normalize the transfection efficiency, the β -galactosidase expression vector pCH110 (Clontech)

was cotransfected (14). The normalized ratio of CAT expression was averaged over at least three independent experiments for each point, with error bars designating standard deviation. A dual-luciferase reporter assay was performed as described previously (35, 36). The pRL-TK plasmid (Promega) expressing *Renilla* luciferase was cotransfected to normalize the transfection efficiency. Both firefly and *Renilla* luciferase activities were measured in a Monolight 2010 luminometer (Analytical Luminescence Laboratory) using a modified mode (30 s). The normalized ratio of firefly luciferase expression was then averaged over at least four independent experiments with error bars designating standard deviations.

Statistics. Statistical comparisons of the control with the treated groups were performed by Student's *t* test. The degree of significance (*P* value) was shown with the asterisk. The accepted level of statistical significance was *P* < 0.05.

RESULTS

Characterization of the Binding Preference of TR4 to Different DRs

We have demonstrated that TR4 may recognize and bind to bipartite repeats of the AGGTCA motif in its target genes as described above. Using cold competitive EMSA, our data suggested the binding preference of TR4 to synthetic DRs (Fig. 1A) is in the order of DR6 > DR5 > DR1 > DR3 > DR2 > DR4 with the IC₅₀ ranging slightly from 0.08 to 0.24 ng (Fig. 2A). For natural DRs, however, TR4 has a dramatically different preference in the order of CRBP II (DR1) > SV40 +55 (DR2) > TRE (DR4) > RARE β (DR5) > VDRE (DR3) > ERE (IR3) with the IC₅₀ varying widely from 0.023 to 85 ng (Fig. 2B). These data suggested that TR4 has the ability to bind to various bipartite repeats of the AGGTCA consensus motif with different affinities.

A		DR6	DR5	DR1	DR3	DR2	DR4
IC ₅₀		0.08	0.12	0.15	0.20	0.22	0.24
B		CRBP II	SV40 +55	TRE	RARE β	VDRE	ERE
		DR1	DR2	DR4	DR5	DR3	Palindromic
IC ₅₀		0.023	0.21	0.38	1.5	2.0	85

FIG. 2. Binding affinity of TR4 to bipartite repeats of synthetic and natural response elements. (A) RBA values for synthetic response elements. Six direct repeats of the consensus AGGTCA sequence spaced by 1 to 6 nucleotides (DR1-6) were serially diluted from 1.6 to 0.00625 ng to compete with 0.1 ng ³²P-labeled DRs for binding to the *in vitro* translated TR4 (1 μ l) in EMSA. The RBA was determined as a percentage of the radioactivity of the DNA-protein complex obtained with the most diluted cold competition. IC₅₀ representing the competitor concentration that inhibits 50% of the RBA was calculated from the curves of competitive EMSA. (B) RBA values for natural response elements. CRBP II, SV40 +55, RARE β , VDRE, TRE, and palindromic ERE containing bipartite repeats of the AGGTCA core sequence were tested under the same conditions as described above.

Binding of TR4 to a Monomeric Response Element

To further explore the possibility that TR4 may also bind to a monomeric response element, TR4 protein was coupled *in vitro* transcribed and translated to produce a protein as shown in Fig. 3A for the source of EMSA study. Using a double-stranded NBRE oligonucleotide as a probe (Fig. 1B), a specific DNA-protein complex was visualized (Fig. 3B, lane 3, arrowhead). This radioactive DNA-protein complex was abolished in the presence of 50-fold molar excesses of unlabeled NBRE (Fig. 3B, lane 4). Moreover, the monoclonal anti-TR4 antibody (G232-416.3) could supershift this specific DNA-protein complex (lane 6, arrow). However, the monoclonal anti-TR4 antibody (G232-85.6) could eliminate such a complex (lane 7). These data suggested that TR4 indeed has the ability to specifically bind to the monomeric NBRE.

To investigate further the ability of TR4 to bind to the natural monomeric response element, we carried out EMSA by using the -228TR4RE of the human 21-OHase gene as a probe (Fig. 1B). As shown in Fig. 3C, similar results as those described above using the NBRE as a probe were obtained. Thus, TR4 can specifically bind to the -228TR4RE of the 21-OHase gene, a natural monomeric HRE.

To determine the DNA-protein binding affinity between TR4 and a monomeric DNA response element in more detail, we performed Scatchard analysis by EMSA. Constant amounts of the *in vitro* expressed TR4 were incubated with different concentrations of the NBRE and the -228TR4RE of the 21-OHase gene, respectively. DNA-protein complexes were resolved in EMSA. Scatchard analysis revealed a single binding component for the specific DNA-protein complexes with dissociation constants (K_d) of 0.2 and 0.4 nM for the NBRE and the -228TR4RE of the 21-OHase gene (data not shown), respectively. These results indicated that TR4 binds to a monomeric DNA response element with high affinity.

Lack of Indication for TR4 to Form Heterodimers with Other Nuclear Receptor Partners

TR4 has been grouped into the homodimeric binding subfamily (2, 4, 18). SHP is an unusual orphan receptor that lacks a conventional DNA-binding domain (32). SHP has been shown to form heterodimers with several members of the nuclear receptor superfamily (32, 37). On the other hand, RXR is a heterodimer partner for several nuclear receptors (4). We, therefore, are interested to know if TR4 might also form heterodimers with either RXR α or SHP by protein-protein interaction. Using *in vitro* expression (Fig. 3A) and EMSA in the presence of the NBRE probe (Fig. 4), a specific DNA-protein complex was seen as before between TR4 and the probe (Fig. 4, lane 3, arrowhead). In

contrast, neither RXR α nor SHP can interact with the NBRE (lanes 4 and 5). Moreover, we were able to detect the original DNA-protein complex when the DNA probe was incubated with the combination of TR4 and either RXR α or SHP (lanes 6 and 7). Subsequently, the monoclonal anti-TR4 antibody (G232-416.3) could further supershift the DNA-protein complex formed by TR4 and the probe (lane 8). These results suggested that heterodimerization does not occur between TR4 and either RXR α or SHP.

Repression of Gene Expression of the Human 21-OHase Gene by TR4

Tissue distribution analysis of TR4 by both Northern blot analysis and *in situ* hybridization showed that TR4 transcripts were expressed in the adrenal cortex (5, 12). To determine whether TR4 plays a role in the 21-OHase gene expression via interaction with the -228TR4RE, we performed a dual-luciferase reporter assay in mouse Y-1 adrenocortical tumor cells. As shown in Fig. 5, adrenal Y-1 cells were cotransfected with a mammalian expression vector containing the full-length TR4 cDNA (pCMX-TR4) and either the parent pGL3-promoter or the pGL3-228 reporter plasmid. The results showed that TR4 can significantly suppress the luciferase reporter activity via the -228TR4RE of the 21-OHase gene. This indicated that TR4 can repress the 21-OHase gene expression via interaction with the -228TR4RE.

To confirm this repression effect of TR4 in the natural 21-OHase promoter, we further used a CAT assay with the cotransfection of mammalian expression vectors and CAT reporter plasmids into Y-1 cells. In Fig. 6, the cotransfection of pCMX-TR4 expression vector and either 100CAT21 (without the -228TR4RE of the 21-OHase gene) or 389CAT21 (with the -228TR4RE) reporter plasmid indicated that TR4 can repress the transcriptional activity of the 21-OHase promoter via the -228TR4RE. This result is in agreement with results obtained above, which illustrate TR4 may function as a repressor of transcriptional activity of the natural 21-OHase promoter by binding to the monomeric -228TR4RE.

DISCUSSION

The high DNA binding affinity of TR4 to the monomeric response element is quite surprising. TR4 has been demonstrated to bind to bipartite repeats of the AGGTCA core sequence in several response targets (10, 12, 14-20). Thus, TR4 plays roles in viral gene expression, and appears to be involved in the hormone signaling pathways. Moreover, it has been shown that TR4 forms a homodimer, but not a monomer, in binding to tandem repeats of the AGGTCA core motif (19). Despite of binding to direct repeats, in the present

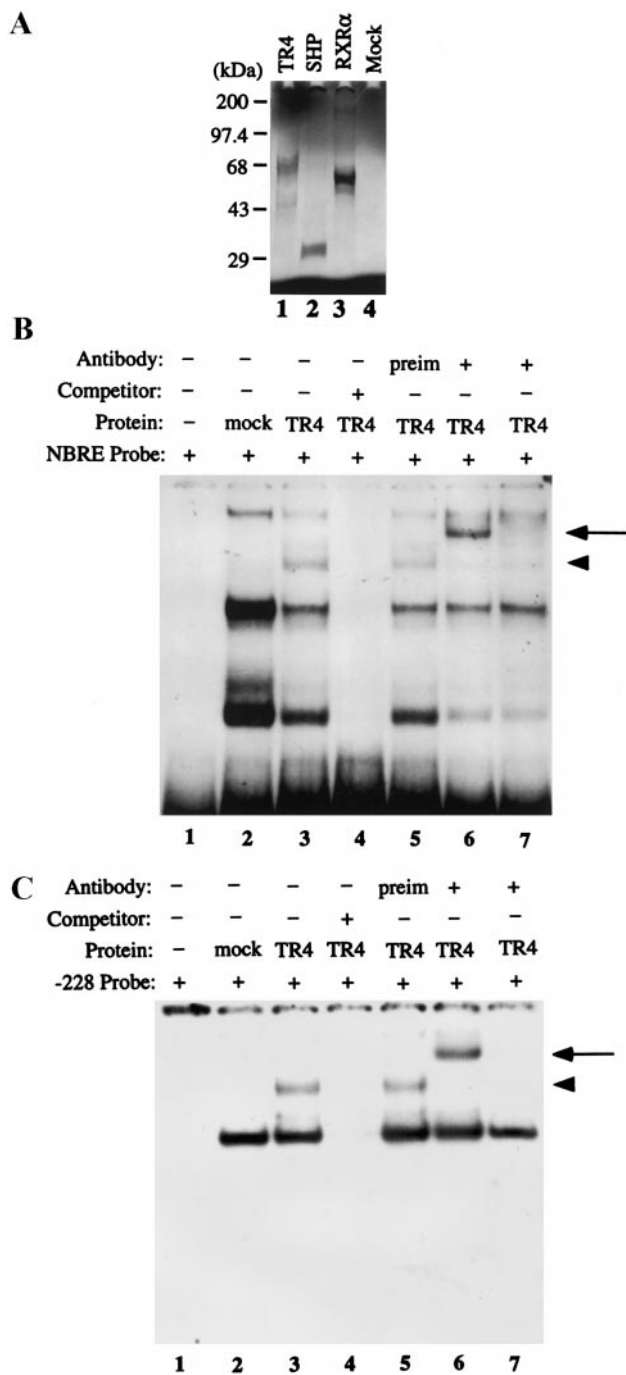


FIG. 3. Binding of the *in vitro* expressed TR4 to the monomeric response elements. (A) Analysis of *in vitro* translated products in SDS-10% polyacrylamide gel electrophoresis. TR4 (67 kDa), SHP (29 kDa), RXR α (51 kDa), and mock-translated products expressed in a coupled transcription-translation system are displayed in lanes 1-4, respectively. (B) Binding of TR4 to the NBRE. EMSA was performed with the *in vitro* expressed TR4 and the 32 P-labeled NBRE probe. Lane 1 displays the probe alone, which contains the 30-bp NBRE sequence. Binding reaction mixtures were incubated with the probe and either the mock-translated product (lane 2), or the *in vitro* synthesized TR4 (lanes 3-7) in the presence of 50-fold molar excesses of unlabeled NBRE (lane 4), preimmune serum (preim, lane 5), monoclonal anti-TR4 antibody (G232-416.3, lane 6), or another monoclonal anti-TR4 antibody (G232-85.6, lane 7). The retarded

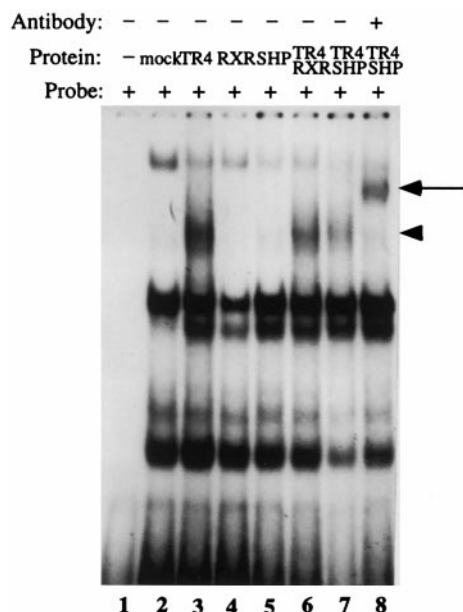


FIG. 4. Binding of the TR4 with other nuclear receptor partners to the -228TR4RE of the 21-OHase gene. Lane 1 displays the probe alone. The complexes resulting from binding reaction mixtures incubated with mock-translated product (lane 2), TR4 (lanes 3 and 6-8), RXR (lanes 4 and 6), or SHP (lanes 5, 7, and 8) in the presence of the monoclonal anti-TR4 antibody (G232-416.3, lane 8) are shown. The retarded and supershift complexes are indicated by an arrowhead and arrow, respectively.

report, we demonstrated that TR4 binds to a monomeric response element of the AGGTCA motif. As a result, TR4 may be a negative modulator of the 21-OHase gene regulation. Collectively, this unique monomeric DNA binding may extensively multiply and complicate the role of TR4 in physiological regulation.

In the comparison of synthetic and natural HREs, TR4 showed different binding preferences toward these DNA response elements (Figs. 1 and 2). In the case of synthetic HREs, there was only a two-fold difference in the binding affinity among DR1 to DR5. This indicates that TR4 has a broad and nondiscriminating ability to recognize synthetic DRs. In contrast, the binding affinity differed dramatically between TR4 and natural response elements, in which CRBPII reached the highest with an 87-fold difference in binding affinity. In addition to direct repeats, TR4 bound to palindromic ERE poorly, suggesting that direct repeats of the AGGTCA consensus motif function more effectively

complexes are indicated by an arrowhead for specific DNA-protein complexes, whereas the supershift band is marked by an arrow for DNA-protein-antibody complexes. Nonspecific complexes appear between the retarded complexes and the free probe at the bottom. (C) Binding of TR4 to the -228TR4RE of the human 21-OHase gene. EMSA was conducted with TR4 and the 32 P-labeled -228TR4RE under the same conditions as described for B.

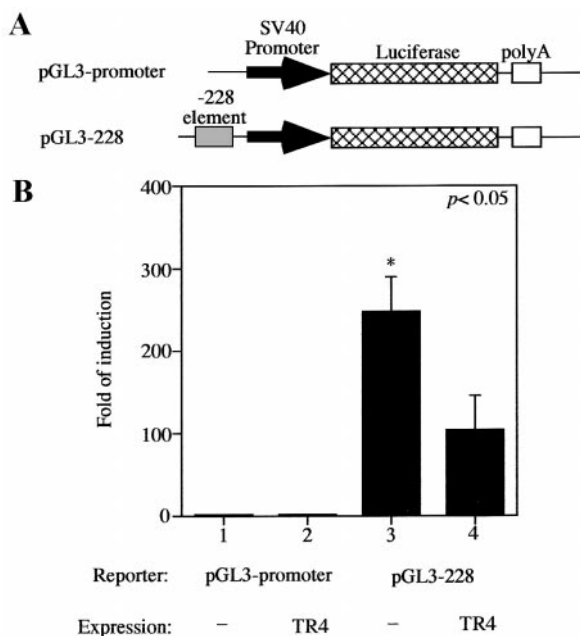


FIG. 5. TR4 represses the 21-OHase gene expression via the -228TR4RE by dual-luciferase reporter assay. (A) Schematic structure of the parent pGL3-promoter vector and pGL3-228 plasmid containing the -228TR4RE. (B) Functional assay. Mouse adrenal Y-1 cells were cotransfected with 3.5 μ g of either the parent pCMX (lanes 1 and 3) or the pCMX-TR4 expression plasmid (lanes 2 and 4), 4 μ g of either the parent pGL3-promoter (lanes 1 and 2) or pGL3-228 (lanes 3 and 4) reporter plasmid, and 1 μ g of pRL-TK. All firefly luciferase activities were normalized with *Renilla* luciferase activities and then averaged over at least three independent experiments with error bars designating standard deviations. Significant ($P < 0.05$) difference between control and experimental groups is marked with an asterisk.

as HREs for TR4. This great variety in binding preference of TR4 to different natural HREs strongly implies that not only the space between the direct repeats, but also the motif sequence contributed to the specific binding of TR4 as described above. These information may help us to understand how individual nuclear receptor differentially controls the specificity of gene networks. Moreover, the binding preference of TR4 for different HREs may also provide a clue for the identification of TR4 target genes.

Detailed analysis of members of the nuclear receptor superfamily has shown that dimerization produces novel complexes that bind DNA with unique properties, and thereby generate diversity in hormone response networks (2). In contrast, little is known about the contributions of each monomer toward the transcriptional regulation of the complex. We herein demonstrated that the monovalent HREs serve as binding targets for TR4. Such behavior would be in sharp contrast with classical family members, which may bind as either homo- or heterodimers with partners to two copies of the core recognition motif. These observations prompted us to ask how TR4 interacts with the mono-

meric DNA element. The Nurr1-RXR heterodimer model raises the possibility that RXR associates with the monomeric HRE-bound Nurr1 in the absence of RXR-specific DNA contacts (38). Based on this structural model, we therefore propose that TR4 may form homodimers in the DNA binding, while only one of them has specific DNA contacts. This hypothesis may reflect the fact that the migration position of the TR4-monovalent HRE complexes is the same as that of the TR4-bivalent HRE complexes (17). In addition, TR4 may form heterodimer with the TR2 orphan receptor, a closed subfamily member (9). However, TR4 does not form heterodimers with either RXR α or PPAR α (19), which is in concert with our finding here that TR4 can not form heterodimers with either RXR α or SHP. Collectively, this alternative pathway of TR4 versus the monovalent DNA response element again further provides diversity in physiological regulation by this orphan receptor.

Comparison of the 5' flanking sequences of the 21-OHase gene among human, mouse, and bovine revealed an important regulatory element which covers the major part of a highly conserved 40-bp region (39). This regulatory region has been demonstrated to be required for the expression of 21-OHase in Y-1 adrenal

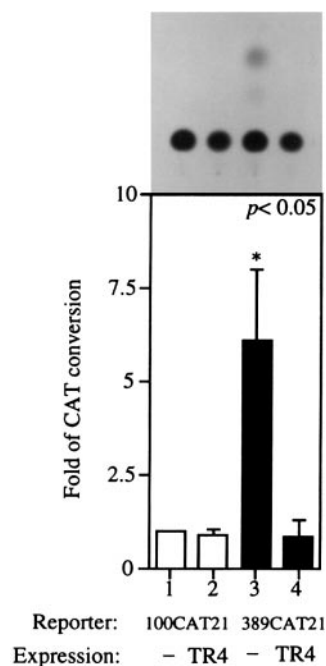


FIG. 6. TR4 represses the transcriptional activity of the 21-OHase promoter via the -228TR4RE by CAT assay. Adrenal Y-1 cells were cotransfected with 3.5 μ g of either the parent pCMX (lanes 1 and 3) or the pCMX-TR4 expression plasmid (lanes 2 and 4), 4 μ g of either the 100CAT21 (lanes 1 and 2) or 389CAT21 (lanes 3 and 4) reporter plasmid, and 1 μ g of pCH110. All CAT activities were normalized with β -galactosidase activities, and then averaged over at least three independent experiments with error bars designating standard deviations. Significant ($P < 0.05$) difference between control and experimental groups is marked with an asterisk.

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Human -250 CTCTGGATGC AGGAAAAGG TCAGGGTTGC
Mouse -227 --GACTGC-A TAC-G-G--- ----CTT
Bovine -191 A-TCT-GCTG TT--G----- ----T

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FIG. 7. Sequence comparison of a regulatory region among 21-OHase genes from different species. The nucleotide sequences of the homologous regions of three 21-OHase genes are shown (39). 21-OHase genes are from human (GenBank Accession Nos. M12792 and M23280), mouse (M15009 and M64933), and bovine (M11267 and M13545), respectively. Identical bases are indicated by a hyphen. The monomeric response element is boxed.

cells. Deletion of this region most probably reflects changes in the decrease in transcriptional rates of 21-OHase transcripts (39). As shown in Fig. 7, this regulatory region in the human gene is located between -250 and -221 nucleotides 5' of the transcriptional initiation site. Noteworthy, the AGGTCA consensus sequence of a monomeric response element is highly conserved in the center of this regulatory region among all three species. In contrast, the perfect monomeric response element is only present in the human gene, while both mouse and bovine genes vary in the AT-rich preceding region (Fig. 7). Recently, there are two important regulatory elements have been characterized in front of the human 21-OHase gene, the proximal (-300 bp) and distal (-4.6 to -5.6 kb) elements (26). Basically, the 300-bp proximal promoter contains several elements that appear to be crucial for both basal and tissue-specific and hormonally induced transcription. As a result, TR4 may have an additional regulatory effect on the human 21-OHase gene expression via this monomeric -228TR4RE within the 300-bp proximal element.

Interaction between nuclear receptors is an important event for the transcriptional regulation of genes controlling cellular functions. SF-1/Ad4BP is a member of the nuclear receptor superfamily, and interacts with conserved AGGTCA promoter elements to regulate the coordinate expression of the cytochrome P450 steroid hydroxylases (40). 21-OHase is one of well-characterized SF-1-responsive genes (26, 27). It raises the possibility that SF-1 and other nuclear receptors (such as NGFI-B, COUP-TF, and TR2) have the great potential for competition (at the same or overlapping AGGTCA half-site) or cooperativity (via homo- or heterodimerization) in the complex regulatory circuits of 21-OHase gene expression. In this regard, further studies will be needed to provide a better understanding of antagonistic or cooperative interactions of these transcriptional regulators on the steroidogenic enzyme expression in adrenocortical cells.

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