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Review

# Recent advances in the TR2 and TR4 orphan receptors of the nuclear receptor superfamily

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## Abstract

The human testicular receptor 2 (TR2) and TR4 orphan receptors are two evolutionarily related proteins belonging to the nuclear receptor superfamily. Numerous TR2 and TR4 variants and homologs have been identified from different species, including vertebrates (e.g. human, murine, rabbit, fish, and amphibian) and invertebrates (e.g. *Drosophila*, sea urchin, and nematode) since TR2 was initially isolated over a decade ago. Specific tissue distribution, genomic organization, and chromosomal assignment of both orphan receptors have been investigated. In order to reveal the physiological functions played by both TR2 and TR4, upstream modulators of TR2 and TR4 gene expression, their downstream target gene regulation, feedback mechanisms, and differential modulation mediated by the recruitment of other nuclear receptors and coregulators have been investigated. Studies summarized in the present report have provided unexpected insights into the TR2 and TR4 functions in a variety of biological processes. The essential and difficult tasks of identifying orphan receptor ligands, agonist/antagonist assignment, their physiological functions, and mechanisms of action will continue to challenge nuclear receptor researchers in the future. © 2002 Elsevier Science Ltd. All rights reserved.

**Keywords:** Steroid and thyroid hormone receptors; Orphan receptors; Gene regulation; Coregulators

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## 1. Introduction

The functions of steroid and thyroid hormones are mediated through the action of specific receptor proteins [1,2]. Nuclear receptors (NRs) comprise a huge family of ligand-dependent transcription factors that regulate complex gene networks in a wide variety of biological processes, such as growth, development, and differentiation [2]. Members of this 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. The functional structure and organization of the NR, as shown in Fig. 1, generally contains six (A–F) domains with a well conserved DNA-binding domain in the central core (C domain) of the protein, which is responsible for DNA recognition and dimerization [1–5]. Distal to the DNA-binding domain is a variable hinge region (D domain) containing a nuclear localization signal. It is assumed that this region may allow the receptor to bend or alter its conformation [5]. The ligand-binding domain, located in the C-terminal region, is relatively large and functionally complex. It is believed that this domain contains regions important for ligand-binding, dimerization, transactivation, and intramolecular repression [3–5]. The N-terminal domain is highly variable in amino acid sequence and in length. This domain contains a transactivation function which regulates gene expression by interacting with the core transcriptional machinery, coactivators, or other transactivators [3–5].

Hormones are synthesized and secreted by endocrine cells [6,7]. These hormones travel via the blood stream to their

target cells, enter these cells by simple or facilitated diffusion, and then bind to specific receptors in the cytoplasm or nucleus (Fig. 2). Upon binding with their respective ligands, the NRs undergo an activation or transformation step. The ligand–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 transcription of the target gene. The target gene will be transcribed, translated into protein product, and ultimately alter cellular function [8].

Orphan receptors comprise a novel group of NR-like proteins [2,3,9,10]. Numerous orphan receptors have been isolated from low-stringency hybridization screening, genetic and molecular cloning techniques [2,9,11]. Due to distinctive homology in their amino acid sequences, orphan receptors have been classified as members of the NR superfamily. Consequently, they have no known ligand and usually no known function when they are initially identified. Thus far, we estimate approximately 170 orphan receptors have been identified from different species and various tissues [3,12]. Nevertheless, it is assumed that most of these orphan receptors have important cellular functions based on the following criteria: (1) they are expressed as proteins in cells, sometimes with cell type and developmental specificity; (2) they are members of a highly evolved family of transcriptional factors; (3) several orphan receptors have been shown to function by regulating known specific target genes and developmental processes; (4) certain orphan receptors have been implicated in the mediation of cellular responses to neurotransmitters, retinoic acids (RAs), peroxisome proliferators, or phosphorylation pathways; and (5) some orphan

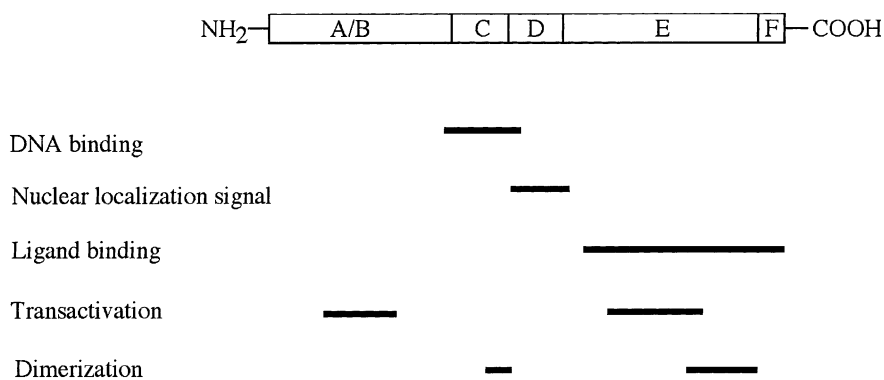


Fig. 1. Structural/functional domains of a typical nuclear receptor.

receptors may function as coregulators to modulate signaling pathways.

Differential recognition of target genes by the NR members, which regulate the transcription of complex gene networks, is determined by at least three properties: protein–DNA interactions, protein–protein interactions, and protein environment. Firstly, protein–DNA interactions are mediated by the highly conserved DNA-binding domain that defines the NR superfamily [13]. The molecular specificity of NRs is achieved by their selective interaction with DNA-binding sites referred to as HREs [14]. The HREs are structurally related but functionally distinct. Based on the finger model, the first zinc finger in the DNA-binding domain of NRs may determine target HRE specificity. As shown in Fig. 3, three amino acids in the C-terminal region of the first zinc finger are marked as the proximal (P) box, which is important in base interaction [14–17].

Consequently, HREs can be classified into two categories of repeat consensus sequences based on the P box, the glucocorticoid receptor (GR) and estrogen receptor (ER) response element subfamilies [16]. The GR subfamily, which includes GR, androgen receptor (AR), progesterone receptor (PR) and the mineralocorticoid receptor (MR) recognizes two AGAACA core consensus half sites. The ER subfamily, which includes ER, testicular receptor (TR) the 1,25-dihydroxyvitamin D<sub>3</sub> receptor (VDR) retinoic acid receptor (RAR), retinoic × receptor (R × R), and many orphan receptors, recognizes two AGGTCA consensus half sites. Based on the P box, TR2 has been grouped with members of the ER subfamily [14]. Therefore, we suspect that TR2 may recognize AGGTCA core consensus motifs. In addition, five amino acids localized to the second zinc finger, referred to as the distal (D) box, are important in dimerization contact formation [14]. Moreover, two elements in the

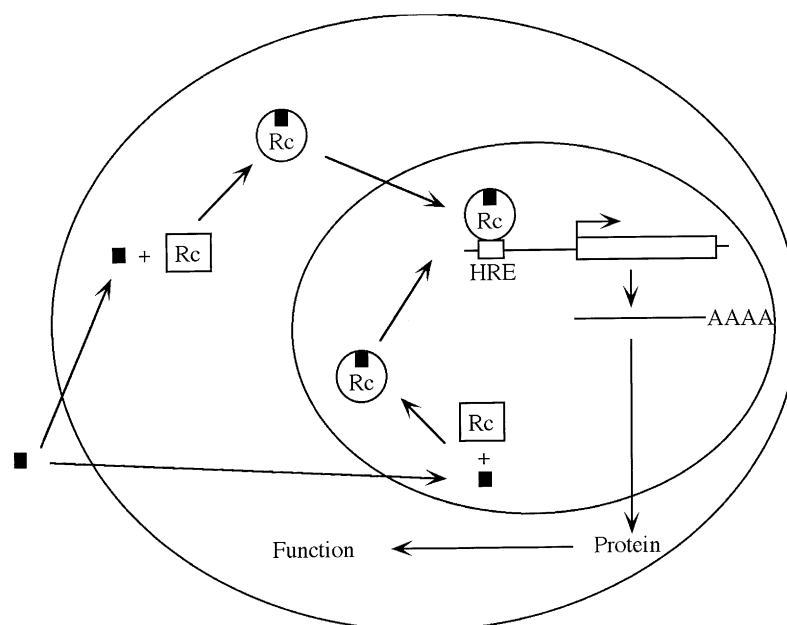


Fig. 2. Molecular mechanism of nuclear receptor action. The closed square and Rc represent the hormone and receptor, respectively.

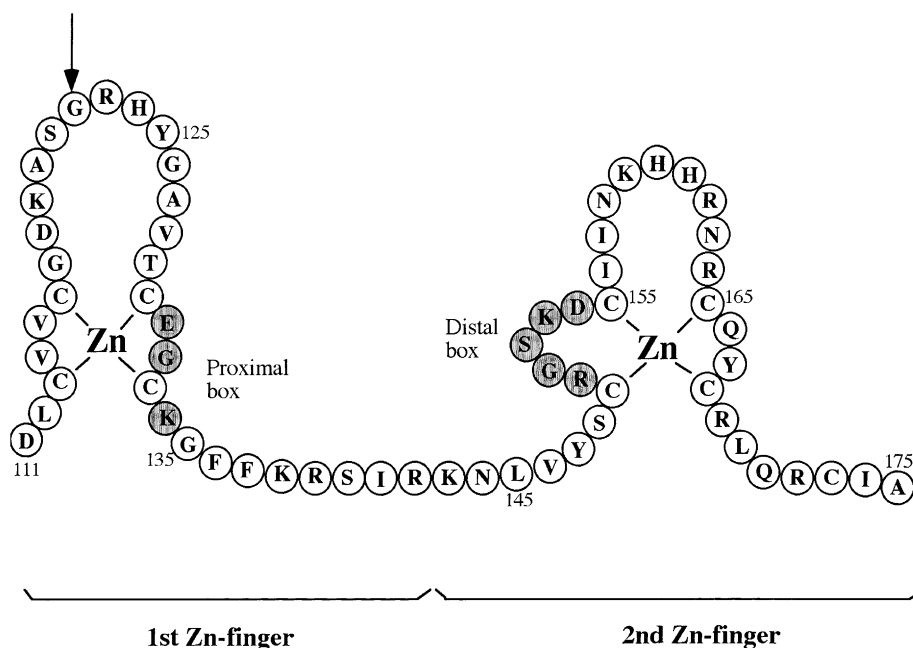


Fig. 3. Structural scheme of the DNA-binding domain of the human TR2 orphan receptor cDNA. Numbers represent amino acid sequence of TR2. The unique RNA splice site is marked by the arrow. Amino acid residues involved in the P (proximal) and the D (distal) boxes are dotted.

C-terminal region of the second zinc finger of the NGFI-B orphan receptor, referred to as the A and T boxes, are also critical for DNA recognition [18–20], highlighting the importance of non-zinc-finger regions in the specificity of DNA binding. On the other hand, several features of a HRE determine the specificity of DNA recognition. These include the precise sequence, the orientation of the core recognition motifs, and the spacing between core motifs [13,14,21,22]. The intrinsic DNA-binding properties of NRs cannot be simply ascribed to sequence, orientation, or spacing rules, as exemplified by the NGFI-B response element (NBRE) in which sequences outside of the core recognition motifs also contribute to the specificity of DNA binding [19,20].

Secondly, protein–protein interactions necessary for the formation of homo and/or heterodimers in solution are mediated by an extensive C-terminal dimerization interface in the ligand-binding domain, an inhibitor of transcription in the ligand-binding domain, and the D box in the DNA-binding domain [13]. The members of the NR superfamily are capable of binding to DNA in monomeric, homodimeric, and heterodimeric modes. A comprehensive analysis of the ligand-binding domain reveals several critical regions for protein–protein interactions. A highly conserved region, referred to as conserved region II or Ti (transcriptional inactivation) plays an important role in the dimerization of TR and other superfamily members [23]. A DNA-supported asymmetric dimerization interface is located within the DNA-binding domain of NRs and selectively promotes DNA binding to cognate direct repeat HREs [24–26]. In addition, the identity (I) box located in an extensive C-terminal dimerization interface in the

ligand-binding domain is structurally similar to the leucine zipper dimerization domain [18,27–30]. The combination of this obligatory I box and an optional dimerization interface in the DNA-binding domain increases the diversity of heterodimeric interactions and high affinity DNA binding [29].

Thirdly, the protein environment also influences selectivity of NR recognition of their target genes [14]. For example, ligand specificity is one of the key parameters contributing to DNA-binding and dimerization. Studies of TR and R × Rs suggest that their respective ligands may affect dimerization and diversity of function [13,31–34]. Furthermore, nuclear accessory factors (coregulators) including coactivators, corepressors, and cointegrators, are thought to serve as bridging molecules or adaptors between NRs and the basal transcriptional machinery [8,35]. Finally, the chromatin (nuclear matrix) structure may facilitate or restrict NR action [36–38]. The nuclear matrix is the structural component of the nucleus that determines nuclear morphology and organizes the DNA in a three-dimensional fashion. The nucleoprotein organization of a variety of hormone responsive regulatory elements and reconstruction of complex chromatin templates are important features in NR action. While the molecular mechanism of the NR regulation of gene expression is currently unclear, evidence suggests that the interplay of the specific three-dimensional organization of the genome, the structural components of the nucleus (nuclear matrix) and histone modification enzymes (histone deacetylases and acetyltransferases) may all contribute to the regulation of gene expression [36–39].

Our long-term goals are to understand the physiological properties of TR2 and TR4, and their possible roles in

cellular responses. In searching for TR2 and TR4 target genes, we may be able to identify new response systems with valuable physiological implications. By revealing the roles of TR2 and TR4 in different physiological pathways, we may ultimately determine their ligand specificity and biological functions.

## 2. Molecular cloning and sequence comparison

### 2.1. The TR2 and TR4 orphan receptors and their homologs

The human TR2, is one of the first orphan receptors identified and it shares structural homology with members of the NR superfamily [40,41]. As is illustrated in Fig. 4, different lengths of human TR2 cDNA variants, TR2-5, -7, -9, and -11 have been isolated [40–43]. Northern blot analysis suggested that the TR2-11 transcript may represent the major forms among TR2 variants, [44,45]. Consequently, we have used the TR2-11 in further investigation of TR2 function. Subsequently, TR2 homologs from different species, such as rat [44], mouse [46–50], *Drosophila* [51–53], sea urchin [54], amphibian [55,56] and nematode [57], have also been cloned. Detailed cloning information, calculated molecular mass, size of the transcripts, and GenBank accession numbers of TR2 variants are summarized in Table 1(a).

The human and rat TR4 orphan receptor cDNAs were initially cloned from human and rat hypothalamus, prostate, and testes libraries [58]. Later, another identical human TR4 cDNA, named TAK1, was also cloned. [59]. Two TR4 transcripts, 7.8 and 2.8 kb, were determined by Northern blot analysis. The 7.8 kb transcript is expressed ubiquitously, while the 2.8 kb transcript is restricted to the testes. Recently new variants of TR4 from different species have also been isolated [60–64] and are summarized in Table 1(b).

### 2.2. Amino acid sequence comparison

The deduced amino acid sequence of the TR2 DNA-binding domain, with the typical type II zinc-finger motif, shows a certain degree (50–54%) of homology with that

of several known NRs, as shown in Fig. 5A. In contrast, amino acid comparison of the putative ligand-binding domain of TR2 shows very low homology (<10%) with classical steroid receptors. Nonetheless, high methionine content is a common feature of the ligand-binding domain of NRs [43], and TR2 also has a high methionine content in this region. Together, these results support the classification of TR2 as a member of the NR superfamily. TR2 ligand binding, however, remains to be demonstrated. The TR2 ligand may or may not be a steroid hormone, or alternatively, this orphan receptor may function without binding to any ligand [51,65–68].

Amino acid comparison of the DNA- and ligand-binding domains of both TR2 and TR4 with those of their homologs shows relatively high homology among these regions (Fig. 5B). TR2 is highly conserved in the DNA-binding domain among the human, murine, and amphibian species, while the *Drosophila* and sea urchin TR2 proteins have 70–80% homology, and the nematode TR2 has <50% homology in this region. Additionally, the human and murine TR2s have about 90% homology in their putative ligand-binding domains. This implies that the potential ligand, if it exists, for this protein may be important for the activation of this receptor during evolution. The amino acid sequences of the ligand-binding domains of the *Drosophila*, sea urchin, and nematode TR2 orphan receptors are less conserved than that of human TR2. However, the amino acid sequence of TR2 is also closely related to that of TR4. This suggests that these two orphan receptors constitute a unique subfamily within the NR superfamily [58]. In the case of TR4, amino acid comparison indicates that this protein is highly conserved in the DNA- and ligand-binding domains among species homologs identified so far (Fig. 5C).

## 3. Tissue distribution

Using Northern and dot blot analyses, the tissue distribution of the human TR2 transcripts was analyzed [40–42]. The results showed that TR2 mRNA is most abundant (per unit of RNA) in the rat androgen-sensitive prostate, and is least abundant in the estrogen-sensitive uterus, with the following

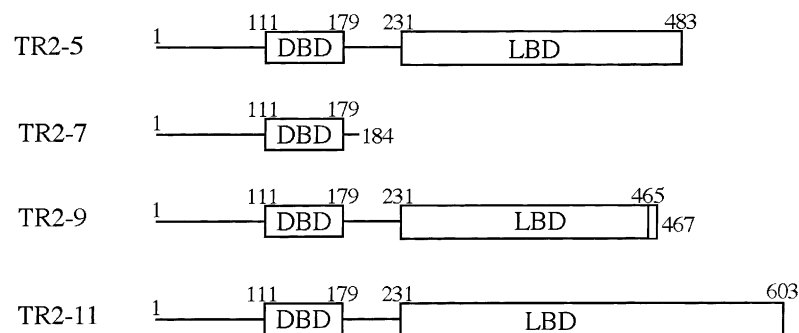


Fig. 4. TR2 orphan receptor variants. Numbers indicate amino acid position. The putative DNA- (DBDs) and ligand-binding domains (LBDs) are boxed.

Table 1  
TR2 and TR4 orphan receptor and its homologs

Isotype	GenBank accession number	Amino acid	Calculated molecular mass	Screening library	Reference
(a) TR2					
Human					
TR2-5	M21985	483	53	Testes	[40–43]
TR2-7	None	184	21	Testes	[40–43]
TR2-9	M29959	467	51	Testes	[40–43]
TR2-11	M29960	603	67	Prostate	[40–43,45]
Rat TR2	L26398	590	66	Prostate	[44]
Mouse					
TR2	U30482	590	66	Testes	[46]
TR2	U28265	590	70	Embryo	[47]
mTR2R1	S75970	629	69	Brain	[48]
Tr2-11	Y11436	590	66	Eye	[49]
TR2-11-t	None	184	20	Testes	[50]
<i>Drosophila</i>					
XR78E/F	U31517	601	66	Imaginal disc	[53]
DHR78	U36791	601	66	Larvae	[51]
TmHR78	AJ005765	489	55	Epidermis	[52]
Sea urchin					
SpSHR2	U38281	583	75	Embryo	[54]
Amphibian					
aDOR1	AF008302	416	45.8	Embryo	[55]
xDOR2	AF013295	542	60	Embryo	[56]
Nematode					
Bp-nhr-2	AF091044	112	14.4	Filariae	[57]
(b) TR4					
Human					
TR4	L27586	615	67	Prostate/testes	[58]
TAK1	U10990	596	65	Raji cells	[59]
TR4a1	M29959	467	51	Brain/placenta/ovary	[60]
TR4a2	M29960	603	67		[60]
Rat					
TR4	L27513	596	65	Prostate/testes	[58]
rTR4-1	None	596	65	Brain	[61]
rTR4-2	None	596	65	Brain	[61]
rTR4-3	None	602	65	Brain	[61]
Mouse					
TR4	U32939	596	67	Testes	[62]
mTAK1	U11688	596	66	Testes	[63]
Rabbit					
TR4-0	None	596	66	Heart	[64]
TR4-1	None	246	33	Heart	[64]

relative amounts in other tissues: ventral prostate as 100%; seminal vesicle, 92%; testes, 42%; submaxillary gland, 18%; uterus, <1%. Additionally, TR2 mRNAs were detected in total RNA isolated from LNCaP cells [43]. LNCaP is an androgen-dependent human prostatic carcinoma cell line originally isolated from a lymph node metastatic lesion. Using the 1.6 kb *EcoRI* restriction fragment of the TR2-11 cDNA as a probe, two bands of 2.5 and 3.0 kb were visualized. To determine whether these two bands represent two TR2 variants in LNCaP cells, RNA blots were probed with a 0.4 kb *SacI*–*EcoRI* fragment from the TR2-5 (or TR2-7 and 9) cDNA, and a 0.6 kb *EcoRI*–*EcoRI* fragment

from the TR2-11 cDNA. Both bands were positive for the TR2-11-specific probe, but negative for the TR2-5-specific probe. These results demonstrated that TR2-11 is the only TR2 form expressed in LNCaP cells. This observation and the fact that TR2-5, 7, and 9 cDNAs were isolated from a testes library, and that the TR2-11 cDNA was isolated from a prostate library suggest that the expression of TR2 variants exhibits a certain degree of tissue specificity. The coexpression of the TR2-5, 7, and 9 variants brings up the possibility of competition between receptor forms for ligand binding. Different receptor forms may bind to different natural ligands, or may bind to the same ligand with different affinities.

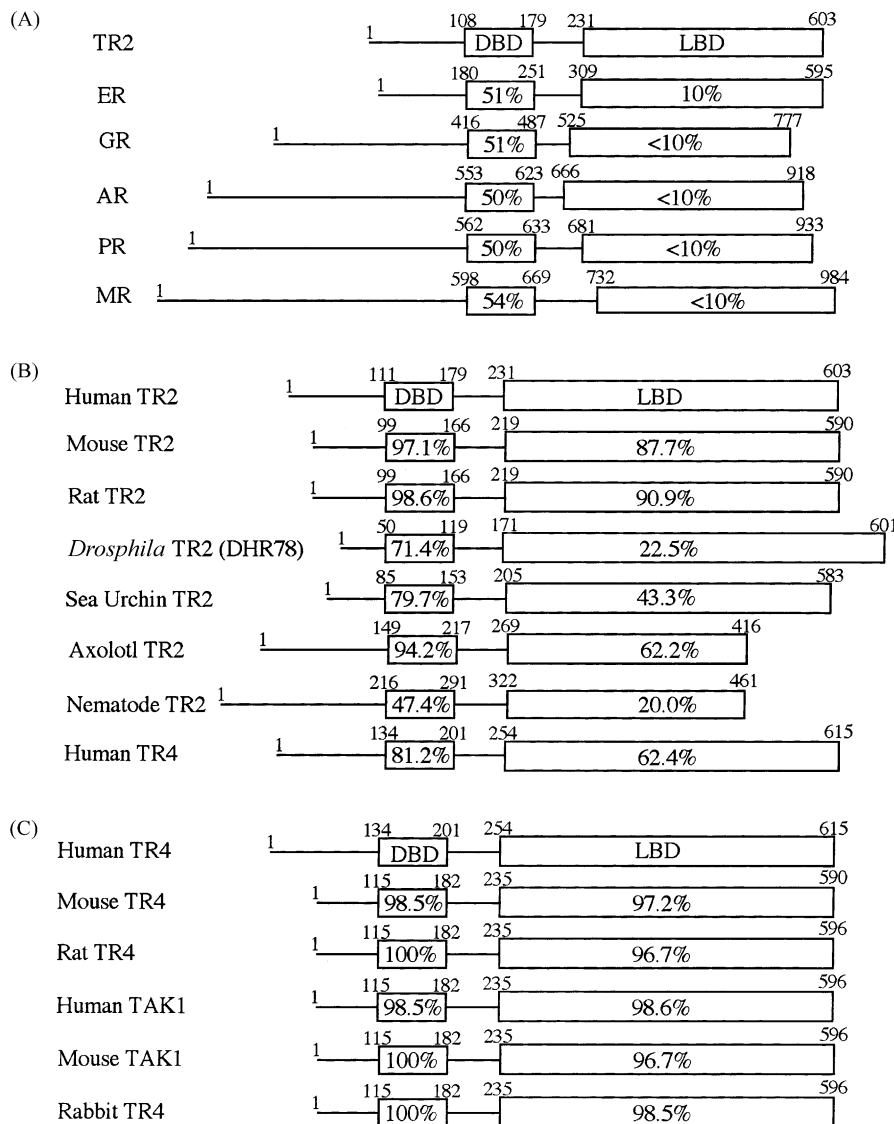


Fig. 5. Amino acid homology between TR2 and classical steroid receptor cDNAs (A) and TR2, TR4, and their homologs cDNAs (B, C) in the putative DNA- and ligand-binding domains (boxed). Numbers indicate amino acid position. Homology is calculated by setting oldpep as the scoring matrix parameter, using the BestFit program of the SeqWeb Version 2.02 of GCG (Genetics Computer Group).

The activation of target genes could therefore, be regulated by control of the expression ratio of variant receptor forms.

Recently, in situ hybridization techniques were applied to determine the developmental expression pattern of TR2 in mouse tissues and embryos [46,68,69]. In general, TR2 is expressed in many mouse tissues, including the brain, kidney, intestines, and other tissues [68]. At embryonic day 16.5 (E16.5) TR2 expression in the liver is reduced significantly, but is still higher than that in the pancreas, which serves as the background level of expression. In contrast, the signals in the kidney and certain regions of the brain remain strong. At higher magnification, TR2 expression in the kidney is localized in the glomeruli, the tubules, and the interstitial tissues between tubules. The expression of TR2 is especially prominent in developing neural areas and in certain non-neural developing organs [46]. At E9, a stage at

which early events of neuronal proliferation and differentiation occur, the TR2 transcripts are expressed abundantly and ubiquitously throughout the neural epithelium. During E11–16, TR2 expression increases, but is gradually restricted to the periventricular zones of the developing brain vesicles, where many cells are undergoing mitosis. High levels of TR2 expression are also found within the primitive cerebellum, neocortex, striatum, and olfactory bulb of the E16 brain. Other brain regions at E16 express few TR2 transcripts. In addition, abundant TR2 transcripts are detected in the developing spinal cord, including the motor neurons during E11–13. Moreover, prominent TR2 expression is found in certain developing ganglia and most targets of sensory innervation. Ganglia with high levels of TR2 expression include the sensory ganglia at the dorsal root and trigeminal, sympathetic, and parasympathetic ganglia. The

neuronal epithelium of the cochlea, nasal cavity, tongue, and retina express high levels of TR2 mRNA as well [68].

The overall TR4 expression pattern in the mouse is similar to that of TR2, as described above [62,63]. In general, TR4 transcripts are expressed in many mouse tissues, including the central nervous system, and peripheral organs, such as the adrenal gland, spleen, thyroid gland, and prostate. A coronal section through the rostral region of the adult mouse forebrain demonstrates the presence of TR4 transcripts within the hippocampus, habenula nuclei, thalamus, and throughout the cortical layers. The most intense signals are found in the cortex, piriform cortex, regions of ammon's horn, and the dentate gyrus. Furthermore, the signal within the dentate gyrus is localized to the granule cells, which undergo active postnatal neurogenesis. TR4 transcripts are detected as early as E9 [62]. Within the nervous system of E9–11 mice, TR4 is initially expressed uniformly throughout the neural tube along both its rostrocaudal and dorsoventral axes. Subsequently, the expression becomes restricted to the ventricular zones of the brain vesicle where cells are rapidly proliferating during E14–16. High levels of TR4 transcript are also observed in the striatum, the actively dividing population of the cerebellar primordium, spinal cord, and spinal motor neurons of the E16 brain. Recently, a detailed study of TR4 expression during brain development was performed in the rat [70]. TR4 transcripts are highly expressed in the central nervous system at E14.5–19.5. In postnatal rats, TR4 transcripts are mainly found in the hippocampus and cerebellum. In the adult rat brain, TR4 transcripts are predominantly expressed in granule cells of both the hippocampus and cerebellum. These results collectively suggest that both TR2 and TR4 are widely and actively involved in many aspects of proliferation and differentiation during early embryonic development.

#### 4. Genomic organization and chromosomal assignment

The human *TR2* gene was mapped to human chromosome 12 at band q22 using fluorescence in situ hybridization combined with a high-resolution G-banding technique [71]. The entire *TR2* gene features 13 introns and 14 exons joined by the consensus splice sequences (GT-AG) at all intron–exon boundaries. It is noteworthy that genomic structures of all known NRs contain a splice site between the first and second zinc fingers in the DNA-binding domain. In contrast, the *TR2* gene has a unique splice site located in the middle of the first zinc finger (Fig. 3). This finding suggests that the *TR2* could be an evolutionary ancestor within the NR superfamily. An S1 RNase protection assay revealed that there are multiple transcription initiation sites (TIS) and that the major one is located at nucleotide 104, upstream from the translation start codon. In addition, sequence analysis of a 2.7 kb 5' flanking fragment suggests that several potential *cis*-acting elements exist in this region, such as the AP-1, HNF-5, GATA1, and GC boxes. Deletion

analyses and chloramphenicol acetyltransferase (CAT) assays further revealed that a minimal promoter, containing a 103 bp DNA fragment of the initiator-like sequences from 65 to –38, is capable of triggering basal level TR2 transcription [72]. Maximal transcriptional activity is achieved by the sequences included within the 65 to –441 region. An important *cis*-regulatory region of TR2 may be localized in the region between –73 and –441. This positive regulatory region can be further narrowed down to a 64 bp fragment (from –263 to –201) termed as the TR2 promoter activating *cis*-element (TR2-PACE). Promoter activity drops to the basal level when the TR2-PACE is internally removed. CAT activity can be enhanced significantly as long as this TR2-PACE is placed upstream of a basal promoter containing either a TATA box or an initiator element fused to a reporter gene in the coding strand orientation. Moreover, electrophoretic mobility shift assay (EMSA) and southwestern analysis showed that unknown nuclear protein(s) may bind to the TR2-PACE. Sequences within this region are protected from DNase I digestion by the addition of nuclear extracts. In summary, detailed genomic structure analysis of the human *TR2* gene has provided a better understanding of how *TR2* gene expression is modulated.

A 19-nucleotide motif composed of two RGGTCA inverted repeats with a spacing of 7 nucleotides (IR7) has been identified upstream of the *TR2* gene from each species tested [73]. Partial cDNAs of TR2 homologs have been recently isolated from rainbow trout (*Oncorhynchus mykiss*) and zebrafish (*Danio rerio*). The IR7 motif is evolutionarily conserved among the *TR2* genes, suggesting that this TR2–IR7 may be an important *cis*-acting element in TR2 expression. In addition, a stretch of 20 amino acid residues, containing a constitutively active nuclear localization signal, was found within the second zinc-finger motif of TR2 [74].

Several important genes have been found around the TR2 locus, including the B-cell translocation gene-1, mast cell growth factor, the transcription factor NF-Y B subunit, and insulin-like growth factor-1. Interestingly, the TR3 orphan receptor was also initially mapped to the same chromosome (12q13.1) [75]. In addition, chromosomal abnormalities including gene loss, in this region are associated with specific forms of neoplasia or loss of potential tumor suppressor genes [76]. Whether there is a potential linkage between the *TR2* gene and its neighbors will be an interesting question to investigate.

The mouse *TR2* gene has been mapped to the distal region of mouse chromosome 10 [77]. This gene spans a distance of more than 50 kb and is organized into 13 exons. The TIS is located at the 158th nucleotide upstream from the translation initiation codon. All the intron–exon junction sequences also follow the GT-AG rule. Several immune response elements were identified within a 500 bp fragment upstream from the TIS, but there is apparently no TATA box.

The human *TR4* gene was mapped to chromosome 3 at band p24.3, which is in a totally different locus from the human *TR2* gene [61,71]. A 7 kb *Xba* I–*Xba* I genomic

fragment from the *TR4* gene was used for chromosome localization, using dual-color fluorescence in situ hybridization combined with G-banding techniques. Interestingly, several members of the NR superfamily have been mapped to the vicinity of the *TR4* gene, including the human peroxisome proliferator activated receptor (*PPAR* $\gamma$ ), *RAR* $\beta$  and thyroid receptor (*TR* $\beta$ ) on chromosomes 3p25, 3p24.2–p24.3, and 3p22–p24.1, respectively [60]. Additionally, a clinical report indicated that a mentally retarded patient with blepharophimosis-ptosis-epicanthus inversus syndrome had a chromosomal deletion of 3p25 [78]. These results suggest that *TR4* may play an important physiological role in the nervous system.

## 5. Regulation of *TR2* and *TR4* expression

### 5.1. Androgen

The expression of human *TR2* mRNA is negatively regulated by androgen in the rat ventral prostate and in the human prostate cancer LNCaP cell line [40,42–44]. Dot blot hybridization showed that *TR2* mRNA in the rat ventral prostate increases about two-fold above normal levels two days after castration. This increased mRNA expression could be reversed by the injection of androgen (5 $\alpha$ -dihydrotestosterone, DHT). In addition, injection of the antiandrogen (flutamide) into intact rats increases *TR2* mRNA levels up to two-fold over normal levels. In human prostate cancer LNCaP cells, DHT can repress *TR2* mRNA to 60% of normal levels, while 18S RNA control is unaffected [44]. This finding suggests that the *TR2* gene may be regulated by AR. As the physiological role of *TR2* is currently not clear, the impact of androgen repression of *TR2* is also unknown. Nevertheless, the repression of *TR2* expression provides an additional model to study negative gene regulation by androgen in the prostate.

### 5.2. Radiation and tumor suppressors

The expression of human *TR2* is down regulated by ionizing irradiation [79]. Moreover, irradiation can repress the expression of *TR2* at both the transcriptional and translational levels. Ionizing irradiation, which damages DNA, induces a p53-dependent G<sub>1</sub> phase cell cycle arrest. The p53 tumor suppressor is involved in both the initiation of apoptosis after radiation-induced DNA damage and transient G<sub>1</sub> cell arrest. p53 protein can also repress *TR2* gene expression, and this repression can be reversed by the presence of the simian virus 40 (SV40) large T-antigen.

Recently, *TR2* expression was completely repressed in surgery-induced cryptorchidism, the failure of the testes to descend into the scrotum at birth, in the rhesus monkey [80]. Furthermore, the p53 and retinoblastoma gene product (Rb) tumor suppressors can repress *TR2* expression via the p53–p21–cyclin-dependent kinases (CDKs)–Rb–E2F signal pathway. From these results it can be concluded that radia-

tion, cryptorchidism, and tumor suppressors p53/Rb can repress *TR2* expression, providing a new pathway to link ionizing radiation and tumor suppressors p53/Rb to members of the NR superfamily.

### 5.3. Retinoids

Mouse *TR2* gene expression is dramatically decreased in Vitamin A-depleted animals [77]. Testis sections from normal and Vitamin A-depleted mice were stained for *TR2* expression by immunohistochemistry using a purified antibody. The expression of *TR2* protein is repressed in Vitamin A-depleted mice compared to the control, suggesting that *TR2* expression is limited to the haploid round and elongating spermatids. The specificity of *TR2* expression to haploid germ cells further indicates a function of this protein in the regulation of spermatogenesis.

Additionally, data supports *TR2* involvement in the RA signal transduction pathway [67,69]. All-*trans*-RA (10<sup>-6</sup> M) can induce the binding of human *TR4* to an R  $\times$  R response element (R  $\times$  RE) in murine F9 teratocarcinoma cells [81]. Northern blot analysis further proved that *TR4* mRNA could be increased by all-*trans*-RA-treatment. These results suggest that the retinoid signaling pathway can regulate the expression of the *TR4* gene. Moreover, an inverted repeat (IRO) element was identified in the P promoter region of the mouse *TR2* gene [82]. This response element was further characterized as a functional retinoic acid response element (RARE). Recently, Western blot analysis further confirmed that RA can increase *TR4* expression at the protein level in human HaCaT keratinocytes [83]. Collectively, these results suggest that the expression of both *TR2* and *TR4* in different species is involved in the retinoid signaling pathway.

### 5.4. Ciliate neurotrophic factor

The expression of both mouse *TR2* and *TR4* is up-regulated by ciliary neurotrophic factor (CNTF) or RA in mouse P19 cells during neuron differentiation [46]. CNTF is a neurocytokine which promotes the survival and differentiation of a variety of neuron cell types, including sensory, sympathetic, parasympathetic, and motor neurons. CNTF does not play a crucial role during embryonic development as determined in studies with mice lacking CNTF, but regulates the maintenance of motor neuron cells in the mouse. It was demonstrated that CNTF can induce the expression of both *TR2* and *TR4*, and potentiates their ability to bind to the CNTFR-DR1 response element of the CNTF receptor (CNTFR $\alpha$ ) gene. This regulation may lead to a continuous supply of CNTFR $\alpha$  in response to CNTF treatment.

### 5.5. Dopamine

Human *TR2* is not constitutively active in transactivational assays [84]. A chimera, PR-h*TR2*, containing the N-terminal and DNA-binding domains of PR fused with the

potential ligand-binding domain of human TR2, was constructed to test TR2 transactivational activity in CV-1 cells grown in serum-free medium. This chimera can be activated in a ligand-independent manner by the catecholamine neurotransmitter dopamine. As dopamine activates intracellular signal transduction cascades, the observed activation of TR2 may involve phosphorylation by a kinase effector of dopamine signaling.

## 5.6. Others

The expression of human TR2 is relatively abundant compared to other members of the NR superfamily by RT-PCR analysis. Degenerative PCR cloning from human umbilical vein endothelial cells (HUVEC) indicates that TR2 and chicken ovalbumin upstream promoter transcription factor I (COUP-TF I) together account for approximately 60% of the total clones [85], and TR2 is the second most abundant transcript among cloned family members.

A high abundance of TR4 transcripts can be found in nervous tissues as previously described. Degenerative PCR cloning from rat supraoptic nuclei (SON) in the hypothalamo-neurophyseal system led to the identification of five NR family members, including TR4 [86]. Dot blot screening of amplified gene fragment analysis showed that TR $\alpha$ , apolipoprotein AI regulatory protein (ARP-1) TR4, and COUP-TF I are the most abundant receptors expressed in the SON region, in the following order: TR $\alpha$  > ARP-1 > TR4 = COUP-TF I. This finding indicates that the peptide-producing magnocellular neurons of the SON, differing from peripheral oxytocin-producing cells, express a specific set of transcriptional factors of the NR superfamily. Moreover, RT-PCR analysis revealed that TR4 is detectable in the brain, placenta, and ovary [60], while the human ovarian cancer PA1 cell line fails to express TR4.

## 6. Target genes and coregulators of the TR2 and TR4 orphan receptors

### 6.1. Viral gene expression

#### 6.1.1. Simian virus 40 major late promoter

The first DNA response element (TR2RE–SV40) for human TR2 was identified in the SV40+ 55 region [66]. EMSA analysis showed specific binding with high affinity (dissociation constant = 9 nM) between TR2 and this TR2RE–SV40 sequence. DNA-swap experiments using the CAT assay demonstrated that androgen can suppress the transcriptional activity of the SV40 early promoter via the interaction between this TR2RE–SV40 and the chimeric receptor AR/TR2/AR (the DNA-binding domain of TR2 flanked by the N-terminal and androgen-binding domains of AR). In addition, this TR2RE–SV40 can function as a repressor to suppress the transcriptional activities of both SV40 early and late promoters. These data suggest the

TR2RE–SV40 may represent the first identified natural DNA response element for TR2, and TR2 functions as a repressor for SV40 gene expression.

The key expression of the SV40 major late promoter can also be repressed by human TR4 via the SV40+ 55 region [87]. EMSA analysis showed specific binding with a dissociation constant of 1.09 nM between TR4 and SV40+ 55 oligonucleotides. Moreover, results from the CAT assay indicated that TR4 can function as a repressor via this element, suppressing the transcriptional activities of both the SV40 early and late promoters. TR4 may therefore, play an important role in the suppression of SV40 gene expression.

#### 6.1.2. Human immunodeficiency virus type 1 long-terminal repeat

The roles of human TR2 and TR4 in the gene regulation of the long-terminal repeat of the human immunodeficiency virus type 1 (HIV-LTR) has been investigated [88]. In gel-retardation assays, both TR2 and TR4 showed high affinity to a palindromic (symmetric) element (PR9) at the 5'-end of the HIV-LTR, with equilibrium dissociation constants of 1.11 and 0.52 nM, respectively. Although both TR2 and TR4 showed no effect in regulating the transcriptional activity of CAT downstream of the HIV-LTR, only TR4 can cross-talk with COUP-TF I and TR $\alpha$ 1 to potentiate the transcriptional activity of the HIV-LTR. These results indicate that TR4, but not TR2, may associate with other NRs in the up-regulation of HIV replication.

#### 6.1.3. Hepatitis B virus core promoter

Infection of the liver by human hepatitis B virus (HBV) leads to significant liver diseases, ranging from acute liver failure to chronic active hepatitis, liver cirrhosis, and hepatocellular carcinoma. EMSA analysis showed that human TR4 is able to bind to an imperfect direct repeat response element with a nucleotide in the spacer (DR1; nucleotides 1757–1769) in the core promoter of HBV (Lin et al., manuscript in preparation). A reporter gene assay revealed that TR4 can enhance the transcriptional activity of the HBV core promoter. This TR4-mediated transactivation may be down regulated by cotransfection of AR. In addition, human TR2 has been shown to bind to this DR1 in the precore promoter, and repress the synthesis of precore RNA [89]. These findings indicate that appropriate TR2 or TR4 ligands may be useful in the treatment of viral infection, e.g. HBV.

#### 6.1.4. Papillomavirus type 16 long control region

Human TR2 may up-regulate the transcriptional activity of the human papillomavirus type 16 (HPV-16) long control region [80,90]. HPV is an epitheliotropic DNA tumor virus associated with the development of cervical carcinoma. The sequences in the HPV-16 long control region regulate cell type and constitutive expression from the promoter p97. The oncogenic properties of HPV-16 are primarily conferred by two viral genes, E6 and E7. E6 can associate with the p53 tumor suppressor, resulting in degradation of p53 protein. TR2

gene expression can be down regulated by p53 and reporter gene assays indicate that E6 can reverse the effect of p53 on TR2 expression [90]. Moreover, TR2 can bind to a DR4 response element in the HPV-16 promoter, and potentiate HPV transcriptional activity in a dose-dependent manner.

## 6.2. Hormone signaling pathways

### 6.2.1. RAR/R × R signal transduction

Human TR2 binds to synthetic HREs consisting of two AGGTCA half sites with various types of spacing in the following order, from greatest to least affinity: DR1 > DR2 > DR5 = DR4 = DR6 > DR3 [69]. TR2 also binds to natural HREs with distinct affinities: cellular retinol-binding protein II promoter region (CRBP<sub>II</sub>p; DR1) > SV40+ 55 region (DR2) > RAREβ (DR5) = NBRE = palindromic TRE. The wide spectrum of genes with HRE in their promoters suggests that TR2 may cross-talk with other signal transduction systems. CAT assays demonstrated that TR2 modulates CRBP<sub>II</sub>p- and RAREβ-CAT gene expression activated by R × Rα and RARα/R × Rα heterodimers, respectively. These data suggest that TR2 may be a master regulator in modulating the expression of two key genes, RAREβ and CRBP<sub>II</sub>p, involved in the RA signal transduction pathway [67,69].

Human TR4 binds specifically to DR1 and DR5 response elements, shown to be present in both RAR and R × R genes by EMSA [81]. CAT reporter gene assay demonstrated that TR4 represses RA-induced transactivation in a dose-dependent manner. Inhibition of the retinoid signaling pathway also occurs through natural response elements found in the CRBP<sub>II</sub>p and RAREβ genes. These results indicate that retinoid signaling can be regulated by TR4 in a negative feedback control mechanism, which may restrict RA signaling to certain elements in a cell-specific fashion. Similar characterization of this negative modulator of gene regulation mediated by the retinoid signaling pathway has been demonstrated in various studies [91–93].

### 6.2.2. TR targets

Human TR2 induces transactivational activity in a DR4–TRE system [94]. TR2 binds to a synthetic DR4–TRE with slightly greater binding affinity than that of the TRα<sub>1</sub>/R × Rα heterodimer, with dissociation constants of 0.5 and 2.3 nM, respectively. Both TR2 and the TRα<sub>1</sub>/R × Rα heterodimer compete with each other for binding to limited numbers of DR4–TRE. TR2 inhibits the suppressive effect of unliganded TRα<sub>1</sub> on CAT reporter activity in a dose-dependent fashion. Conversely, ER and the chimeric TR2/ARp/TR2 fail to bind to DR4 and therefore, do not influence the inhibitory effect of unliganded TRα<sub>1</sub>. When triiodothyronine (T<sub>3</sub>) is supplemented, estradiol-ER inhibits TRα<sub>1</sub>/R × Rα-mediated CAT activity, while TR2 has an additive effect on TRα<sub>1</sub>/R × Rα transactivation. These results indicate that DNA binding is essential for TR2 to take action, while fully functional liganded TRα<sub>1</sub> may rely on common factors shared with ER but not TR2.

Human TR4 induces the transcriptional activity of a reporter gene containing a DR4 response element, in a TR HRE [95]. EMSA and Scatchard analysis indicated a strong binding affinity (dissociation constant = 2 nM) between TR4 and the DR4. TR4 also enhances the expression of the α-myosin heavy-chain and rat *S14* genes, which contain the DR4 or DR4-like motifs. Furthermore, this transcriptional activation by TR4 is dose and DR4 sequence-dependent. These data suggest that this DR4 may be a positive regulatory element for TR4, and that TR4 may induce the transcriptional activity of genes containing such HREs.

### 6.2.3. VDR targets

Cell transfection and in situ hybridization analyses demonstrated that the expression of the Vitamin D<sub>3</sub> target gene, 25-hydroxyvitamin D<sub>3</sub> 24-hydroxylase, can be repressed by human TR4 through high affinity binding (dissociation constant = 1.32 nM) to the DR3–VDRE [96]. EMSA and proteolytic analysis showed that TR4 adopts to different conformations once bound to DR3–VDRE or DR4–TRE. These different TR4-DR3–VDRE and TR4-DR4–TRE conformations may allow TR4 to recruit different coregulators.

### 6.2.4. PPAR targets

Human TR4 (TAK1) binds as a homodimer to the peroxisome proliferator response elements (PPREs) present in the promoter of the PPARα target genes rat enoyl-CoA hydratase and peroxisomal fatty acyl-CoA oxidase [97]. TR4 represses PPARα-mediated transactivation through these PPREs in a dose-dependent manner, indicating that TR4 plays a role in PPARα signaling.

### 6.2.5. Luteinizing hormone receptor gene

Yeast one-hybrid screening and EMSA showed that the human TR4 binds to an imperfect DR element in the promoter of the human luteinizing hormone receptor (LHR) gene [98]. Functional analysis in CV-1 cells revealed that human TR4 may activate LHR promoter activity through the imperfect DR *cis*-element. These findings suggest that TR4 is able to contribute to the differential expression of the LHR gene in gonadal and non-gonadal tissues.

### 6.2.6. Steroid 21-hydroxylase gene

Human TR4 binds as a homodimer to a DNA response element containing two direct repeats of the AGGTCA consensus motif [93]. Surprisingly, the expression of the human steroid 21-hydroxylase (21-OHase) gene is repressed by TR4 via the monomeric AGGTCA motif (–228TR4RE) in its 5′ flanking region [99]. EMSA showed specific binding with a dissociation constant of 0.2 nM between TR4 and the monomeric –228TR4RE. Additionally, both dual luciferase and CAT reporter assays demonstrated that TR4 can function as a repressor via the –228TR4RE of the 21-OHase gene. These data suggest that TR4 may play an important role in 21-OHase gene expression by binding to a monomeric DNA response element.

### 6.2.7. Erythropoietin gene

A TR2RE-EPO has been identified in the 3' minimal hypoxia-inducible enhancer of the human erythropoietin (EPO) gene [68]. EPO is an essential survival and growth factor for erythrocytic progenitor cells in the bone marrow. EPO is synthesized mainly in the kidney and fetal liver in response to hypoxia in mammals. EMSA showed specific binding with high affinity (dissociation constant = 0.14 nM) between TR2 and the TR2RE-EPO. EMSA with different TR2 truncation variants further indicated that this specific binding is not due to the homodimerization of TR2. In addition, TR2 may suppress CAT activity via the TR2RE-EPO in the human hepatoma HepG2 cells under hypoxic or normoxic conditions. These results suggest that the human EPO gene may be the first human target gene found to be regulated directly by TR2.

## 6.3. Membrane receptor genes

### 6.3.1. Histamine H<sub>1</sub> receptor gene

A DNA response element (TR2RE-HR) in the 3' flanking region of the human histamine H<sub>1</sub> receptor (HR) gene was identified as a target for human TR2 [100]. Tetracycline-inducible and improved differential display systems were both used to isolate a cDNA fragment differentially regulated by TR2. Northern blotting and sequence analysis further confirmed that the expression of the HR gene was differentially repressed by TR2. EMSA also revealed a specific binding (dissociation constant = 47.8 nM) between TR2 and the TR2RE-HR. Furthermore, reporter gene assays indicated that TR2 may suppress the expression of luciferase activity via the TR2RE-HR in HeLa cells. These results indicate that the human HR gene may be directly regulated by human TR2, and support the use of tetracycline-inducible and improved differential display systems to study target genes regulated by orphan receptors.

### 6.3.2. CNTFR $\alpha$ gene

A CNTFR-DR1 was identified in the fifth intron of the alpha component of the human CNTFR $\alpha$  gene [62]. EMSA showed a specific high affinity binding (dissociation constant = 0.066 nM) between TR4 and the CNTFR-DR1. A CAT reporter gene assay demonstrated that the fifth intron of the CNTFR $\alpha$  gene has enhancer activity, which could be induced by TR4 in a dose-dependent manner. These data suggest that the human CNTFR $\alpha$  gene is a neural-specific gene induced by TR4. Furthermore, it has also demonstrated that human TR2 induces CNTFR $\alpha$  transcriptional activity through binding to the same response element [46].

## 6.4. The muscle system

### 6.4.1. Aldolase A gene

Human TR2 was recently identified as an M1 site binding protein of the muscle-specific pM promoter in the human

aldolase A gene [101]. EMSA showed specific binding with high affinity (dissociation constant = 4.6 nM) between TR2 and the M1 element. A circular permutation assay revealed that localized DNA flexibility is induced by TR2 binding, with an estimated bend angle of  $73 \pm 2^\circ$ . Furthermore, a dual-luciferase reporter gene assay demonstrated that TR2 enhances luciferase activity via the wild-type M1 site but not the mutant M1 element in QM7 myoblasts. In conclusion, our data demonstrate that TR2 may be a transcriptional activator of muscle-specific aldolase A gene expression.

### 6.4.2. Actin gene

Sea urchin TR2 (SpSHR2) is encoded by a maternal RNA that persists throughout embryonic development to the pluteus stage [102,103]. TR2 binds to the C1R response element in the upstream promoter of the *CyIIIb* actin gene. Although further studies are required to confirm these results, preliminary observations suggest that the actin *CyIIIb* gene is a TR2 target gene in the sea urchin embryo.

## 6.5. Apoptosis

The repression of human TR2 gene expression by irradiation is mediated by p53, a key regulator of Bcl-2 in apoptotic pathways. Both TR2 and TR4 can induce the expression of Bcl-2 in many cells (Kim et al. manuscript in preparation). Other NRs, e.g. AR and coregulators, e.g. receptor-interacting protein 140 (RIP140) can suppress this TR4-mediated Bcl-2 gene expression. Moreover, data indicate that over-expression of mouse TR2 in mouse P19 cells induces cellular apoptosis in the absence of RA [104]. These results imply that the linkage of TR2/TR4 and Bcl-2 is important in the processes controlling cell survival and death.

## 6.6. Coregulators

NRs are able to either directly interact with components of the general transcriptional machinery, or indirectly interact through heterodimerization or interaction with nuclear coregulators (coactivators, corepressors, and coin-tegrators) [35,105]. A mouse TR2 corepressor, RIP140, has been identified from a mouse embryonic cDNA library [106]. The receptor-interacting domain of RIP140 was mapped to regions containing the LXXLL motif, and the RIP140-interacting domain of TR2 was mapped to its C-terminal 10- to 20-amino-acid sequence of the putative activation function 2 (AF-2) region. RIP140 functions as a repressor of TR2-mediated transcriptional regulation. A TR4 coregulator (TRA16) was isolated from a human testes library by yeast two-hybrid screening (Yang et al., manuscript submitted). TRA16 has a molecular mass 16 kDa and has novel sequences unlike any known genes/proteins. Reporter assays showed that TRA16 could suppress the transactivation of both TR2 and TR4 in a dose-dependent manner.

Several NRs have also been shown to influence the transcriptional activity of TR2 and TR4. Yeast and mammalian two-hybrid assays, the GST pull-down assay, and EMSA have demonstrated that TR2 and TR4 form heterodimers [107]. This dimerization is mediated by the ligand-binding domains, and three leucine residues in helix 10 are critical for this interaction. In addition, coexpression of these two orphan receptors exerts a stronger repressive activity than expressing either receptor alone in a reporter gene assay. The AR can also heterodimerize with TR4, and function as a repressor to down-regulate *TR4* target genes by preventing the binding of TR4 to those genes [108]. Reciprocally, TR4 can repress AR target gene expression. However,  $R \times R$ , a common heterodimerization partner of NRs, cannot interact with either TR2 or TR4, making TR2 and TR4 distinct from the  $R \times R$ -heterodimer NR subfamily [107,108]. These data reveal a unique cross-talk pathway within the NR superfamily based on heterodimerization between TR4 and AR. Interestingly, ER can also heterodimerize with TR4 and TR2, and function as a repressor of their target genes, respectively [109], Hu et al., in press). TR2 and TR4 may also function as repressors of ER-mediated target gene regulation.

## 7. Autoregulation and feedback mechanisms

### 7.1. Retinoids

As we described previously, RA can increase the expression of human TR4 at the mRNA and protein levels [81,83]. On the other hand, TR2/TR4 can repress gene regulation mediated by the retinoid signaling pathway. We hypothesize that the retinoid signaling pathway can be regulated by TR2/TR4 through a negative feedback control mechanism, which may restrict RA signaling in a cell-specific fashion [81]. Thus, RA may have at least two ways to regulate its targets. One way is to bind with RAR/ $R \times R$  and positively regulate RA targets. Another is to increase the expression of TR2/TR4, resulting in negative regulation of RA targets.

### 7.2. CNTF

The roles of both TR2 and TR4 in CNTF signaling were studied, as previously described [46]. The expression of both mouse TR2 and TR4 is up-regulated by CNTF in mouse P19 cells during neuron differentiation. Additionally, mouse TR4 induces the expression of the *CNTFR $\alpha$*  gene via the CNTFR-DR1 response element [62]. These results suggest the existence of an autoregulation mechanism within the CNTF signaling pathway. The function of this neurocytokine, CNTF, to promote the survival and differentiation of a variety of neuronal cells, is mediated through its receptor, CNTFR $\alpha$ . CNTF can also increase the expression of TR2 and TR4, resulting in increased expression of its own receptor, CNTFR $\alpha$ . Thus, an autoregulation cycle amplifies neurocytokine signals through these orphan receptors.

### 7.3. Viral and tumor suppression pathways

p53 functions as a tumor suppressor by arresting cells at the  $G_1$  stage of the cell cycle in response to DNA damage induced by radiation or other stimuli. The expression of TR2 can be down regulated by ionizing irradiation and p53 [79]. This p53-mediated repression can be reversed by the SV40 T-antigen. Both TR2 and TR4 can repress *SV40* gene expression via a DR2 response element [66,87]. The expression of TR2 is also down regulated by the Rb tumor suppressor [80], and TR2 induces expression of the *HPV-16 E6* gene, which also inactivates p53 [90]. These results indicate that TR2 is a target of negative growth regulatory proteins, and may even inhibit viral oncoproteins to maintain the activity of these regulatory proteins. Finally, the downstream effectors of p53 include p21 (Waf-1/Cip-1) and CDKs ( $G_1$  arrest) for negatively cell cycle regulation, and Bax/Bcl-2 (apoptosis) pathways, respectively.

## 8. Functional mechanisms of target gene regulation

There are many studies investigating the role of NRs in the transcriptional activation of eukaryotic promoters [6,7].

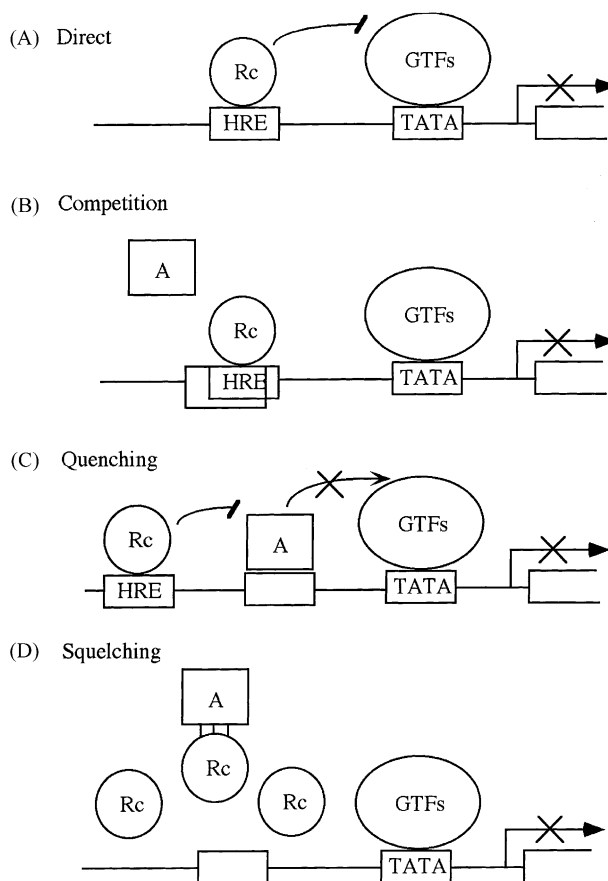


Fig. 6. Models for the molecular basis of transcriptional repression by members of the nuclear receptor superfamily in (A) direct repression of basal transcription; (B) competitive repression; (C) quenching repression; and (D) squelching repression. GTFs represents general transcription factors, Rc for activated receptor, and A for activator or coregulator.

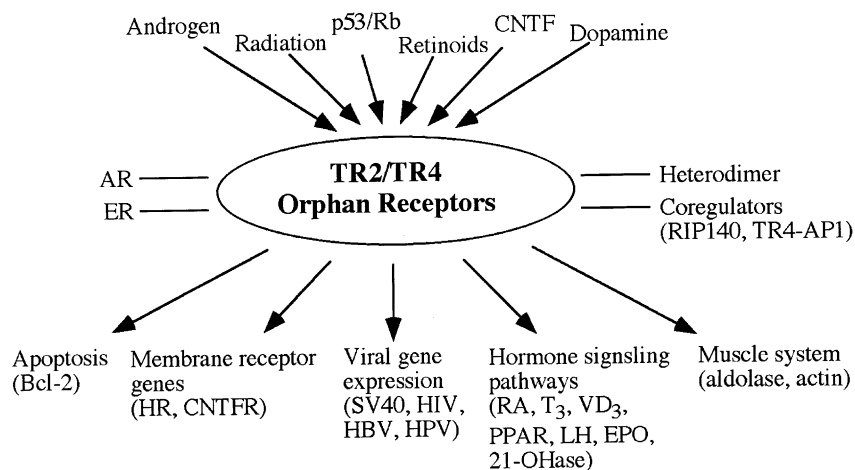


Fig. 7. TR2 and TR4 signaling pathways.

Considerably less is known about the molecular mechanisms of transcriptional repression by NRs [110,111]. As illustrated in Fig. 6A, a direct repression mechanism has been proposed whereby an activated receptor, a repressor, binds to a HRE at some distance from the target promoter and interferes with the activity of the basal transcription machinery [111]. In the competitive repression mechanism a repressive receptor binds to overlapping or closely linked sequences, preventing the binding of an upstream activating protein (Fig. 6B). According to the quenching mechanism, the repressive receptor and activating protein bind to adjacent and non-overlapping DNA sequences (Fig. 6C). The receptor may then inhibit the activity of the activating protein by protein–protein interactions. The last model is the squelching mechanism (Fig. 6D) where the receptor is over-expressed and sequesters the activator protein without binding to DNA sequences.

In this review article, we have described how TR2 and TR4 bind to specific HREs to repress gene expression. Direct and/or competitive repression therefore, explain the mechanism of transcriptional repression by TR2 and TR4. The squelching mechanism may more appropriately represent the repression mechanism used by other receptor members, e.g. AR and ER, or corepressors, e.g. RIP140 and TR4-AP1, to suppress TR2 and TR4. The upstream modulators of *TR2/TR4* gene expression, their downstream target genes, and the influence of recruitment other NRs and coregulators are summarized in Fig. 7.

## 9. Future perspectives

Gene regulation by NRs is initiated by binding of their cognate ligands. Ligand activation of orphan receptors remains controversial, since their ligands have not yet been identified. Several ligands or synthetic compounds reportedly activate orphan receptors [112]. The identification of

ligands for orphan receptors has basically relied on in vitro biochemical methods, including proteolysis, photocrosslinking, chemical extraction, radiolabeled-ligand binding, affinity chromatography, and the yeast three-hybrid system. Among these, the most frequently used approach is the transactivation assay using transient transfection of reporter and target gene constructs in cultured mammalian cells. In these reporter gene assays, hybrid receptor constructs, with the potential ligand-binding domain fused with a known DNA-binding domain, have often been used [113]. To date, there is no general method for the identification of new ligands for orphan receptors in vivo. While various attempts have been made to identify potential ligands for TR2 and TR4 (Lee and Chang, personal communication) it is currently unclear whether TR2 and TR4 are ligand-independent (constitutively activated) or ligand-dependent transcription factors [51,65–68]. During the study of TR2 and TR4 regulation of SV40, we, for the first time, identified potential ligands (or activators) of both orphan receptors present in serum [66,87]. Independently, this phenomenon was confirmed by the study of natural RZR/ROR response elements, which suggested that normal serum may contain a ligand or an activator for COUP-TF [114]. In addition, Wei's group showed that the expression of TR2 is limited to the haploid round and elongating spermatids [77]. They proposed that the TR2 ligand is most likely synthesized and secreted by sertoli cells, since the haploid germ cells are isolated between adjacent sertoli cells. Furthermore, Conneely's group reported identifying a potential TR2 activator molecule [84]. They have tested more than 150 candidate compounds, including both natural and synthetic steroids, isoprenoids, prostaglandins, T<sub>3</sub>, thyroxine (T<sub>4</sub>) fat soluble vitamins, and lipid/water soluble tissue extracts. None of these compounds affect CAT activity. However, recently, Thummel's group proposed that *Drosophila* TR2 (DHR78) is a ligand-activated receptor, since DHR78 can be induced by 20-hydroxyecdysone in cultured larval organs [51,52,65]. The identification of ligands for both

orphan receptors may facilitate the development of antagonists or agonists as new therapeutic agents for clinical applications.

Gene knock-out (KO) animals are one of the approaches used to define the biological function of orphan receptors. Currently, we are conducting both *TR2* and *TR4* gene KO experiments in mice. Genomic organization of both orphan receptors has previously been characterized allowing targeted disruption of the DNA-binding domain of TR2 or TR4 by the insertion of a neomycin resistance gene cassette along with the *lacZ* gene. ES cell electroporation and the generation of heterozygous mutant mice were undertaken with the collaboration of the Lexicon Genetics Inc. Results from gene *KO/lacZ* knock-in studies may shed some light on the physiological functions of these two orphan receptors. Following generation of KO animals, we characterized the TR2 and TR4 KO mice. To our surprise, we found that the mice lacking TR2 are viable and have no serious developmental defects. Both female and male are fertile [115]. On the other hand, from our preliminary observation we found that the TR4 KO mice display a growth defect, and reduced fertility or abnormal mating frequency/behavior. Female TR4 KO mice display abnormal maternal behavior, and animals of either sex display hypersensitivity to manipulation. Additional areas where evidence for defects among TR4 KO animals exist include bone and muscle development, the development or differentiation and migration of specific neuronal nuclei, as well as aspects of spinal cord development and function. Studies to confirm the initial phenotypic observations of the TR4 KO animals, as well as to explore aspects of TR4 function in more depth, are ongoing.

The specificity of gene regulation by NRs can be modulated by protein–DNA and protein–protein interactions. Differential NR-mediated gene expression may involve the recruitment of different coregulators [8]. Thus, we surmise that both TR2 and TR4 may also require accessory factors, in addition to potential ligands, to function properly. Indeed, several receptors associate with TR2 and TR4, and modulate their activity. Further studies of orphan receptor-associated proteins may increase our understanding of the complexity of NR action.

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