Gene expression of the androgen repressed rat TR2 orphan receptor: a member of steroid receptor superfamily

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A full-length rat cDNA clone was obtained from the TR2 orphan receptor, a member of the steroid receptor superfamily, using cDNA library screening and 3′ RACE-PCR technology. Under these conditions, only the TR2-11 form of the TR2 orphan receptor, the major form found in prostate, was identified. The overall amino acid homology between human and rat TR2-11 orphan receptors was near 90% with one amino acid difference in the DNA-binding domain sequence. Northern blot analysis identified multiple forms of the TR2 orphan receptor mRNAs expressed in human and rat prostates. Androgens repressed TR2 orphan receptor mRNA levels in human prostate LNCaP cells and rat ventral prostate. Polyclonal anti-TR2 orphan receptor antibodies raised from a unique TR2 orphan receptor 20 amino acid peptide were used to localize the TR2 orphan receptor in the nuclei of prostate and epididymis epithelium cells. Together, these data demonstrate that the TR2 orphan receptor can be expressed at mRNA and protein levels in the human and rat prostrates and may have some potential function in mediating androgen action in these tissues.

Keywords: orphan receptor; prostate; androgen; LNCaP cells

Introduction

The discoveries of orphan receptors (Chang & Kokontis, 1988; Giguere et al., 1988) have expanded our knowledge of steroid hormone receptors. So far at least 25 orphan receptors have been identified from various tissues of different species (Fuller, 1991; Segraves, 1991; Laudet et al., 1992; Chang et al., 1994). With the identification of 9-cis retinoic acid for the ligand of RXR orphan receptor (Levin et al., 1992; Mangelsdorf et al., 1992) and the discoveries that COUP-TF and RXR orphan receptors could function as an accessory factor for the other steroid receptors (Kliewer et al., 1992a; Leid et al., 1992), the impact of orphan receptors has become more significant.

The cDNAs of human TR2 and TR3 orphan receptors were isolated from the screening of human testis and prostate cDNA libraries for androgen receptors using an oligonucleotide probe homologous to the highly conserved part of the DNA-binding domain of glucocorticoid receptor (Chang & Kokontis, 1988; Chang et al., 1989a,b). The in vitro translated TR2 orphan receptor did not bind to any known steroids such as androgens, estrogens, progesterones and glucocorticoids (Chang et al., 1989a).

Attempts to identify the ligand for TR2 orphan receptor through the construct of chimeric receptors have revealed some clues as to how it is activated. For example, the

chimeric receptor, TR2/AR/TR2 receptor, with the N-terminal domain and C-terminal putative ligand-binding domain of the TR2 orphan receptor and DNA-binding domain of the androgen receptor, is not constitutively active when expressed in monkey kidney COS-1 and human prostate PC-3 cells. In contrast, another chimeric receptor, TR3/AR/TR3 receptor, can constitutively activate CAT (with ARE in promoter region) expression in the absence of any added factors (Kokontis et al., 1991). Lydon et al. (1992) also reported that a chimera of TR2 orphan receptor can be activated in the ligand-independent manner through a signal transduction pathway initiated at the cell membrane by the neurotransmitter dopamine.

Together, these data suggest that TR2 orphan receptor may regulate gene expression by different pathways of activation. Four TR2 orphan receptors have been isolated from human testis (TR2-5, 7, 9) and prostate (TR2-11) libraries (Chang et al., 1989a). Based on our genomic structure analysis, TR2-7 orphan receptor may due to an unspliced exon at right after DNA-binding domain (Chang et al., unpublished data). For the TR2-5, 9 and 11 orphan receptors, they differ in length of the C-terminal domain: a region that has been shown for other members of the steroid hormone receptor family to be the ligand-binding domain. In this report, we determined which of these variants represented the major form of TR2 orphan receptors in prostate. Using a RACE-PCR technique and two primers to amplify all potential 3′ ends of TR2 orphan receptors in human and rat prostates we obtained only the TR2-11 orphan receptor. However, using human and rat TR2-11 cDNAs as probes, we could still detect multiple TR2 orphan receptor transcripts in human prostate LNCaP cells and rat ventral prostate. In addition to identifying the major form of TR2 orphan receptor in prostate, we also report on its mRNA level regulation by androgens and on its cellular localization using antibodies derived from a TR2 orphan receptor peptide.

Results

Cloning and sequencing analysis of the rat TR2-11 orphan receptor cDNA

Using a 1.7 kb EcoRI DNA fragment encoding the 5′ end of the human TR2-11 orphan receptor as a probe, we isolated a 1.7 kb clone from a rat prostate λgt 11 library. Sequence analysis showed the rat TR2 orphan receptor 1.7 kb clone consisted of 5′-untranslated region of 188 bp and an open reading frame with extensive homology to human TR2-11 orphan receptor (Figures 1 and 2).

Using rat prostate RNA and one set of primers (C1 plus oligo (dT) and C2, a common sequence appearing in TR2-5, 9, and 11), we applied a RACE (rapid amplification of cDNA ends) -PCR method (Graham et al., 1991; Chang et al., 1994) to clone the missing 3′ end of the rat TR2 orphan receptor (Figures 1 and 2). Using this method we only
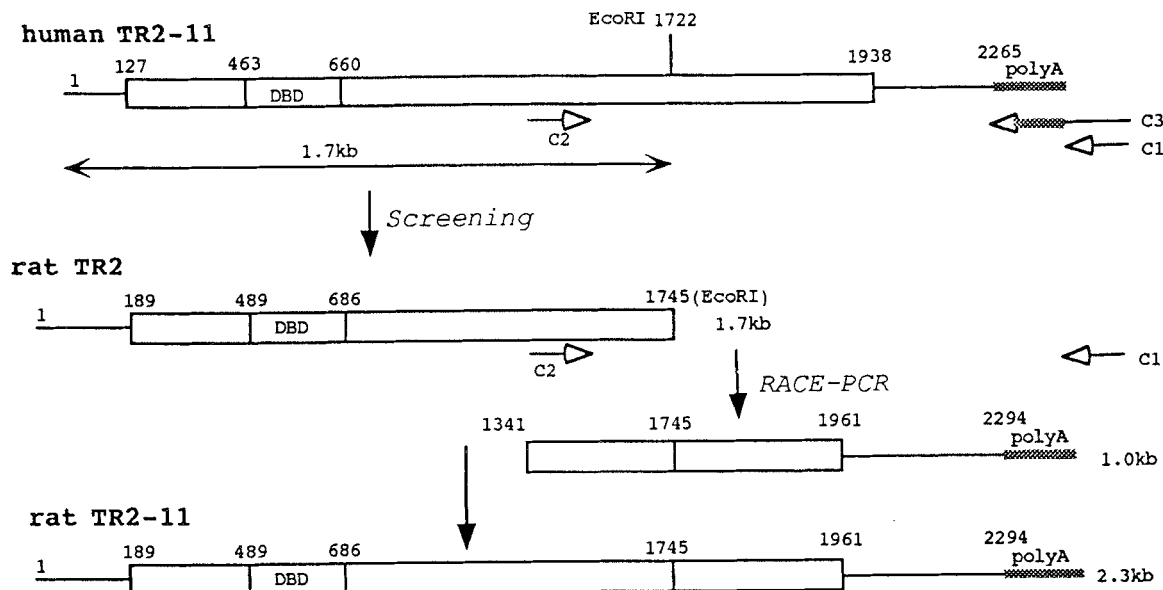
Figure 1 Strategy for the cloning of full-length TR2 orphan receptor cDNA. The 1.7 kb EcoRI DNA fragment encoding the 5' end of hTR2-11 orphan receptor cDNA was used as a probe to clone the rat 1.7 kb DNA fragment encoding the 5' end of TR2-11 orphan receptor. Using rat prostate cDNA as template, together with two primers (C2 and C1), a 1.0 kb 3' end of rat TR2 orphan receptor was cloned. All individual DNA fragments were then ligated into pSK plasmid for sequencing. DBD: DNA binding domain

obtained a 1.0 kb PCR product, whose size would be predicted as a product from the rat TR2-11 form of the TR2 orphan receptor cDNA.

Sequence analysis further proved the 1.0 kb RACE-PCR product covered the entire C terminal portion of rat TR2-11 orphan receptor cDNA (Figure 2). Since our strategy using RACE-PCR should have allowed us to amplify all potential TR2 orphan receptor forms with difference at 3' end, and only the TR2-11 DNA fragment was amplified, we can infer that TR2-11 orphan receptor may, therefore, represent the major form of TR2 orphan receptors in rat prostate.

Each segment of the rat TR2-11 orphan receptor cDNA clones was then ligated into pSK plasmid to get the full length rat TR2-11 orphan receptor cDNA. The open reading frame between the first ATG (nucleotide number 189) and TGA (nucleotide number 1962) encoded 590 amino acids, with a calculated molecular weight of 65.5 kd for the rat TR2-11 orphan receptor. In the 3'-untranslated region, a eukaryotic polyadenylation signal AATAAA was found between the nucleotide numbers 2271 and 2277 bp in rat TR2-11 orphan receptor.

Sequence comparison between DNA-binding domains of human and rat TR2-11 orphan receptors indicated that they have only one difference in amino acid sequence. This DNA-binding domain shares 50–60% homology with all other known steroid receptors. The amino acid sequence homology at the C-terminal region, a putative ligand-binding domain, between human and rat TR2-11 orphan receptors is 86%. In the N-terminal region, about 95% of amino acids are identical between human and rat TR2-11 orphan receptors. The overall amino acid sequence homology between human and rat TR2-11 orphan receptors is 90%.

Multiple species of TR2 orphan receptor mRNA expressed in the human prostate LNCaP cells and rat ventral prostate

To determine the size of the TR2 orphan receptor mRNA, Northern blot analysis was carried out with total RNA isolated from human prostate LNCaP cells and rat prostate. In LNCaP cells, at least two mRNA bands of approximately 2.9 kb and 2.5 kb were detected (Figure 3) if we used a ^{32}P-labeled 1.7 kb EcoRI DNA fragment encoding the 5' end

of the human TR2-11 orphan receptor cDNA as probe. The multiple TR2 orphan receptor mRNA bands may explain, at least partially, our previous human TR2 receptor cDNA sequencing results (Chang & Kokonits, 1988; Chang et al., 1989a) which suggested four different types of human TR2 orphan receptor cDNAs may exist. In the rat ventral prostate, we could detect a strong 2.4 kb band and a weaker 2.9 kb mRNA band (Figure 3) if we used a ^{32}P-labeled 1.7 kb EcoRI DNA fragment encoding the 5' end of the rat TR2-11 orphan receptor cDNA as probe. These results suggest that human and rat might have multiple TR2 orphan receptor mRNAs which could be derived from alternative splicing of single or multiple TR2 orphan receptor genes. Using testis as 100%, our Northern blot analysis also showed the following relative amounts of TR2 mRNA in other rat tissues: epididymis, 115%; thyroid, 70%; submaxillary gland, 62%; lung, 62%; seminal vesicle, 51%; cortex, 47%; liver, 42%; heart, 39%.

Androgen repression of TR2 orphan receptor mRNA

Northern blot analysis was used to study the androgen regulation of TR2 orphan receptor mRNA levels in human prostate LNCaP cells and rat ventral prostate. Total RNA was isolated from rat ventral prostate (normal, 3 days castration, and 3 day castration plus 3 days DHT injection) or human LNCaP cells treated with or without 20 nM DHT for 24 h. Northern blot probes of this RNA was hybridized with the above-mentioned rat (or human) TR2 orphan receptor cDNAs. As shown in Figure 3, the TR2 orphan receptor mRNA level per unit of RNA in rat ventral prostate increased 7-fold after androgen withdrawal by castration, while DHT injection reversed this increase to 2-fold of normal. In human prostate LNCaP cells, DHT could also repress TR2 orphan receptor mRNA to 60% of normal (with 18S RNA as control, data not shown).

Production of TR2 orphan receptor polyclonal antibody

A unique peptide containing a 20 amino acid sequence (NH₂-Ser-Ile-Arg-Lys-Asn-Leu-Val-Tyr-Ser-Cys-Arg-Gly-Ser-Lys-Asp-Cys-Ile-Ile-Asn-Lys-COOH) in the DNA-binding domain

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r-TR2    1:  GAATTCGGGGCAGGCGCCAAGAGCACCGCGCACCGCCAAGCCGAGGGTGGGGTCACGAACTCTGACCC
r-TR2   69:  CCACCCCCAAATACACAAACCCAGAAAGCTCTCCTGTCCCCTTGGCCTCGGGTCCCCTCGCCTCTGCTCTCTCCAGCCCCAGCGGCCGCCACGGCGGGAGGGATATTTTGACAATTAGATC

r-TR2    1:  GAATTCGGGGC
r-TR2   12:  CCGTCGGCTTTCTTCAACCCTCTCTTCCCGGAGCGCCCCCAATCCACGAGTGGCAGCCGCGGGACTGTCGCGTCGGCGCCCGACGCGGAGTCAGCAGGGGCGAAAAGCGGTAGATC

r-TR2    1:  met ala thr ile glu glu ile ala his gln ile ile asp gln gln met gly glu ile val thr glu gln gln thr gly gln lys ile gln
r-TR2  189:  ATG GCG ACC ATA GAA GAA ATT GCA CAT CAG ATT ATT GAC CAA CAG ATG GGT GAG ATT GTT ACA GAG CAG CAG ACT GGA CAG AAA ATC CAG
h-TR2  127:  --- --A --- --- --- --- --- --- --- --A --- --- --A --- --- --- --A --- --- --- --- --- --A --- --G --- --- --- ---
h-TR2    1:  -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -

r-TR2   31:  ile val thr ala leu asp his ser thr gln gly lys gln phe ile leu ala asn his glu gly ser thr pro *** *** *** *** *** ***
r-TR2  279:  ATC GTG ACA GCA CTT GAC CAC AGC ACA CAG GGC AAG CAG TTC ATT CTA GCA AAC CAT GAG GGA TCC ACC CCA *** *** *** *** *** ***
h-TR2  217:  --T --- --- --- --- --T --T -AT --C --A --- --- --- --- --- --G A-- --T --C --C --C --T --T --- AGC AAA GTC ATT CTG GCC
h-TR2   31:  -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -

r-TR2   55:  *** *** *** *** *** *** gly lys val phe leu thr thr pro asp ala ala gly val asn gln leu phe phe ala ser pro asp leu ser
r-TR2  351:  *** *** *** *** *** *** GGG AAA GTC TTC CTC ACG ACA CCA GAT GCT GCA GGT GTC AAC CAG TTG TTT TTT GCC AGT CCT GAT CTG TCC
h-TR2  307:  AGG CAA GAT TCC ACT CCG --A --- --T --- --T --A --T --- --- --A --- --- --- --- --- --A --- --- A-- -C- --- --- --- --T
h-TR2   61:  -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   thr -   -   -   -   -

r-TR2   79:  thr pro his leu gln leu leu thr glu asn ser pro asp gln gly pro asn lys val phe |asp leu cys val val cys gly asp lys ala|
r-TR2  423:  ACA CCA CAC CTC CAG CTC TTA ACA GAA AAT TCT CCT GAC CAG GGA CCA AAT AAG GTC TTT |GAC CTT TGC GTT GTG TGT GGA GAC AAA GCA|
h-TR2  397:  G-- -A- --- --T --- --- C-- --- --T --- --- --A --- --A --- --- --- --- --- --T |--T --- --- --A --A --- --- --- ---|
h-TR2   91:  ala -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   |-   -   -   -   -   -   -   -   -|

r-TR2  109: |ser gly arg his tyr gly ala ile thr cys glu gly cys lys gly phe phe lys arg ser ile arg lys asn leu val tyr ser cys arg|
r-TR2  513: |TCA GGC CGC CAT TAC GGA GCA ATA ACT TGT GAG GGC TGC AAA GGG TTT TTT AAA AGA AGC ATC CGG AAG AAT CTA GTG TAC TCG TGC CGG|
h-TR2  487: |--- --A --T --- --T --- --- G-- --- --- --A --- --- --- --A --- --- --- --- --- --- --A --A --- T-- --A --T --A --T --A|
h-TR2  121: |-   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -|

r-TR2  139: |gly ser lys asp cys ile ile asn lys his his arg asn arg cys gln tyr cys arg leu gln arg cys ile ala phe gly met|lys gln
r-TR2  603: |GGG TCC AAA GAC TGC ATC ATT AAC AAG CAC CAC CGG AAC CGC TGC CAA TAC TGC CGG CTG CAG CGG TGC ATA GCC TTC GGG ATG|AAG CAA
h-TR2  577: |--A --A --G --T --T --T --- --T --- --- --- --A --- --- --T --- --- A-- T-A --- A-A --T --T --G --T --A ---|--- ---
h-TR2  151: |-   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -  '-   -   -|-   -

r-TR2  169:  asp ser val gln cys glu arg lys pro ile glu val ser arg glu lys ser ser asn cys ala ala ser thr glu lys ile tyr ile arg
r-TR2  693:  GAC TCC GTT CAG TGT GAA AGA AAG CCC ATT GAA GTG TCC CGA GAA AAG TCT TCC AAC TGT GCA GCT TCT ACA GAG AAA ATC TAC ATC CGC
h-TR2  607:  --- --T --C --A --- --- --- --A --- --- --- --A --A --- --- --A --- --- --- --C --- --A --- --A --- --- --T --- --A
h-TR2  181:  -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -

r-TR2  199:  lys asp leu arg ser pro leu ala ala thr pro thr phe val thr asp ser glu thr ala arg ser thr gly leu leu asp ser gly met
r-TR2  783:  AAA GAC CTC CGA AGC CCT CTG GCT GCA ACG CCA ACC TTT GTA ACG GAC AGC GAG ACT GCC AGG TCA ACT GGA CTG TTG GAT TCG GGG ATG
h-TR2  757:  --G --- --T --T --- --A T-A A-- --- --T --- --T --- --- --A --T --T --A -G- A-A --- --- --A --- --- --A --- --A --A ---
h-TR2  211:  -   -   -   -   -   -   -   thr -   -   -   -   -   -   -   -   -   -   -   -   -   thr -   -   -   -   -   -   -   -

r-TR2  229:  phe val asn ile his pro ser gly ile lys thr glu pro ala leu leu met thr pro asp lys ala glu ser cys gln gly asp leu gly
r-TR2  873:  TTT GTG AAT ATT CAT CCA TCT GGA ATA AAG ACA GAA CCA GCT CTG CTA ATG ACA CCA GAT AAG GCT GAG TCC TGT CAG GGA GAC TTG GGC
h-TR2  847:  --C A-- --- --- --- --- --- --- G-- --A --T --G T-- --- G-- --G --- --- T-- --- --- --A --A --- --- --- --T --A A-T
h-TR2  241:  -   -   -   -   -   -   -   -   -   -   ser -   val -   -   -   ser -   -   -   -   -   -   -   -   -   -   -   ser

r-TR2  259:  thr leu ala ser val val thr ser leu ala asn leu gly lys ala lys asp leu ser his cys gly gly asp leu pro val val gln ser
r-TR2  463:  ACA TTG GCC AGT GTC GTC ACG TCA TTA GCG AAT CTC GGC AAG GCT AAA GAC CTT TCT CAC TGT GGT GGC GAT CTG CCT GTG GTG CAG AGC
h-TR2  937:  --- --- --- --A --- --T --A --- --- --- --T --A --A A-- --- --T --- --- --A AA- A-- AAT --A A-- T-- A-- A-T G-A ---
h-TR2  271:  -   -   -   -   -   -   -   thr -   -   -   -   -   -   ser ser asn -   met -   -   ile glu -

r-TR2  289:  leu arg asn gly asp thr ser phe gly ala phe his gln asp ile gln thr asn gly asp val ser arg ala phe asp asn leu ala lys
r-TR2 1053:  TTG CGC AAT GGA GAT ACA TCC TTT GGT GCA TTT CAT CAG GAT ATC CAG ACC AAT GGT GAT GTG TCA AGG GCA TTT GAC AAT CTT GCA AAA
h-TR2 1027:  --A A-- --- -AT --- --C --T --G T-- -A- --- *** --A --A --G --- --- --C --- --- --T --- --- --- --- --- -C- --- ---
h-TR2  301:  -   ser -   asp -   -   -   -   cys glu -  *** his -   -   -   -   -   -   -   -   -   -   thr -   -

r-TR2  318:  ala leu thr pro gly glu asn pro ala cys gln ser pro gly glu ser met glu gly ser thr his leu ile ala gly glu pro ser ***
r-TR2 1143:  GCA TTG ACT CCC GGA GAA AAC CCA GCC TGC CAG AGC CCA GGA GAG AGC ATG GAA GGA AGC ACA CAT CTA ATC GCT GGA GAG CCA AGC ***
h-TR2  991:  --- C-- -A- --T --- --G -G- A-- --- --- T-- -T- -C- G-- --- --- --T GT- --C --- --- T-- --T T-- --- ATA
h-TR2  331:  -   -   asn -   -   -   ser thr -   -   -   ser ala gly -   -   -   val -   -   thr -   -   ser -   ile

r-TR2  348:  *** cys met glu arg glu gly pro leu leu ser asp ser his val val phe arg leu thr met pro ser pro met pro glu tyr leu asn
r-TR2 1230:  *** TGC ATG GAG AGA GAG GGG CCA CTG CTC AGC GAT TCG CAT GTC GTT TTC AGG CTC ACC ATG CCT TCT CCT ATG CCC GAG TAC CTG AAT
h-TR2 1204:  AAT -A- -CC --A -A- --- --- --- --T --- --- --A --C --A -C- --- --- --- --- --- --- --- --T --- --- ---
h-TR2  360:  asn tyr thr -   lys -   -   -   -   -   ala -   -   -   -   -   -   -   -   -   -   -   -   -   -

r-TR2  377:  val his tyr ile gly glu ser ala ser arg leu leu phe leu ser met his trp ala leu ser ile pro ser phe gln ala leu gly gln
r-TR2 1320:  GTG CAC TAC ATT GGG GAG TCG GCC TCC AGA CTG CTG TTC TTA TCG ATG CAC TGG GCG CTT TCC ATA CCT TCT TTC CAG GCG CTC GGG CAA
h-TR2 1294:  --- --- --- --- --- --- --T --- --- --- --- --- --A --- --G --T --- --- --- --A --- --G --T --- --- --T --A --- ---
h-TR2  390:  -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -
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                                Primer C2
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r-TR2  407:  glu asn ser ile ser leu val lys ala tyr trp asn glu leu phe thr leu gly leu ala gln cys trp gln val met asn val ala thr
r-TR2 1410:  GAA AAC AGC ATA TCA CTG GTA AAA GCC TAT TGG AAT GAA CTC TTT ACC CTC GGT CTC GCC CAG TGC TGG CAA GTG ATG AAC GTC GCA ACT
h-TR2 1384:  --- --- --- --- --- --- --G --- --T --C --- --- --- --T --- --T --- --T --- --- --- --- --- --- --- --T --A --- ---
h-TR2  420:  -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -

r-TR2  437:  ile leu ala thr phe val asn cys leu his asn ser leu gln gln gln asp lys lys met ser pro glu arg arg lys leu leu met glu his ile
r-TR2 1500:  ATA TTA GCA ACG TTT GTC AAC TGT CTT CAC AAT AGC CTT CAG CAA GAT AAA ATG TCC CCG GAG AGG AGG AAG TTG TTG ATG GAG CAC ATC
h-TR2 1474:  --- --- --- --A --- --- --T --- --- --- --- --T --- --A --- --- --- --- --A A-A --A --A --A --A -A- --- --- --- ---
h-TR2  450:  -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   ser -   -   -   -   -   -   -

r-TR2  467:  phe lys leu gln glu phe cys asn ser met val lys leu cys ile asp gly his glu tyr ala tyr leu lys ala ile val leu phe ser
r-TR2 1590:  TTC AAG CTG CAG GAG TTC TGC AAT AGC ATG GTG AAG CTC TGC ATA GAC GGG CAT GAG TAC GCC TAC CTC AAG GCC ATC GTT CTC TTC AGT
h-TR2 1564:  --- --A --A --- --- --T --- --C --- --- --T --A --- --- --T --T --A T-C --A --T --- --- --G --- --A --A --A --- ---
h-TR2  480:  -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   ser -   -   -   -

r-TR2  497:  pro asp his pro gly leu glu asn met glu leu ile glu lys phe gln glu lys ala tyr val|glu phe|gln asp tyr ile thr arg thr
r-TR2 1680:  CCC GAC CAT CCA GGC CTG GAG AAC ATG GAG CTG ATT GAG AAA TTT CAG GAA AAG GCC TAT GTG|GAA TTC|CAA GAT TAC ATC ACC AGG ACA
h-TR2 1654:  --A --T --- --- A-- --A --A --- --- --A --- --A --- --- --- --- --T --- ---|--- --|--- --- --T --A --- -AA ---
h-TR2  510:  -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -|-   -|-   -   -   -   -   -   -
                                                                                        Eco RI

r-TR2  527:  tyr pro asp asp thr tyr arg leu ser arg leu leu leu arg leu pro ala leu arg leu met asn ala thr ile thr glu glu leu phe
r-TR2 1770:  TAT CCA GAT GAC ACC TAC AGG CTG TCT AGA TTA CTT CTG CCG GCC TTA CGG CTG ATG AAT GCC ACC ATC ACT GAG GAA TTG TTC
h-TR2 1744:  --- --- --- --- --- --- T-A --C --- C-- --A --- --- T-- --A --T --A A-A --- --- --T --- --T --- --A --- --- --T
h-TR2  540:  -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -

r-TR2  557:  phe lys gly leu ile gly asn val arg ile asp ser val ile pro his ile leu lys met glu pro ala asp tyr asn ser gln ile ile
r-TR2 1899:  TTC AAA GGT CTC ATT GGC AAC GTA CGG ATC GAC AGT GTC ATC CCA CAC ATT TTA AAA ATG GAG CCT GCA GAT TAC AAC TCT CAA ATA ATT
h-TR2 1834:  --- --- --- --- --- C-- --T A-- --A --T --- --- --T --- --- --T --- --G --- --- --- --- --- --- --T --- --- --- ---
h-TR2  570:  -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -

r-TR2  587:  gly his ser leu stop
r-TR2 1950:  GGT CAC AGC CTT TGA
h-TR2 1924:  --- --- --- A-- ---
h-TR2  600:  -   -   -   -   -
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r-TR2 1965: AAGTGGAGGTCACCCTGCTCCAGGCTAAGT TTACCGTTCCTTCTCGGAACACATGAAACCA AATCGAACTGTTTCTTCCAGGGCATCTGCG AATATTAACTTTAAGAGTAACCAAAATCAA
r-TR2 2085: AAGTATTTTTCTTTTGGATTCCTTAAGAAG AATTTAAGTGACCAGGCAGGGGTCAGGAATT ATACTACCCTGTCTGTCCTATAGGAATGAA TCAACAATGGTACGGGGCTGTTCTTGGGGA
r-TR2 2205: GGATGACACTTAAGACTCTCTCCTCCTGAC TGTCTACACTAAGCTTTCTCATTCTATTTTA TAAAACAAATAAACTAGTCTTTCCCCCCTG AAAAAAAAAAAAAAAAAAAAAAAAAAAAA

h-TR2 1939: AAACTGTGACTGCAGTGCTGTAAACTTAAC TGTTCTTTGCCAGAACACAAGACACCAAAT TGAACTCACTGCTTTTGAGGCATCTGGAAA TTTTTACTTTAAAAAGTAACCAGAATCCAA
h-TR2 2059: GGTATTTTTATTTTAGCTTCCCTTAAGAAT TTTTGAAGTGACTGGGCAGGCAGCAGAAAT TAAATGAATTTTTCTTCCTGATTCCTTTAA ATGAATATGAAACAACTACAAATTTATTCTT
h-TR2 2179: GGTGAAGATGATACCTGAAGCTGTCACCTC TTGATTATCTAAACTAAGCGCTCATTCTAT TTTATAAAACAAATAAATTAGTCTCTTTTT TCTGAAAAAAAAAAAAAAAAACCC
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Figure 2 cDNA sequences and deduced amino acid sequences of rTR2-11 and hTR2-11 orphan receptors. The nucleotide sequences and the deduced amino acid sequences are numbered on the left. The hTR2-11 orphan receptor residues that are identical to rTR2-11 orphan receptor residues are shown by hyphens. The sequences in the DNA-binding domain of the TR2-11 orphan receptors are boxed. The sequences of each primer used in Figure 1 were underlined. The EcoRI site that separate 5' end 1.7 kb DNA fragment was also boxed. The 20 amino acid sequence for the production of anti-TR2 orphan receptor antibodies were also underlined. Sequence data from this article have been deposited with the EMBL/Genbank Data Libraries under Accession No. L26398 for rat TR2-11 orphan receptor
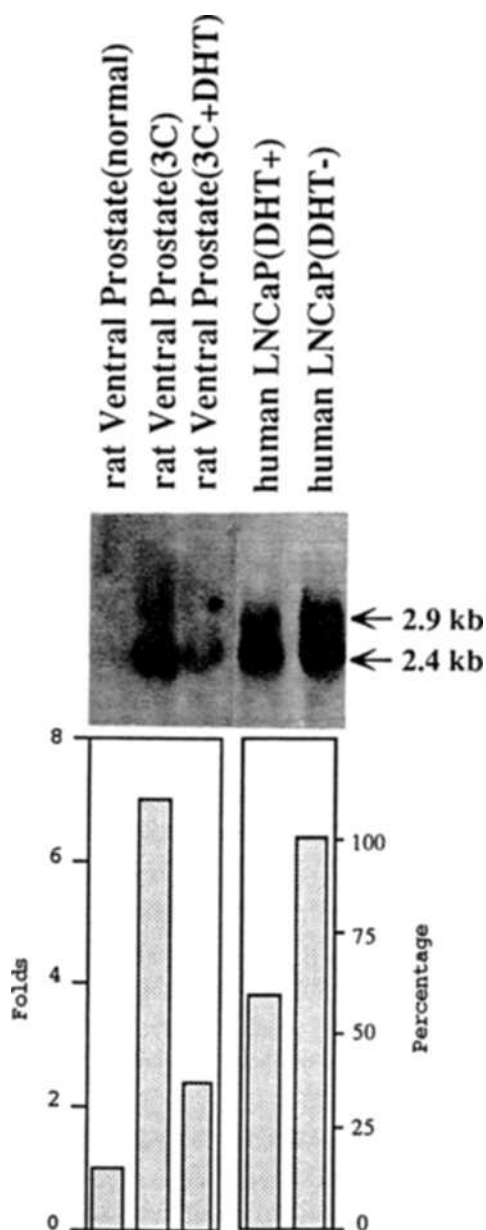
Figure 3 Androgen repression of TR2 orphan receptor mRNAs. Total RNA (20 µg) from rat ventral prostate (normal, 3 days castration, and 3 days castration plus 3 days DHT injection) and human prostate LNCaP cells treated with or without DHT for 24 h were isolated, separated by electrophoresis on a 1% agarose gel, transferred to a nylon filter and hybridized with ^{32}P-labeled 1.7 Kb 5' end TR2-11 orphan receptor cDNAs as described in the Materials and methods. Relative levels of TR2 orphan receptor mRNA suppressed by androgens in rat ventral prostate and human prostate LNCaP cells were quantitated by densitometric scanning

of human and rat TR2 orphan receptors (Figure 2) was synthesized to be used as antigen for the production of polyclonal anti-TR2 orphan receptor antibodies. The positive serum from rat was further purified as described in Materials and methods, and the specificity was confirmed by a double-antibody precipitation method. As shown by SDS–PAGE (Figure 4), the *in vitro* transcription/translation of the 70 kd human TR2-11 orphan receptor could be precipitated by the purified antisera. There also appears a very faint immunoprecipitated band at 48 kd which could be a degradation product of TR2-11 orphan receptor. No immunoprecipitated protein complex could be visualized using sera obtained from nonimmunized rat (data not shown) or with samples
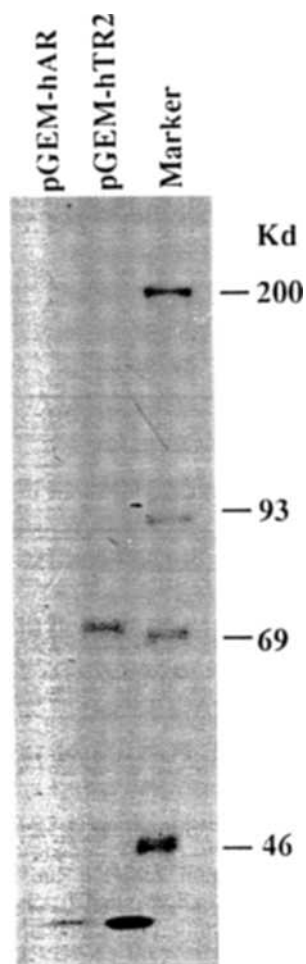
Figure 4 SDS–PAGE analysis of antibody precipitated *in vitro* transcription/translation of TR2-11 orphan receptor. TR2-11 orphan receptor was *in vitro* transcribed, translated, reacted with polyclonal anti-TR2 orphan receptor antibodies and run on SDS–PAGE system as described in Materials and methods

obtained from the *in vitro* transcription/translation of human androgen receptor (Figure 4).

Immunohistochemical localization of TR2 orphan receptors

Polyclonal anti-TR2 orphan receptor antibody was used to stain mouse tissue sections to study the inter- and intracellular localization of TR2 orphan receptor protein. Male accessory reproductive organs such as prostate (Figure 5a and b) and epididymis (Figure 5c and d) were stained with the antibody. As with other members of the steroid receptor superfamily, TR2 orphan receptor protein was located predominantly in nuclei but not in cytoplasm. In those accessory organs, positive cells were confined to the epithelium while no stromal cells were positive for staining. The control sections with antigen-absorbed serum showed no nuclear staining.

Discussion

Previously, we reported the isolation from prostate and testis cDNA libraries (Chang & Kokonits, 1988; Chang *et al.*, 1989a), of four human TR2 orphan receptor cDNAs with differences in the C-terminal lengths of the putative ligand-binding domain. To determine the major form of TR2 orphan receptor, we applied the RACE-PCR technique together with two primers (C1 plus an oligo dT and C2, a
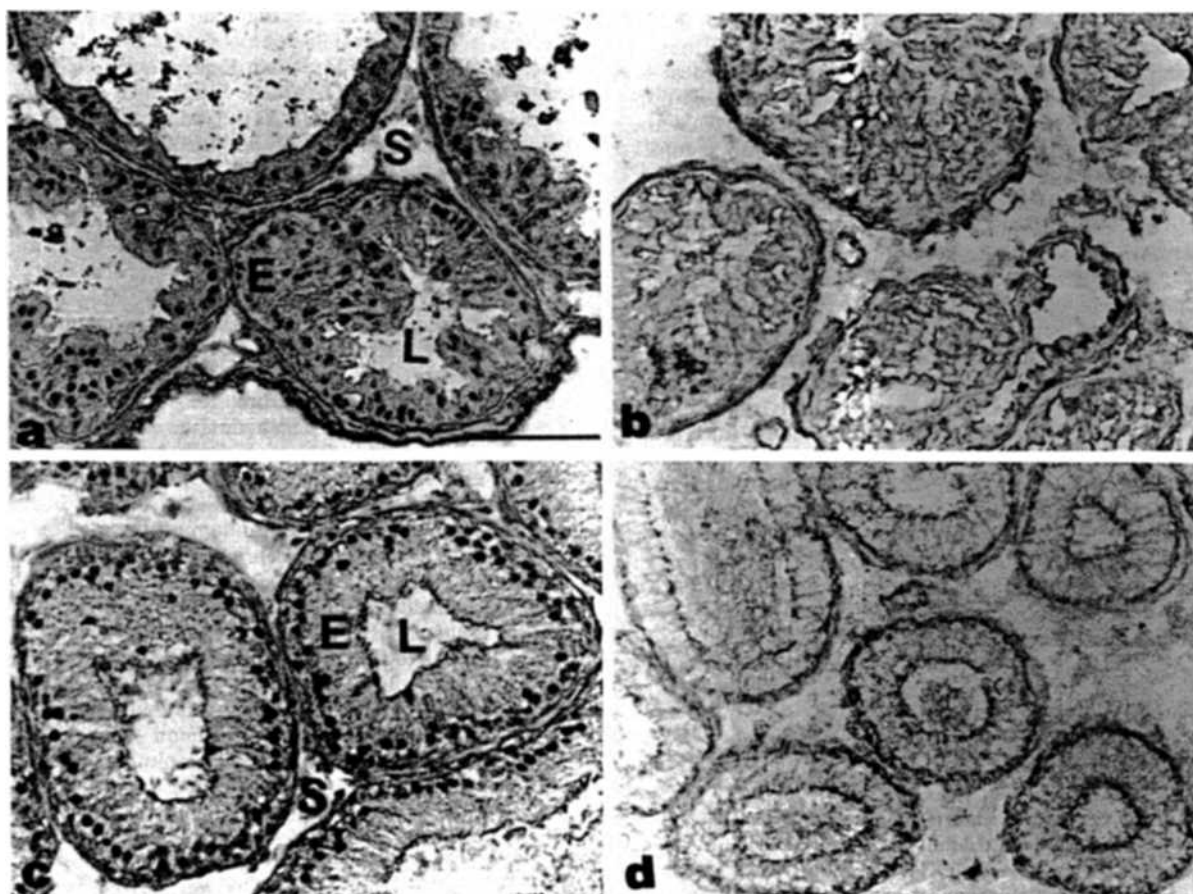
Figure 5 Immunohistochemical localization of TR2 orphan receptor protein in mouse prostate gland (a and b) and epididymis (c and d). The positive reactions were found in nuclei of epithelial cells while stromal cells were negative for staining. The control sections were stained with the antigen-absorbed serum and show no nuclear staining (b and d). E: epithelium; L: lumen; S: stroma; Bar = 100 μm

common sequence appearing in TR2-5, TR2-9 and TR2-11 orphan receptor cDNAs) to amplify TR2 orphan receptor mRNAs from rat prostate. As shown in Figures 1 and 2, the only amplified DNA fragment we were able to isolate from RACE-PCR was the 3' end of TR2-11 orphan receptor. These results indicated that TR2-11 orphan receptor was the major form of identified TR2 orphan receptors in rat prostate.

When a 1.7 kb EcoRI DNA fragment encoding the 5' end of human TR2-11 orphan receptor cDNA was used as a probe in Northern analysis of prostate LNCaP cells, we were able to get at least two different sizes (2.9 kb and 2.5 kb) of human TR2 orphan receptor mRNA (Figure 3). This suggests an unidentified 2.9 kb form of TR2 orphan receptor mRNA exists in human prostate LNCaP cells. Since the length of all identified TR2 orphan receptor cDNAs (TR2-5, 7, 9 and 11) was less than 2.5 kb and 3' end DNA fragments (last 600 bp) of TR2-11 orphan receptor can also hybridize to this 2.9 kb human TR2 orphan receptor mRNA. We believe that the 2.9 kb mRNA may represent a 5' variant form of TR2 orphan receptor mRNA. Using 5' RACE-PCR technique, we are in the process of cloning this 2.9 kb TR2 orphan receptor mRNA from human prostate LNCaP cells.

In summary, our Northern blot data clearly suggests that multiple species of TR2 orphan receptors can be expressed in the human prostate LNCaP cells and rat ventral prostate. The significance of these multiple forms of TR2 orphan receptors in prostate, however, remains unclear.

Multiple species of mRNAs also appear in the thyroid hormone receptor, another member of the steroid hormone

receptor superfamily. So far, at least six forms of thyroid hormone receptors (TR) have been identified: TRα1, α2, α3, β1, β2 and Rev-erb Aα (Lazar, 1993). TRα and TRβ are the products of different genes, located at human chromosome 17 and 3, respectively. Additional isoforms named by number are generated from α and β genes by an alternative splicing mechanism. In addition, Rev-erb Aα is encoded by the noncoding (anti-sense) strand of TRα gene. Since TRα2 and 3 cannot bind to thyroid hormones, they may form heterodimers with other TR isoforms and have dominant negative effects on the thyroid hormone functions (Lazar, 1993).

While many steroid hormones can induce their target genes, we can also find several genes (and their products) repressed by steroid hormones which regulate cell growth and function (Chang et al., 1987). Our Northern blot analysis in Figure 3 clearly demonstrates that castration can increase TR2 orphan receptor mRNA levels and the addition of androgens can reverse this increase in the rat ventral prostate. The results from human prostate LNCaP cells further confirm that androgens can repress the TR2 orphan receptor mRNA levels. As the physiological role of TR2 orphan receptor is not clear at this moment, the impact of androgen repression of TR2 orphan receptor is, therefore, also unknown.

Nevertheless, repression of TR2 orphan receptor by androgen in prostate provides us an additional important model to study the negative mechanism of regulation of gene expression by androgen in prostate. Whether this negative regulation mechanism is dependent on a direct interaction of the androgen-receptor complex with other transcriptional

factors is not clear. Several models have been proposed to explain the negative regulations by thyroid/steroid hormones: for example, if thyroid hormone response element is near or overlapped with the transcription start site, the thyroid hormone receptor complex may act by interfering with the assembly or procession of the basal transcription complex and, therefore, represses gene expression (Lazar, 1993). For the glucocorticoids, the interaction of GR with AP1 has been reported to be an essential step for the induction/repression. Furthermore, the subunit composition of AP1, homodimer of c-Jun/c-Jun or heterodimer of c-Jun/c-Fos can also specify the enhancement or repression, respectively by GR complex (Pearce & Yamamoto, 1993).

In male accessory organs, the positive-stained cells using TR2 orphan receptor antibody were found mainly in the epithelium. Few stromal cells showed positively stained. This pattern of staining was in contrast to that of AR during the development of the mouse prostate gland. Our previous studies (Takeda & Chang, 1991) demonstrated that both fetal epithelial and stromal cells were positively stained with anti-AR antibody, suggesting that androgens are required for both compartments to keep their function. TR2 orphan receptor protein and its unknown ligand might be necessary for the reproductive organ to maintain the function or differentiation of their epithelium.

Materials and methods

Animals, tissues and cell line

Six-week-old Lewis rats were purchased from Taconic (Germantowin, NY, USA) and adult male Crl:CD-1 (ICR) mice were from Charles River Inc. (Wilmington, Massachusetts, USA). For immunohistochemical analysis, fresh tissues were placed in OCT compound (Miles Laboratories Inc. IN, USA) and immediately frozen on solid CO_2. The frozen tissues were stored at $-70°C$ until use. The LNCaP cell line was derived from culture explants of needle biopsy material taken from a lymph node metastatis of prostate cancer (Horoszewicz *et al.*, 1983).

Screening and sequencing of TR2 orphan receptor cDNAs

The rat prostate λgt11 library was screened with a 1.7 kb EcoRI DNA fragment of human TR2-11 orphan receptor cDNA. The conditions of hybridization were 25% formamide, 5 × Denhardt's solution (0.1% Ficoll 400, 0.1% polyvinylpyrolidone, 0.1% bovine serum albumin), 0.1% SDS, 5 × SSC (1 × SSC is 150 mM NaCl, 15 mM sodium citrate), 100 μg/ml denatured salmon sperm DNA and 1 μg/ml poly(A) at 42°C. Filters were washed with a solution containing 0.1% SDS and 0.4 × SSC at 52°C. Clones were subcloned into vector pBluescript. Double strand sequencing on plasmid constructs was performed using dideoxy chain termination method.

RACE-PCR of 3' end of rat TR2 orphan receptor cDNAs

Total RNAs from male rat prostate were isolated by acid guanidinium phenol chloroform method (Chomczynski & Sacchi, 1987). RNAs were denatured by heating 90°C for 5 min and quickly chilled in ice water. First strand cDNAs were synthesized from 1.25 μg of denatured total RNA using 0.75 nmol of 36 mer oligonucleotide C1 (dT) 16: 5'd(AAGG-ATCCGTCGACATCGAT-TTTTTTTTTTTTTTTT) as the primer for reverse transcription and reaction was carried out in 25 μl of RT buffer (5 mM $MgCl_2$, 1 × PCR buffer, 1 mM dA,G,C,TTP, 1 unit/μl of human placental ribonuclease inhibitor, 2.5 units/μl of MMLV reverse transcriptase; RT-PCR kit, Perkin Elmer Cetus) by incubating 23°C for 15 min, 42°C for 60 min, 52°C for 30 min then 95°C for 5 min. The reaction mixture was diluted to 100 μl with 1 mM EDTA

(pH 8.0) and stored at 4°C as the first cDNA pool. Hot start PCR was used here to omit the possibility of miss-annealing of primers (Ampliwax TM, Perkin Elmer Cetus). PCR primers were C1 = 5'-AAGGATCCGTCGACATCGAT-3' and C2 = - 5'-TCCAGACTGCTGTTCTTATC-3' which are the sequences at nucleotide position 1341 to 1360 in rat TR2-11 orphan receptor.

Northern blot analysis of TR2 orphan receptor mRNA in LNCaP cells and rat tissues

Total RNAs were isolated from human LNCaP cells and rat tissues by acid guanidnium thiocyanate-phenol-chloroform extraction (Chomczynski & Sacchi, 1987). Total RNAs (20 μg in each lane) were treated with formaldehyde/formamide, separated by electrophoresis on a 1.0% agarose gel containing 2.2 M formaldehyde (the ethidium bromide staining was then applied here to confirm the equal loading of RNA sample, using 18S RNA as control) and transferred to a nylon filter (Hybond-N, Amersham). Radiolabelling of the probes was performed by a random primer method (Random primer labeling kit, Boehringer Mannheim) with α-$[^{32}P]dCTP$ (Amersham). The specific activity of these labeled probes were about $5 × 10^8$ d.p.m./μg. The hybridization conditions were 5 × SSPE (1 × SSPE is 180 mM NaCl, 1 mM EDTA, 10 mM Na_2PO_4), 5 × Denhardt's solution (1 × Denhardt's solution is 0.1% Ficoll 400, 0.1% polyvinyl-pyrrolidone, 0.1% bovine serum albumin), 0.3% SDS (sodium dodecyl sulfate), 50 μg/ml salmon sperm DNA and $1 × 10^6$ d.p.m. probe/ml hybridization solution at 68°C for 16 h. The filter was washed with a solution containing 1 × SSC (150 mM NaCl, 15 mM sodium citrate), 1% SDS at room temperature for 3 × 15 min, and 0.2 × SSC, 1% SDS at 65°C for 2 × 30 min. Hybridization bands were detected by auto radiography with an intensifying screen at $-80°C$.

Androgen repression of TR2 orphan receptor mRNA in human LNCaP cells and rat ventral prostate

Male Sprague-Dawley rats (400 g) were castrated via the scrotal route with the use of diethyl ether anesthesia. 5α-dihydrotesterone (DHT) (5 mg/rat/day for 3 days) was administered subcutaneously in rats castrated for 3 days prior to injection. The control group received sesame oil over the same period.

Human prostate LNCaP cells were cultured in DMEM/-F-12 medium containing 5% fetal calf serum for 48 h to facilitate the attachment of LNCaP cells to plates. Later, the medium was replaced by fresh charcoal-stripped medium (DMEM/F-12 containing 5% charcoal-stripped fetal calf serum) with or without the addition of 20 nM DHT and incubated at 37°C for another 24 h. LNCaP cells were then harvested for RNA isolation as described above.

Production of TR2 orphan receptors antibodies

The peptide sequence (NH$_2$-Ser-Ile-Arg-Lys-Asn-Leu-Val-Tyr-Ser-Cys-Arg-Gly-Ser-Lys-Asp-Cys-Ile-Ile-Asn-Lys-COOH) is the same in both human and rat TR2-11 orphan receptors and is unique for the TR2 orphan receptor. Peptide was dissolved in PBS and coupled to the carrier protein keyhole limpet hemocyanin (KLH; Sigma Chemical Co., St. Louis, MO). For the primary immunization, conjugated peptide (100 μg) was emulsified with Freund's complete adjuvant and injected intradermally to 8-week-old female Lewis rats. This was followed by 10 days intradermal boosts using the incomplete adjuvant mixture. For the last boost, the conjugated peptide was injected intravenously in saline. The rat serum was diluted (1:3200) and tested by ELISA assay using the peptide as the antigen. The positive serum was further purified and the specificity was confirmed by a double antibody precipitation method.

Linealized pGEM-3Z (Promega, Madison, WI) vector containing the human TR2-11 orphan receptor cDNA was transcribed by SP6 RNA polymerase (Promega) and RNA was translated in a rabbit reticulocyte lysate (Promega) as described previous (Chang *et al.*, 1989b). Four μl of translation reaction was diluted with 0.1% Nonidet P-40 in phosphate-buffered saline (pH 7.4), and incubated for 1 h at 4°C with 3 μl of purified TR2 polyclonal antibody at final total volume of 200 μl, followed by the incubation with 100 μl of goat-anti-rat IgG + IgM (dilute 1:100) for 1 h. After centrifugation, the pellet was washed extensively with lysis buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholic acid, 0.1% sodium dodecyl sulfate (SDS), 50 mM Tris-HCl (pH 7.5)) and incubated at 85°C for 10 min in sample buffer (2% SDS, 10% glycerol, 100 mM dithiothreitol (DTT), 5% β-mercaptoethanol, 60 mM Tris-HCl (pH 6.8) and 0.001% bromophenol blue). The eluted proteins were separated by 8% SDS-polyacrylamide gel electrophoresis. The gel was treated with 20% 2,5-diphenyllozaxazole (PPO, New England Nuclear, Boston, MA) in acetic acid, dried, and autoradiographed.

Immunohistochemical staining of TR2 orphan receptor

Since the polyclonal antibody was made in rat, mouse prostate and epididymis were stained with polyclonal antibody.

Frozen sections, 7 μm thick, from unfixed tissues were fixed in a 4% paraformaldehyde solution. After washing with phosphate buffer and blocking with 1% bovine serum albumin, the sections were incubated with the first antibody (15 μg/ml) at 4°C overnight. They were then incubated with second antibody (biotinylated anti-rat IgG, Zymed Laboratories Inc., San Francisco, CA) at room temperature. This was followed by the incubation with peroxidase-streptavidin (Zymed) at room temperature. Finally, the sections were incubated with the substrate solution (0.01% H_2O_2, 0.05% 3,3-diaminobenzidine tetrahydrochloride; Sigma). For control experiments, the immune serum which was absorbed with antigen (peptide) was used as first antibody.

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