

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 prostates 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; Segaves, 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 (Kliwer *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 λ gt 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

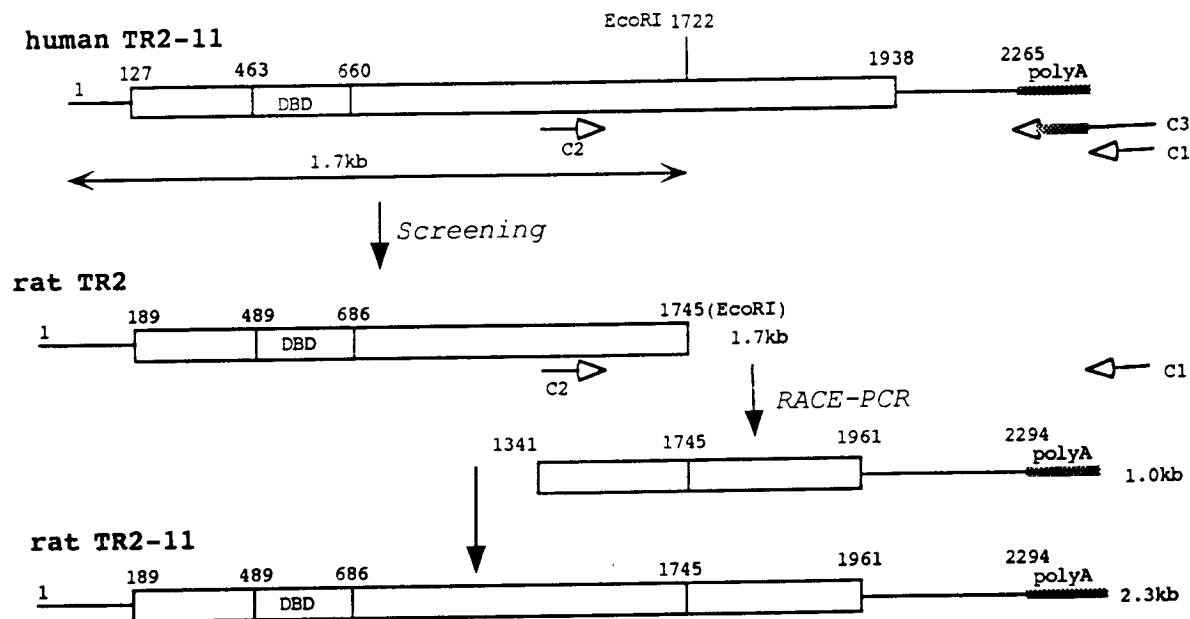


Figure 1 Strategy for the cloning of full-length TR2 orphan receptor cDNA. The 1.7 kb EcoRI DNA fragment encoding the 5' end of hTR2-11 orphan receptor cDNA was used as a probe to clone the rat 1.7 kb DNA fragment encoding the 5' end of TR2-11 orphan receptor. Using rat prostate cDNA as template, together 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 ³²P-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 ³²P-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

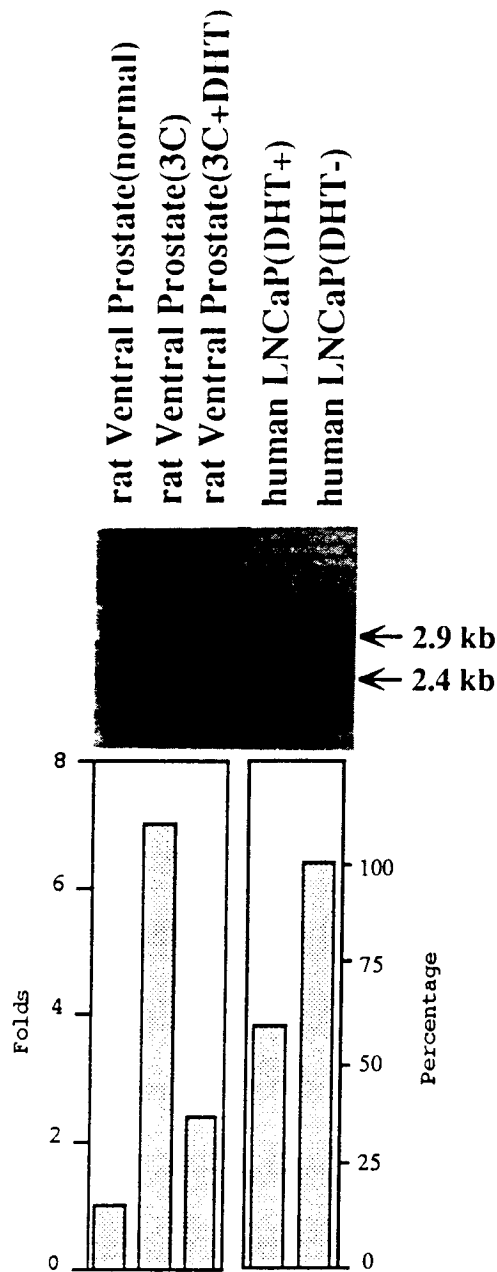


Figure 3 Androgen repression of TR2 orphan receptor mRNAs. Total RNA (20 µg) from rat ventral prostate (normal, 3 days castration, and 3 days castration plus 3 days DHT injection) and human prostate LNCaP cells treated with or without DHT for 24 h were isolated, separated by electrophoresis on a 1% agarose gel, transferred to a nylon filter and hybridized with ³²P-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

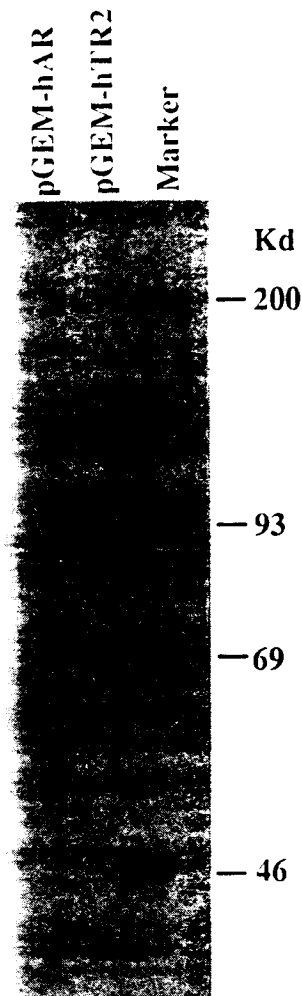


Figure 4 SDS-PAGE analysis of antibody precipitated *in vitro* transcription-translation of TR2-11 orphan receptor. TR2-11 orphan receptor was *in vitro* transcribed, translated, reacted with polyclonal anti-TR2 orphan receptor antibodies and run on SDS-PAGE system as described in Materials and methods

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

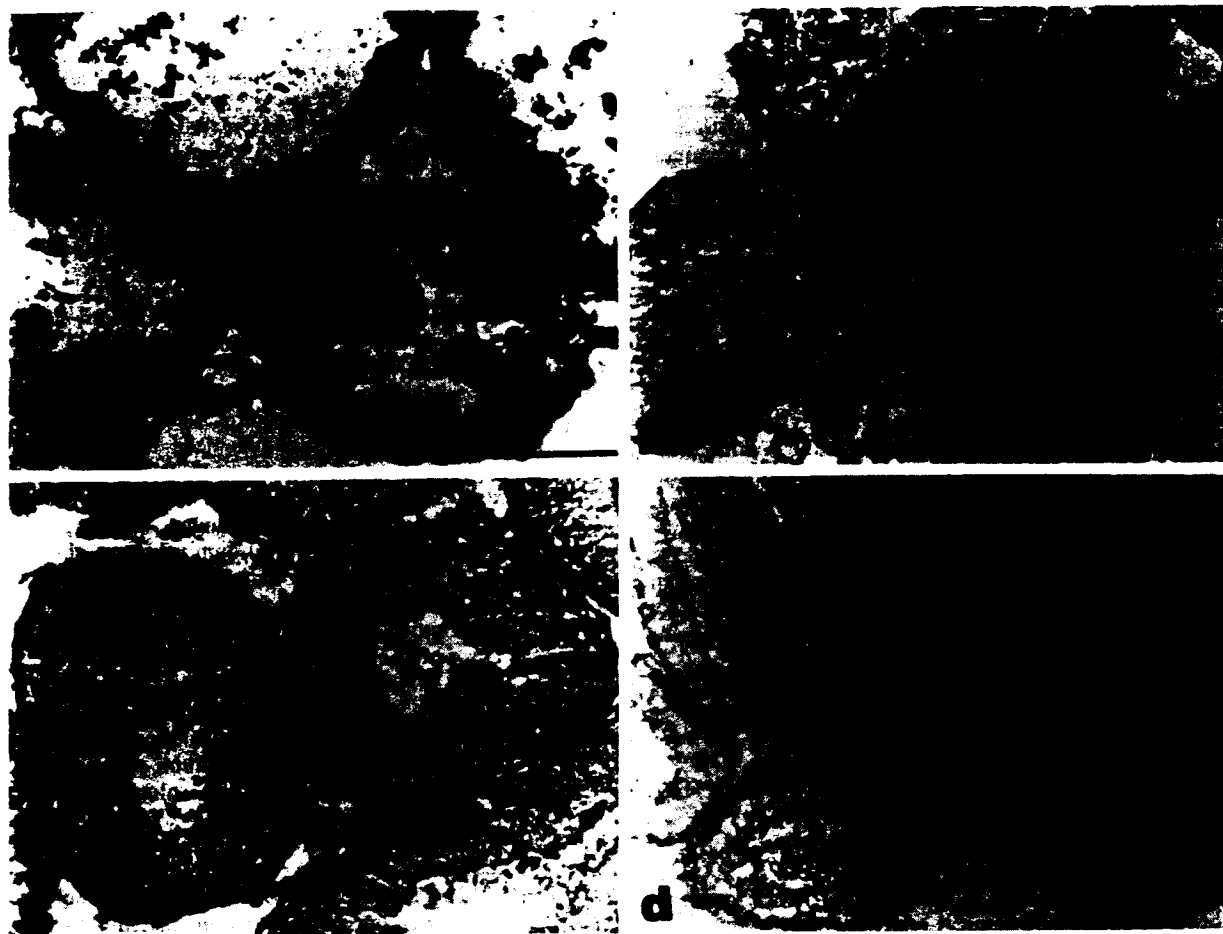


Figure 5 Immunohistochemical localization of TR2 orphan receptor protein in mouse prostate gland (a and b) and epididymis (c and d). The positive reactions were found in nuclei of epithelial cells while stromal cells were negative for staining. The control sections were stained with the antigen-absorbed serum and show no nuclear staining (b and d). E: epithelium; L: lumen; S: stroma; Bar = 100 μ m

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: TR α 1, α 2, α 3, β 1, β 2 and Rev-erb A α (Lazar, 1993). TR α and TR β 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 A α is encoded by the non-coding (anti-sense) strand of TR α gene. Since TR α 2 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 (Germantown, NY, USA) and adult male Cr1: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 CO₂. 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 metastasis of prostate cancer (Horoszewicz *et al.*, 1983).

Screening and sequencing of TR2 orphan receptor cDNAs

The rat prostate λ gt11 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 \times Denhardt's solution (0.1% Ficoll 400, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin), 0.1% SDS, 5 \times SSC (1 \times SSC is 150 mM NaCl, 15 mM sodium citrate), 100 μ g/ml denatured salmon sperm DNA and 1 μ g/ml poly(A) at 42°C. Filters were washed with a solution containing 0.1% SDS and 0.4 \times 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 μ g 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 μ l of RT buffer (5 mM MgCl₂, 1 \times PCR buffer, 1 mM dA.G.C.TTP, 1 unit μ l of human placental ribonuclease inhibitor, 2.5 units μ l 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 μ l 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 guanidinium thiocyanate-phenol-chloroform extraction (Chomczynski & Sacchi, 1987). Total RNAs (20 μ g 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 -[³²P]dCTP (Amersham). The specific activity of these labeled probes were about 5 \times 10⁸ d.p.m./ μ g. The hybridization conditions were 5 \times SSPE (1 \times SSPE is 180 mM NaCl, 1 mM EDTA, 10 mM Na₂PO₄), 5 \times Denhardt's solution (1 \times Denhardt's solution is 0.1% Ficoll 400, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin), 0.3% SDS (sodium dodecyl sulfate), 50 μ g/ml salmon sperm DNA and 1 \times 10⁶ d.p.m. probe/ml hybridization solution at 68°C for 16 h. The filter was washed with a solution containing 1 \times SSC (150 mM NaCl, 15 mM sodium citrate), 1% SDS at room temperature for 3 \times 15 min, and 0.2 \times SSC, 1% SDS at 65°C for 2 \times 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. 5 α -dihydrotestosterone (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 (NH₂-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 μ g) 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.

Linearized 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 μ l 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 μ l of purified TR2 polyclonal antibody at final total volume of 200 μ l, followed by the incubation with 100 μ l 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-diphenylloxazole (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.

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