*Gene Expression*, Vol. 4, pp. 77–84 © 1994
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1052-2166/94 $3.00 + .00


# Molecular Cloning of a Novel Member of the Nuclear Receptor Superfamily Related to the Orphan Receptor, TR2

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We have cloned a novel member (mTR2R1) of the nuclear receptor superfamily of transcription factors from a mouse brain cDNA library. The cDNA sequence predicts a protein primary structure of 629 amino acids with a calculated molecular weight of 68.8 kDa. The amino acid sequence of the protein contains three regions of consensus sequence that are conserved throughout the nuclear receptor superfamily. One of these regions encodes a type II zinc finger DNA binding domain that is characteristic of this family of transcription factors. Comparison of the amino acid sequence of mTR2R1 with other nuclear receptors indicates that it is most closely related to the orphan receptor, hTR2, and suggests that these proteins constitute a novel subfamily within the nuclear receptor superfamily. mTR2R1 is encoded by two mRNAs that show different but overlapping spatial patterns of expression in the adult mouse. The identification of a subfamily of TR2 receptors together with the existence of variant mRNAs for these receptors prompts a close examination of the tissue-specific regulatory role of this subfamily of nuclear receptors.

NUCLEAR receptors comprise a superfamily of transcription factors that regulate developmental, physiological, and behavioral programs of gene expression in response to a variety of chemical signals (Tsai and O'Malley, 1991; Beato, 1989; O'Malley and Conneely, 1992; Tsai et al., 1991). The most extensively characterized members of the superfamily encode ligand-activated receptors for steroids, retinoids, thyroid hormone, and the fat soluble vitamin $D_3$ (Evans, 1988; O'Malley et al., 1991). These receptors are intracellular proteins that become active dimeric transcription factors upon specific binding of their cognate ligand (Tsai et al., 1988; Kumar and Chambon, 1988). The activated receptor dimers then bind to selected *cis*-acting enhancer DNA elements and form a functional transcription initiation complex at the promoter of target genes (Yamamoto, 1985; Tsai et al., 1989; Bagchi et al., 1990). The superfamily also contains some members for which activities can be modulated by intracellular signaling pathways in response to extracellular stimuli in the absence of direct ligand binding (Denner et al., 1990; Power et al., 1991a; Power et al., 1991b; Ignar-Trowbridge et al., 1992) in addition to members for which transcription activation functions may be constitutively active (Lydon et al., 1992; Davis et al., 1991; Paulsen et al., 1992) or for which expression can be regulated by extracellular stimuli (Milbrandt, 1988; Law et al., 1992).

The high degree of sequence conservation within the DNA binding domains of the nuclear receptors has been exploited to generate DNA probes to isolate genes encoding numerous additional members of the receptor superfamily using low-stringency hybridization techniques. There are now in excess of 30 additional members of the superfamily that have been isolated. They are termed orphan receptors because the identity of possible cognate ligands or mechanisms of activation and physiological function remain unknown. Nuclear receptor family members are expressed with overlapping temporal developmental and tissue distribution and show overlapping specificity of interaction with *cis*-acting enhancer elements on target genes (Tsai et al., 1991; Umesono and Evans, 1989) revealing the potential for interactive and/or redundant levels of regulation

Received July 21, 1993; revision accepted July 5, 1994.
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of gene expression by multiple receptors in a given tissue (Li et al., 1993; Lufkin et al., 1993).

To examine the complexity of the nuclear receptor superfamily, we have attempted to identify novel orphans from mouse brain cDNA libraries because of the high complexity of brain mRNA (Sutcliffe, 1988). We report the isolation and characterization of a novel orphan receptor referred to as mTR2R1. The cDNA encodes a protein that is most closely related to the orphan receptor, hTR2, that was originally isolated from human testis (Chang and Kokontis, 1988). The identification of this cDNA together with the demonstration of its expression in male reproductive tissues underlies the existence of a subfamily of hTR2-related orphan receptors that may function interactively to regulate gene expression in these tissues.

## MATERIALS AND METHODS

### *Materials*

The mouse neonatal brain cDNA library was purchased from Stratagene, Inc. (La Jolla, CA). Restriction enzymes were from Promega Corp. (Madison, WI). Taq DNA polymerase was from Perkin Elmer Corp. (Norwalk, CT).

### *Library Screening*

A Uni-ZAP-XR λ phage cDNA library (Catalog No. 937301; Stratagene) derived from BALB/c mouse neonatal brain mRNA was screened with a $^{32}$P-labeled cDNA probe containing the DNA binding domain of COUP-TF1 (Wang et al., 1989). The library screening procedure was essentially the same as described previously for the identification of the orphan receptor NURR1 (Law et al., 1992). Candidate clones were further discriminated by screening with other orphan receptor cDNAs. Clones that were negative to known orphan receptors and positive to the COUP-TF cDNA were pooled and converted into plasmid DNA for PCR amplification of the DNA binding domains and were then subcloned. A λ DR2 cDNA library derived from human prostate mRNA was purchased from Clontech Laboratory, Inc. (Palo Alto, CA). This library was screened with a $^{32}$P-labeled cDNA probe containing only the DNA binding domain of mTR2R1, which was identified from the neonatal mouse brain cDNA library. The cDNA library screening procedure was the same as described for mTR2R1 except the final filter wash was carried out at 60°C in 0.1 × SSC and 0.1% SDS for 20 min. Positive clones were plaque purified and then converted into plasmids for DNA sequence determination.

### *Primer Design and PCR*

Degenerate oligodeoxynucleotides containing all possible codons encoding the two most conserved regions of the DNA binding domain of the nuclear receptor superfamily members were synthesized by the phosphoramidite chemical method (Genosys, Houston, TX and NBI, Plymouth, MN) and used as universal primers. The 5′-primer sequence was 5′-TTT GGA TCC DSN TGY GAR GGC TGY AAA-3′ (IUB group codes were used where D = A-G-T; S = G-G;N = A-G-C-T;Y = C-T;R = A-G′K = G-T). The 3′-primer sequence was 5′-AC RKC NRA NKT YKY NAC CCT AGG TTT-3′. PCR amplification of candidate orphan receptor clones from the mouse cDNA library was carried out in the Perkin-Elmer DNA thermocycler. A typical reaction contained 1 nmol of each degenerate primer, 1 × PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM magnesium chloride, and 0.001% gelatin), 200 μM of each dNTP, and 0.25 units of native Taq DNA polymerase in a final volume of 50 μl. The amount of template DNA used per reaction ranged from 1–1000 ng depending on the complexity of each template. The amplification program was set for 30 cycles at 94°C for 2 min, 55°C for 3 min, and 72°C for 3 min. PCR products were analyzed by 2% SeaKrm GTG agarose in TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA, pH 8.0), and the amplified 138-base pair DNA fragments were isolated from agarose gels by Geneclean (B10101, La Jolla, CA). After restriction enzyme digestion with GamH1, the DAN was ligated into the BamH1 site of a pGEM4 vector (Promega Corp., Madison, WI) for sequencing. PCR primers for mTR2R1 DNA binding domain were 5′-TGY GTN GTN TGY GGN GAY AA-3′ (CVVCGDK) and 5′-CAT NCC CAT YTC NAR RCA YTT-3′ (KCLEMGM). All sequencing reactions used miniprep plasmid DNA prepared by the alkali lysis procedure as described (Sambrook et al., 1989), except that lysozyme was omitted. Plasmid DNA was denatured and annealed to sequencing primer, as described by Toneguzzo et al. (1988). Labeling and termination reactions were carried out as described by the manufacturer's protocol (U.S. Biochemicals, Cleveland, OH). The first 800 bases from the 5′ end of the mouse clone pBS35, which includes the DNA binding domain, were sequenced in this laboratory. Final completion of the entire reading frame of the mTR2R1 cDNA was carried out by Lark Sequencing Technology, Inc. (Houston, TX).

*Northern Blot Analysis*

Northern blot analysis of male reproductive tissues was carried out with total RNAs isolated from freshly killed mice. Total RNAs were extracted by-RNAzol B (BIOTECX Laboratories, Inc., Houston, TX) using the protocol recommended by the manufacturer. Total RNA (20 μg each lane) was fractionated by electrophoresis for 3 h at 70 V in a 1.2% agarose gel containing 1.1% formaldehyde in MOPS (3-[*N*-morpholino]propane sulfonic acid) buffer (1 × MOPS buffer = 0.02 M MOPS, 0.005 M sodium acetate, 0.001 M EDTA, pH 7.0). RNA molecular size marker (5 μg per lane) was purchased from Gibco/BRL (Bethesda, MD). After electrophoresis, the RNA was transferred to a nitrocellulose membrane filter (Schleicher & Schuell, Keene,NH) by capillary action and was processed as described (Seldon, 1989). A 1.0 kb cDNA fragment encoding the least conserved N-terminal domain of mTR2R1 was amplified by PCR and labeled by random priming to a specific activity of 0.5–1.0 × $10^9$ cpm/μg DNA (Feinberg and Vogelstein, 1983) to probe the mouse RNA filters. For tissue distribution analysis of mouse TR2R1, we used multiple tissue RNA blots from CLONTECH Laboratories, Inc. (Palo Alto, CA).

## RESULTS AND DISCUSSION

*Isolation and Characterization of mTR2R1*

A neonatal mouse brain cDNA library was screened by hybridization at low and high stringency with a $^{32}$P-labeled cDNA probe encoding the DNA binding domain of the orphan receptor COUP-TF1 (Wang et al., 1989). Plaques that produced positive signals selectively under low-stringency hybridization conditions were further discriminated by screening with other orphan receptor cDNAs. The candidate clones were then converted to plasmids by in vivo excision and were used as templates to amplify sequences encoding nuclear receptor DNA binding domains using the polymerase chain reaction. The sequences were amplified using degenerate oligonucleotide primers corresponding to the most conserved regions at the base of the first and second fingers of the DNA binding domains (see the Materials and Methods section). The amplified sequences were subcloned and sequenced by the dideoxy method to identify sequences encoding DNA binding domain fragments of novel orphan receptors. One such subclone appeared to encode a DNA binding domain that was related to, but distinct from, other members of the nuclear receptor superfamily identified to date. To identify the original cDNA in the library from which the subclone was derived, the cDNA fragment of this subclone was used as a $^{32}$P-labeled probe for additional rounds of screening of the candidate cDNA clones. Analysis of the purified plasmid pBS35 showed that it contained a 3.5 kb cDNA that was sequenced to establish the predicted amino acid sequence of the novel orphan receptor.

The sequence of the entire open reading frame of the purified clone (mTR2R1) is shown in Fig. 1. The cDNA contains an open reading frame of 1887 nucleotides encoding a protein of 629 amino acids in length and a predicted molecular weight of 68.8 kDa. The open reading frame is preceded by a 5′ leader sequence of 418 nucleotides and is followed by a 3′ untranslated region of approximately 1.1 kb.

*mTR2R1 Is a Member of the Nuclear Receptor Superfamily and Is Closely Related to the Orphan Receptor, hTR2*

Comparison of the sequence of mTR2R1 with published receptors showed that it encodes a novel orphan member of the nuclear receptor superfamily. The appearance of a characteristic type II zinc finger DNA binding domain sequence in the cDNA (Tsai et al., 1991; Evans, 1988) indicated that, like other nuclear receptors, the protein encodes a putative transcription factor. One sequence within this region referred to as the P box has been reported to play a role in determining the sequence specificity of enhancer DNA element half-sites recognized by some nuclear receptors (Umesono and Evans, 1989). Analysis of this sequence within the DNA binding domain of mTR2R1 suggests that, like most orphan receptors, mTR2R1 may interact with upstream enhancer DNA elements that are based on spatial variations of AGGTCA sequence motifs to regulate transcription of target genes (Umesono and Evans, 1989).

Two additional hallmarks of nuclear receptors were found within the carboxy half of the mTR2R1 molecule (Fig. 1). They were originally termed consensus regions II and III because of the general conservation of these regions between nuclear receptors (McDonnell et al., 1987). Although they are located in the ligand binding domain of the ligand-activated members of the superfamily, they are also contained in a similar region of members that have been shown to function as constitutively active transcription factors (Lydon et al., 1992; Davis et al., 1991; Paulsen et al., 1992). These regions have been implicated in ligand binding (Tsai et al., 1991; Evans, 1988), interaction of receptors with heat shock pro-

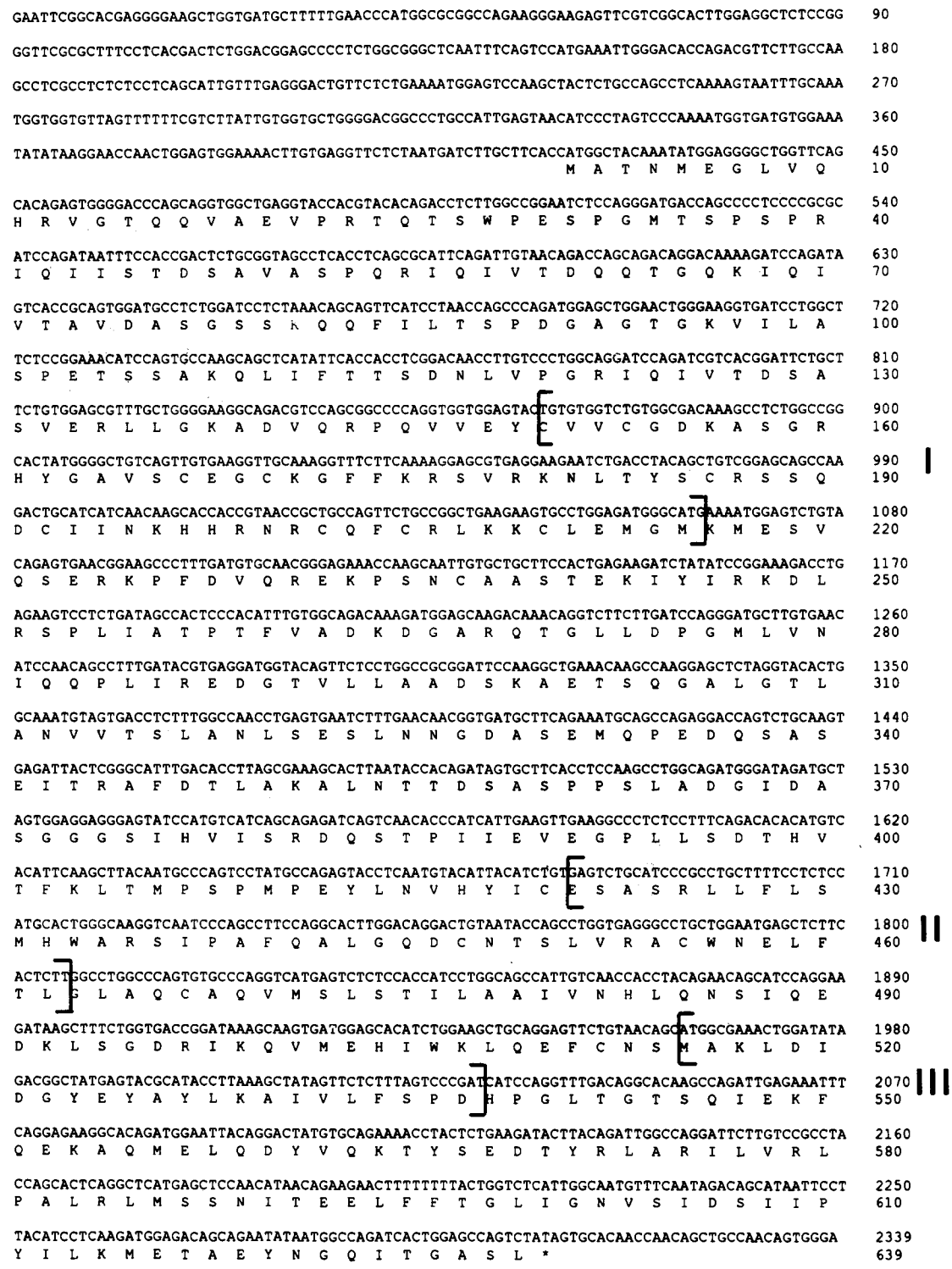


FIG. 1. The nucleotide and predicted amino acid sequence of the mTR2R1 cDNA. The DNA binding domain CI and regions CII and CIII are highlighted.

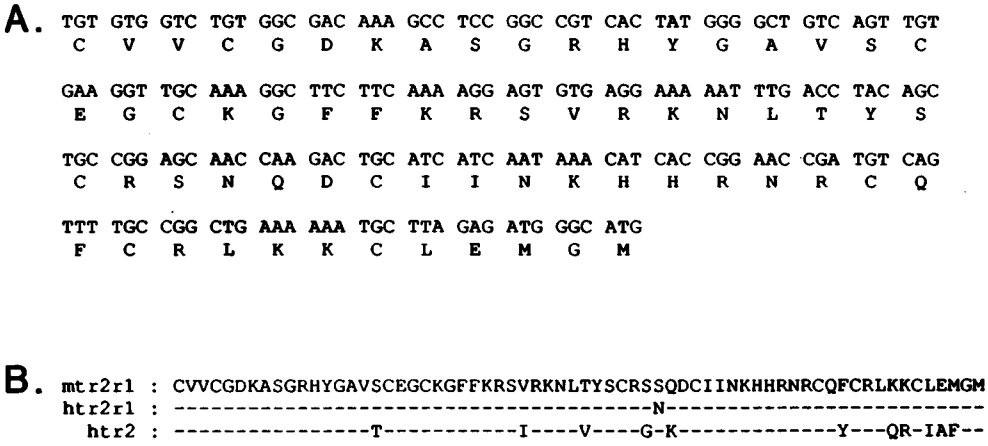


FIG. 2. Nucleotide and deduced amino acid sequence of the DNA binding domain of hTR2R1 (A) and amino acid conservation with mTR2R1 and hTR2 (B).

teins and nuclear transcription proteins (Shanjaard et al., 1991; O'Donnell and Koenig, 1990; Cadepond et al., 1991), and in receptor dimerization (Fawell et al., 1990). Comparison of the sequence of mTR2R1 with all documented members of the nuclear receptor superfamily also revealed that this novel orphan receptor is most closely related to an orphan receptor isolated from a human testis cDNA library, hTR2 (Chang and Kokontis, 1988). Hence, the orphan receptor isolated in the present study has been referred to as TR2-related receptor 1 (mTR2R1). To confirm that mTR2R1 does not correspond to the mouse counterpart of hTR2, we used the mTR2R1 cDNA as probe to isolate human TR2R1 from a human prostate cDNA library. The sequence of the DNA binding domain of hTR2R1 and its alignment with hTR2 and mTR2R1 are shown in Fig. 2 A and B. These data demonstrate that a human counterpart of mTR2R1 can be identified that is distinct from hTR2, confirming that the mTR2R1 clone we have isolated encodes a novel member of the nuclear receptor superfamily that is related to, but distinct from, TR2. This was further substantiated when the three consensus regions of homology among nuclear receptors were compared with mTR2R1 (Table 1).

Four variants of hTR2 have been identified (Chang et al., 1989). Their amino acid sequences are identical throughout the N-terminal and DNA binding domains, whereas the carboxy-terminal ends vary in length and amino acid sequence. A comparison of the regional homologies of these variants with mTR2R1 is shown in Fig. 3. The N-terminal and DNA binding domains of mTR2R1 show a 29% and 82% amino acid conservation with all of the hTR2 variants. Within the DNA binding domain, the sequence of the P box is identical between mTR2R1 and the hTR2 variants, suggesting that these proteins, like most orphan receptors, may interact with some spatial arrangements of the AGGTCA half-site sequence motif.

The carboxy-terminal region is most closely related to that of the hTR2 variant, hTR2-11. Whereas mTR2R1 has a 65% amino acid conservation with three of the hTR2 variants in this region, the hTR2-11

TABLE 1

DOMAIN COMPARISON BETWEEN mTR2R1, hTR2R1, hTR2, hCOUP-TF, mNURR1, AND hPR

| | I | II | III |
|---|---|---|---|
| mTR2R1 | 100% | 100% | 100% |
| hTR2R1 | 98.5% | 100% | 100% |
| hTR2 | 83.3% | 81% | 91% |
| hCOUP-TF | 71% | 50% | 52% |
| mNURR1 | 57.6% | 26% | 13% |
| hPR | 48% | 26% | 22% |

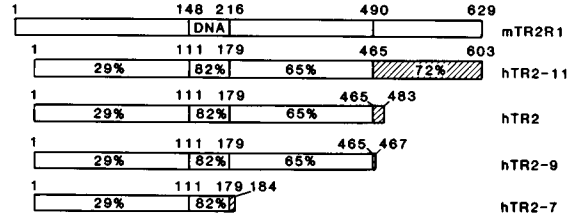


FIG. 3. Schematic alignment of the mTR2R1 polypeptide sequence with that of the previously isolated hTR2 variants (Chang et al., 1989). The variable carboxy-terminal regions within the hTR2 variants are depicted by the shaded areas. The percentage figures given in each box refer to the regional amino acid identity with mTR2R1 for each variant.

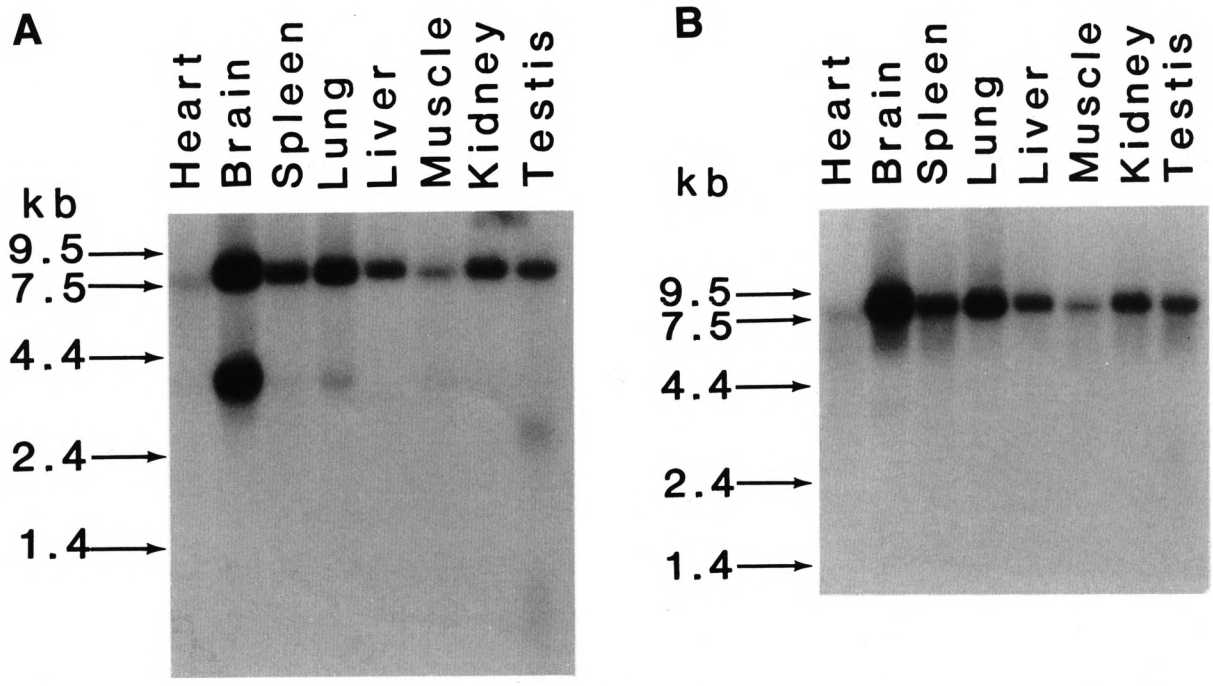


FIG. 4. Mouse multiple tissue blot analysis of the expression of mTR2R1 mRNA. (a) Probe with the entire 3.5 kb mTR2R1 cDNA in clone pBS35. (b) Probe with the 3′ noncoding region of mTR2R1 in clone pBS35.

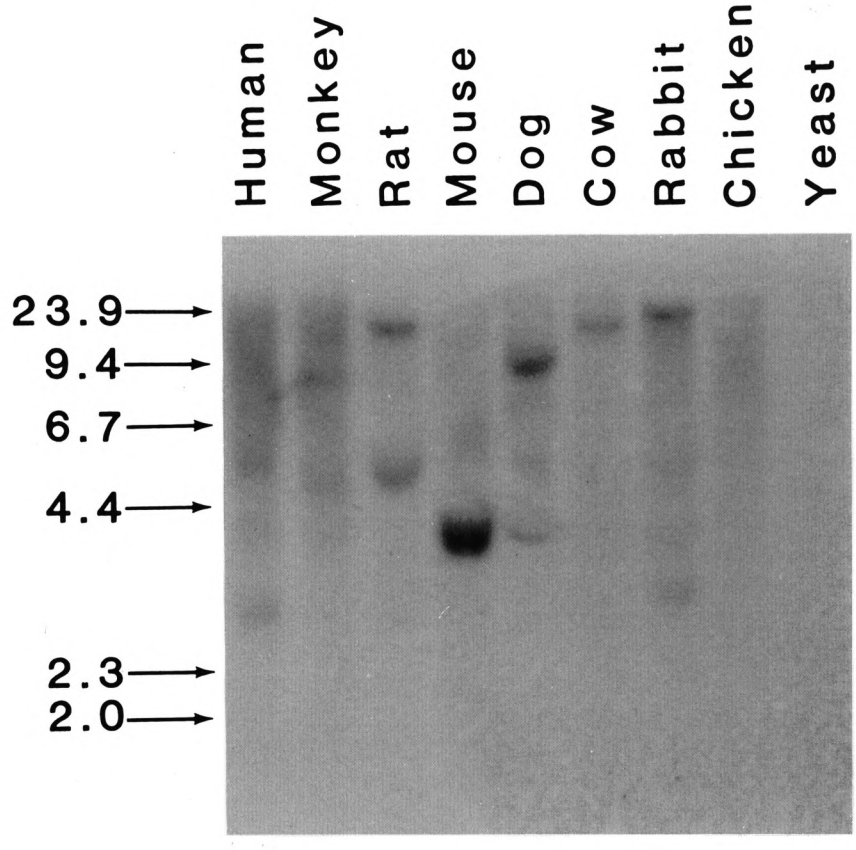


FIG. 5. EcoR1 digested multiple species genomic DNA Southern blot. The positions of the HindIII digested λ DNA markers are indicated.

variant contains a carboxy-terminal extension relative to the other TR2 variants that contains consensus region III and has 72% amino acid identity with mTR2R1 and generates a carboxy-terminal domain that is of similar size to mTR2R1. The high degree of conservation between hTR2-11 and mTR2R1 in the carboxy-terminal region of the proteins suggests a possible overlapping modulation of the functional activities of these proteins. This region encodes the ligand binding domain of the ligand-activated members of the superfamily (Tsai et al., 1991; Evans, 1988). It has not been established whether hTR2 encodes a ligand-activated transcription factor. We have shown that the carboxy-terminal domain of hTR2 does not contain a constitutively active transactivation function, but, like several ligand-activated members of the nuclear receptor superfamily, it can be activated by agents that stimulate intracellular signaling pathways involving phosphorylation events (Lydon et al., 1992). Thus, it is likely that the function of mTR2R1 may be activated also by direct ligand binding, by ligand independent signaling pathways, or by both mechanisms.

### *mTR2R1 mRNA Exists in Two Forms: A Ubiquitous Form and a Tissue-Restricted Form*

To define the tissue expression pattern of this novel orphan receptor mTR2R1, we carried out Northern blot analysis of mouse tissue mRNAs using the entire 3.5 kb cDNA in clone pBS35 as a $^{32}$P-labeled probe. The results are shown in Fig. 4. Two distinct mRNAs were detected (Fig. 4a). There is an 8.0 kb form that is present in every tissue tested and a 3.5 kb form that is predominantly expressed in the brain with only a low level detected in lung tissue. These mRNA expression patterns demonstrate that variant forms of mTR2R1 are expressed at the mRNA level in a tissue-specific manner. In contrast to mTR2R1, only a single hTR2 mRNA (2.5 kb) has been detected to date. The mRNA is expressed most abundantly in male reproductive tissues, including prostate, seminal vesicles, and testis (Chang and Kokontis, 1988). Although four variant hTR2 cDNAs have been cloned, the size and expression pattern of the mRNAs encoding these variants has not been documented. However, all forms were isolated from testis with the exception of hTR2-11, which was isolated from a prostate cDNA library (Chang et al., 1989). Northern blot analysis of total RNA from mouse reproductive tissues demonstrated the expression of mTR2R1 in the prostate, epididymis, and testis (Law et al., unpublished). The differential expression of mTR2R1 and hTR2 suggests that these proteins may function independently to regulate gene expression with the exception of male reproductive tissues, where they may regulate overlapping target genes.

### *The Two TR2R1 mRNAs Are Most Likely the Products of a Single Copy Gene*

To better define the relationship between the two forms of mTR2R1, we reprobed the multiple tissue Northern blot with a $^{32}$P-labeled cDNA derived from the 3′ noncoding region probe hybridized only to the 8.0 kb mRNA. The faint hybridization signal seen at the 3.5 kb region of the filter is due to incomplete removal of the full-length cDNA probe from the previous hybridization experiment. Therefore, the cDNA in pBS35 is derived from the larger 8.0 kb mRNA. The relationship of the two mTR2R1 mRNAs was further evaluated by Southern blot analysis of genomic DNA using a 3′ coding region probe that hybridized to both mRNAs. Results are shown in Fig. 5. There is only one hybridization band at the EcoR1-digested mouse DNA lane, suggesting mTR2R1 is a single copy gene. Thus, the smaller mRNA is most likely derived from tissue-specific posttranscriptional processing. In addition, the mTR2R1 cDNA cross-hybridized to all vertebrate genomic DNAs in this study, which seems to suggest a functional conservation for this novel orphan receptor.

The identification of mTR2R1 underlies the existence of yet an additional subfamily of putative transcription factors within the nuclear receptor superfamily and prompts a close examination of the physiological role of this orphan receptor subfamily.

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