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RTR: a new member of the nuclear receptor superfamily that is highly expressed in murine testis

(Orphan receptor; RXR; spermatogenesis; transcription factor; spermatids; PCR)

Takahisa Hirose^a, Deborah A. O'Brien^b and Anton M. Jetten^a

^aCell Biology Section, Laboratory of Pulmonary Pathobiology, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, NC 27709, USA; and ^bLaboratories for Reproductive Biology and Department of Pediatrics and Cell Biology and Anatomy, University of North Carolina, Chapel Hill, NC 27599, USA

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SUMMARY

We have identified and cloned a novel member of the nuclear receptor superfamily from murine testis, referred to as retinoid receptor-related testis-associated receptor or RTR. Degenerate PCR primers homologous to two conserved regions of the DNA-binding domain of members of this superfamily were employed to identify this gene. The amino-acid sequence of RTR is most closely related to that of the mouse RXRs with an overall identity of 32–34%; the highest similarity (61%) is observed in the DNA-binding domain. Northern blot analysis using RNA from multiple tissues showed that RTR is predominantly expressed in the testis. Northern blot analysis using RNA from different testicular cell types showed that RTR mRNA is not expressed in early germ cells or Sertoli cells but is most abundant in round spermatids. Our observations suggest that this putative transcription factor plays a role in the regulation of gene expression particularly during the post-meiotic phase of spermatogenesis.

INTRODUCTION

The nuclear receptors comprise a large family of ligand-dependent transcriptional factors that regulate gene expression during specific developmental processes, cell growth and differentiation (Yamamoto et al., 1985).

This family includes the steroid and thyroid hormone receptors, retinoid receptors, peroxisome proliferator-activated receptors and several orphan receptors for which the ligand has not yet been identified. Members of this nuclear receptor family share several structural and functional characteristics (Green and Chambon, 1988; Evans, 1988). This superfamily appears to be defined in particular by the DNA-binding domain which is the most highly conserved among the family members and contains two zinc-finger motifs (Laudet et al., 1992).

Correspondence to: Dr. A.M. Jetten, Cell Biology Section, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, NC 27709, USA. Tel. (1-919) 541-2768; Fax (1-919) 541-4133; e-mail: jetten@niehs.nih.gov

Abbreviations: aa, amino acid(s); COUP-TF, chicken ovalbumin upstream promoter transcription factor; Mu-MLV, murine Moloney leukemia virus; nt, nucleotide(s); PCR, polymerase chain reaction; PPAR, peroxisome proliferator-activated receptor; RAR, retinoic acid receptor; RTR, retinoid receptor-related testis-associated receptor; RTR, gene (DNA, RNA) encoding RTR; RXR, retinoid X receptor.

In this study, we report the isolation and partial characterization of an additional member of this nuclear receptor superfamily which we refer to as RTR. We demonstrate that this receptor is highly expressed in the testis and particularly in round spermatids, suggesting a role for RTR in the regulation of gene expression during the post-meiotic phase of spermatogenesis.

GAATTCCTCAGGGGATGTGCGTGGCAGCGGGCGGTGACGTCAGAGGAGGGAGCTGGC 58
 CAGTCTGAGGGGGTCTGGCGGGAGGGGGGGGAGCGGGGGTTCAGGGGGCCAGAGA
 GTCCAGACTCCCGGAGCCCGCCCTCCCGCGCCCTCGGGCCGCCAGTCCCGGGTCTCT
 GTGCGGGGGCAGAGGCTCCGGCCCTGACAGCCCTCCCGCCCTGGAAGACCAGGA
 CACCGCCGGCTCTCGGACGGCCGGGACTCTCGGGGAGGGGGGACCTTCTGGTCT
 CGACGACTACGAAGGCCAAGTCAATGCGGAGCAGCGAAGCCCGAGAGGGCCCTGAGCAC 238

CGCCGATGGAGCGGACGAACCGGCCACCTAGCGGAGGGGGAGGCGGGGGGCTCGGGC 298
 M E R D E R P P S G G G G G G S A 18

GGTTCCTGGAGCGCCCGCGCTCCCTCCCGCCCGCGCAACGTTTCTGTGAGGAT 358
 G F L E P P A A L P P P P R N G F C Q D 38

GAATGGCAGAGCTTGATCCAGGCACTAATGGAGAGACTGACAGTTTAACACTTGGCCAA 418
 E L A E L D P G T N G E T D S L T L G Q 58

GGCCATATACCTGTTCCGTCACAGATGATCGAGTGAACAACAACTGTCTCATCTGT 478
 G H I P V S V P D R A E Q R T C L I C 78

GGGACCCCGCTACGGCTTGCACATGSGAICATCTCTCGAGGGCTGCAAGGGGTTT 538
 G D R A T G L H Y G I I S C E G C K G F 98

TTCAAGAGGAGCATTTGCAACAACCGGTGTATCGGTGACGTCGIGACAAGAACTGTGTC 598
 F K R S I C N K R V Y R C S R D K N C V 118

ATGTCCCGAAGCAGGAACAGATGTGACTGCTGCTCAAGTGTCTCCAGATG 658
 M S R K Q R N R C Q Y C R L L K C L Q M 138

GGCATGAACAGGAAGGTATCAGAGAAGTGGCATGCTGGAGGCGGAAACAGAGCATT 718
 G M N R K A I R E D G M P G G R N K S I 158

GGACCATCCAGATATCAGAAGAAGAAATGAAGAATCATGTCTGGACAGGATTTGAG 778
 G P V Q T S E E E I E R I M S G Q E F E 178

GAAGAAGCAATCACTGGAGCAACCATGGTGACAGCGACCAAGTCCCTGGGAACAGG 838
 E E A N H W S N H G D S D H S S P G N R 198

GCTTCAGAGGCAACCGCCCTCACCAGGCTCCACACTATCATCCAGTAGGCTGTGGAA 898
 A S E S N Q P S P S T L S S S R S V E 218

CTAAATGGATTGATGGCATTAGGGATCAGTACATGGGATGTCAGTGCCTCCACATTAT 958
 L N G F M A F R D Q Y M G M S V P P H Y 238

CAATACATACCACACCTTTTATGCTATTCGCGCACTCACCACCTTTTGGCCCCACAAGCT 1018
 Q Y I P H L F S Y S G H S P L L P P Q A 258

CGAAGCCTGGACCCCTCAGTCTCAGCTGATTCATCAGTGTGATGTCAGCGGAAGCCTG 1078
 R S L D P Q S Y S L I Y L M S A E D L 278

GAGCCATGGGCACACCTATGTTGATGAAGATGGGTATGCTGTGACACAGGAGAAGT 1138
 E F L G T P M L I E D G Y A V T Q A E L 298

TTGCTCTGCTTTGCGCCCTGGCCGACGAGTGTCTTTAGGCAGATTGCCTGGATCAAG 1198
 F A L L C R L A D E L L F R Q I A W I K 318

AAGTGCCTTTCTTCTGCGAGCTCTCAATCAAGGATTACACGTCGCTCTTGGAGCTTACG 1258
 K L P F F C E L I S I K D Y T C L L S T 338

TGGCAGGAGTAACTCTGCTCTCCCTCAGAGTACAGCAAGCAGATCTTTGGGGAG 1318
 W Q E L I L L S S L T V Y S K Q I F G E 358

CTGGCTGATGTCACAGCAAGTACTCACCCTCTGATGAAGAACTCCACAGATTTAGTGAT 1378
 L A D V T A K Y S P S D E E L H R F S D 378

GAAGGGATGGAGGTGATGAACGACTCATCTACCTATATCACAAGTCCATCAGCTGAAG 1438
 E G M E V I E R L I Y L Y H K F H Q L K 398

GTCAGCAACGAGGAGTACGCATGCATGAAAGCAATTAACCTTCTGAATCAAGATATCAGG 1498
 V S N E E Y A C M K A I N F L N Q D I R 418

GGTCTGACCACTGCTCAGCTGGAACAACCTGAACAAGCGGTATTGGTACATTTGTCAG 1558
 G L T S A S Q L E Q L N K R Y W Y I C Q 438

GATTTCACTGAATATAAATACACATCAGCCAAACCGCTTTTCTGATCTTATGATGTC 1618
 D F T E Y K Y T H Q P N R F P D L M M C 458

TTGCCAGAGATCCGATACATCGCAGGCAAGATGGTGAATGTGCCCTGGAGCAGCTGCC 1678
 L P E I R Y I A G K M V N V P L E Q L P 478

CTCCTTTAAGGTGGTGTGACTCTGCAAGACAAGTACGGTGAAGGAGTGA 1732
 L L F K V V L H S C K T S T V K E * 495

CTGTGCCCTGCACCTCTTGGGCCACCCACAGTGCCTTGGGTAGGCAGCACAGGCTCCAG 2006
 AGGAAAGAGCCAGAGACCAAGATGGAGACTGTGGAGCAGCTACCTCCATCACAAGAAGAA
 TTTGTTGTTGCTGTTTTAACTCATTTTCTATATATTATTCACGACAGAGTTG
 AATGTATGGCCTTCAACATGATGCACATGCTTTTGTGTGAATGACGAGATGCATTTCTT
 TGCAGTTACAGAATGTGAAGATGTTAATGTTA

Fig. 1. Nucleotide sequence of *RTR* and deduced aa sequence. The putative start codon is at nt 245. DNA-binding domain is underlined. The stop codon (nt 1730) at the end of *RTR* aa sequence is indicated as *. This sequence data have been deposited with EMBL/GenBank Data Libraries under accession No. U09563. **Methods:** (a) **PCR amplification:** A set of degenerate primers were designed according to the most highly conserved sequence of the DNA-binding domain of members of the human nuclear receptor family (Laudet et al., 1992). The sense-strand primer R-P1 was 5'-TGYGARGGNTGYAARGGYTTYTT-3'; (CEGCKGFF).

EXPERIMENTAL AND DISCUSSION

(a) Cloning of *RTR* cDNA

Highly degenerate primers were designed according to the two best-conserved aa sequences present in the DNA-binding domain of members of the nuclear receptor family. These primers were then employed in amplification reactions with single-stranded cDNA from human lung poly(A)⁺ RNA in order to identify DNA-binding domains of novel nuclear receptors. The amplified fragments of the expected size (130 bp) were cloned and sequenced. Most of the cDNA sequences encoded DNA-binding domains of known receptors including those of RAR α (Petkovich et al., 1987; Giguere et al., 1987), RXR β (Yu et al., 1991), RZR α (Becker-Andre et al., 1993), COUP-TF (Wang et al., 1989) and PPAR γ (Zhu et al., 1993). Among these cDNAs, we identified one which encoded a unique sequence not previously described. The aa predicted by this cDNA sequence suggested that it encoded the DNA-binding domain of a novel member of the nuclear receptor superfamily which we refer to as *RTR*. With the help of the 5'-RACE method we obtained a 280-bp fragment of the *RTR* sequence that was subsequently used as a probe in screening a mouse testis cDNA library. After screening 3×10^5 independent plaques, we obtained 4 positive clones. One of these clones contained an insert of 2006 bp. Analysis of this *RTR* sequence

The antisense strand primer R-P2 was 5'-RCAYTTCTKNCGY-CAGTAYTGRCA-3' (CQYCR(L/K/Q)KC), where K=G or T, N=A or C or G or T, R=A or G and Y=C or T. Human lung poly(A)⁺ RNA (Clontech, San Diego, CA, USA) was reverse transcribed into single strand cDNA with Mu-MLV reverse transcriptase and oligo d(T) primer (Stratagene, La Jolla, CA, USA). The R-P1 and R-P2 primers were employed in amplification reactions using the Amplitaq kit and a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, CT, USA). We carried out the following amplification cycles; 1 min denaturation at 94°C followed by a 1 min annealing at 50°C and 3 min extension at 72°C for 30 cycles. The amplification products were separated on a 2% low temperature melting agarose gel (Sigma, St. Louis, MO, USA) and the DNA products around 130 bp were isolated from the gel and directly ligated into the TA cloning vector (Invitrogen, San Diego, CA, USA). The DNA inserts of 30 clones were analyzed by double-strand dideoxy DNA sequencing using Sequenase (US Biochemical, Cleveland, OH, USA). (b) **Anchor PCR:** To isolate a greater region of the gene encoding *RTR*, we used the 5'-RACE kit (BRL) in the same manner as described previously (Loh et al., 1989). Two sequential antisense primers were designed from the sequence of the original *RTR* PCR fragment. Single-stranded cDNA primed with specific primer was synthesized from 1 μ g of human lung poly(A)⁺ RNA with Superscript reverse transcriptase according to manufacturer's recommendation (BRL). The amplification products were cloned into pBluescript II and sequenced. (c) **cDNA library screening:** Uni-ZAP XR cDNA libraries from mouse testis (Stratagene) were screened with multiprimed cDNA probe from anchor PCR products with moderately stringent conditions. cDNA inserts from several positive clones were subcloned into the *EcoRI* and *XhoI* site of pBluescript II. The complete DNA sequences of both strands were determined as described for PCR products.

revealed a long open reading frame which starts with a putative start codon at nt 245 and terminates with a putative stop codon at 1730 (Fig. 1). On this basis, *RTR* encodes a protein of 495 aa with a predicted molecular mass of 60 kDa. The DNA-binding domain (aa 75–140) of *RTR* contains 10 Cys residues, eight of which participate in the formation of two zinc-finger motifs.

(b) Homologies of *RTR* with other nuclear receptors

Comparison of aa sequence of *RTR* with those of other members of nuclear receptor superfamily revealed that *RTR* does not belong to any of the subfamilies of nuclear receptors reported previously. *RTR* exhibits a 32–34% overall identity with the mouse *RXR* receptors (Mangelsdorf et al., 1992; Leid et al., 1992). The DNA-binding domain of *RTR* showed the highest identity (61%) with that of the mouse *RXR*, the human *RZR* α and mouse *RAR* γ receptors (Fig. 2a). The P-box sequence which constitutes the aa sequence present in the C-terminal region of the first zinc-finger is key in classifying the DNA-binding specificity of nuclear receptors (Forman and Samuels, 1990). In *RTR*, this sequence is

CEGCKG, indicating that *RTR* may recognize enhancer sequences similar to those of the retinoid, thyroid hormone, vitamin D and peroxisome proliferator activating receptors (Laudet et al., 1992). Comparison of the aa sequence of the ligand-binding domain indicated little homology between *RTR* and other nuclear receptors (Fig. 2b). The highest identity (26%) was found between the ligand binding domain of *RTR* and that of mouse *COUP-TFII* (Qiu et al., 1994). The ligand-binding domain of mouse *RXR* α exhibited a 20% identity with that of *RTR*.

(c) Tissue distribution of *RTR* mRNA

To study the tissue distribution of *RTR* mRNA, we performed Northern blot analysis on poly(A)⁺RNA from a variety of mouse tissues (Fig. 3). The full-length *RTR* probe hybridized to a 7.4-kb and a 2.3-kb transcript in testis. The 7.4-kb transcript was much less in brain, lung, liver and kidney than testis and the 2.3-kb transcript was not detected. The expression of transcripts of distinct size has been reported for several other orphan receptors. For example, the orphan receptor *RZR* α has a 15-kb

a.

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RTR (75) CLICGDRATGLHYGIIISCEGCKGFFKRSICNKRVRCSRDKNVMSRKQRNRCQYCRLLKCLQMGM
RXR $\alpha$  (135) -A-----SS-K---VY-----TVRKDLT-T-RDN-D-LIDKR-----YQ---A--- 61%
RZR $\alpha$  (18) -K---KSS-I---V-T-----R--QSNAT-S-P-Q---LID-TS-----H---Q---AV-- 61%
RAR $\gamma$  (91) -FV-Q-KSS-Y---VS-----R--QKNM--T-H-----IINKVT-----Q---FDV-- 61%
RAR $\alpha$  (88) -FV-Q-KSS-Y---VSA-----R--QKNM--T-H-----IINKVT-----Q---FEV-- 59%
TAK1 (117) -VV---K-S-R---AV-----VRKNLT-S-RSNQD-IINKHH-----F---K---E--- 58%
ER (189) -AV-N-Y-S-Y---VW-----A-----QGHND-M-PATNQ-TIDKNR-KS--A---R---YEV-- 50%
AR (558) -----E-S-C---ALT-GS--V---AAEG-QK-L-ASRND-TIDKFR-KN-PS---R---YEA-- 47%

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b.

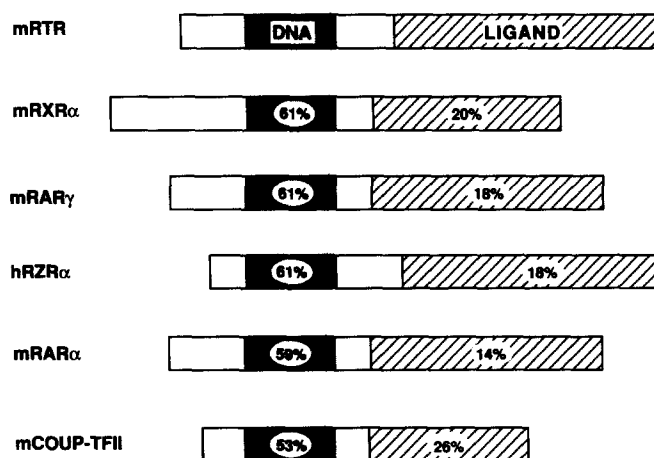


Fig. 2. Schematic aa comparison of *RTR* and several members of the steroid hormone receptor superfamily. (a) The aa sequence alignment of the DNA-binding domain of *RTR* and members of the steroid hormone receptor superfamily. The number in parentheses represent the positions of aa residues in the individual receptors. The aa conserved with *RTR* are indicated with a bar. The numbers on the right of aa sequences indicate the percentage identity with *RTR*. (b) Alignment of aa sequences of DNA-binding domain and ligand-binding domain. The numbers in each box indicate the percentage of identity with *RTR*. The DNA- and ligand-binding domains are indicated. Specific references are mRXR α (Mangelsdorf et al., 1992), mRAR γ (Zelent et al., 1989), hRZR α (Becker-Andre et al., 1993), mRAR α (Zelent et al., 1989), mCOUP-TFII (Qiu et al., 1994), hTAK1 (Hirose et al., 1994), estrogen receptor (mER, White et al., 1987) and androgen receptor (hAR, Lubahn et al., 1988). The prefixes h and m denote human and mouse, respectively.

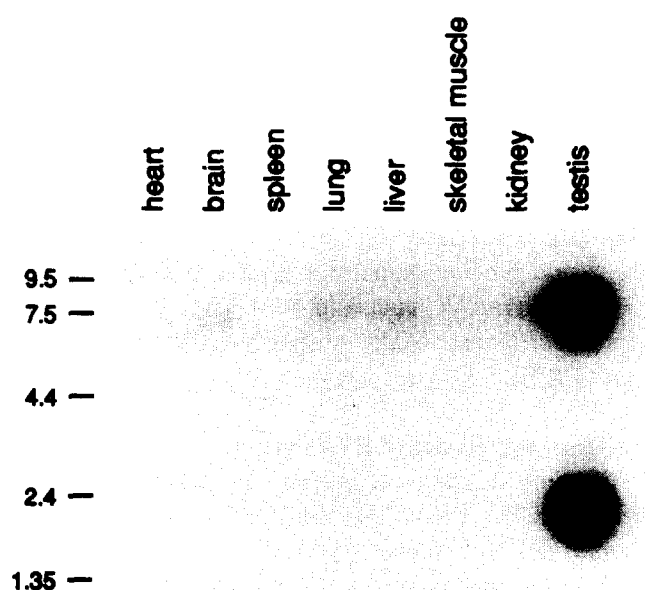


Fig. 3. Tissue specificity of *RTR* expression. Poly(A)⁺ RNA (2 µg) isolated from different Balb/c mouse tissues was analyzed by Northern blot analysis using a ³²P-labeled *RTR* probe. RNA size markers (kb) are indicated on the left side of blot. Full-length cDNA of *RTR* labeled with ³²P by random priming was used to detect *RTR* mRNA. **Methods:** Mouse multiple tissue Northern blots (1.2% agarose-2.2 M HCOH) were purchased from Clontech. The blots were hybridized with a ³²P-labeled *RTR* probe encoding nt 300 to 2014 as described previously (Hirose et al., 1990).

transcript that is expressed ubiquitously and a 2.3-kb transcript expressed predominantly in leukocytes, testis, lung and liver (Becker-Andre et al., 1993). The unrelated orphan receptor *TAK1* (Fig. 2a) which exhibits 26% identity with *RTR* has also two distinct transcripts, with the shorter one specifically expressed in testis (Hirose et al., 1994). The different transcripts of *TAK1* and *RTR* may be generated by alternative splicing.

(d) Cell-type-specific expression of *RTR* in mouse testis

Mature testis is composed of many cell types, including Sertoli cells, Leydig cells and a variety of germ cells (Russel et al., 1990). To investigate which cells in the testis expressed *RTR*, we performed Northern blot analysis using mRNA prepared from different testicular cells (Fig. 4). The full-length *RTR* cDNA probe hybridized to mRNA from whole testis and mixed germ cells but not to mRNA from Sertoli cells (Fig. 4a). The ratio between the 2.3 and 7.4-kb transcripts in testis of CD1 mice was different from that of Balb/c mice. Mixed germ cells contain spermatogonia, several kinds of spermatocytes and haploid spermatids. Therefore, mixed germ cells were separated into distinct cell populations and subsequently analyzed for *RTR* expression (Fig. 4b). *RTR* mRNA was highly expressed in round spermatids and was not detectable in germ cells at an earlier phase of spermatogenesis.

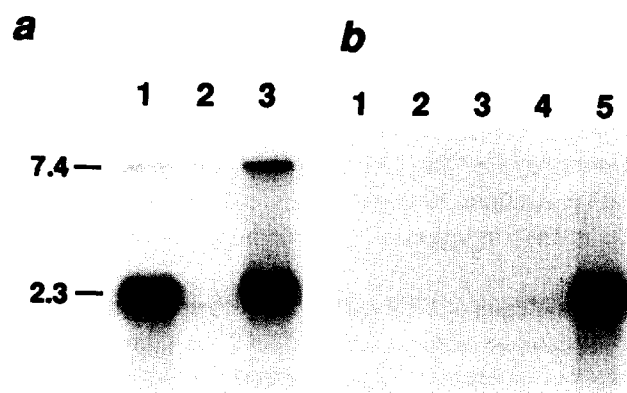


Fig. 4. Specificity of *RTR* expression in testis. Poly(A)⁺ RNA (8 µg) isolated from different testicular cells was analyzed by Northern blot analysis using a ³²P-labeled *RTR* probe. (a) mRNA from whole testis (1), Sertoli cells (2) and mixed germ cells (3). (b) mRNA from different germ cells; mRNA from type B spermatogonia (1), preleptotene spermatocytes (2), leptotene- and zygotene spermatocytes (3), pachytene spermatocytes (4) and round spermatids (5). Equal amounts of RNA were loaded as determined by residual ribosomal RNA content. **Methods:** Mixed germ cell suspensions were prepared from adult CD-1 mice by enzymatic dissociation of seminiferous tubules as described previously (O'Brien, 1987). Germ cells at defined stages of differentiation were isolated from mixed cell suspensions by unit gravity sedimentation (Bellve et al., 1977; O'Brien, 1987). Sertoli cells from 17-day-old CD-1 mice were isolated and maintained in culture for 7 days as described previously (O'Brien et al., 1989). Northern blot analysis (1.0% agarose-2.2 M HCOH) was performed as described in the legend to Fig. 3.

These observations indicate that *RTR* transcripts are most abundant in haploid germ cells and suggest that *RTR* may regulate gene expression in particular during the post-meiotic phase of spermatogenesis. A number of other genes have been reported to be up-regulated during the post-meiotic phase of spermatogenesis, including protamines (Kleene et al., 1983), the proto-oncogenes *c-abl* and *c-mos* (Iwaoki et al., 1993), and glyceraldehyde 3-phosphate dehydrogenase (Mori et al., 1992). Some of these genes may be candidates for regulation by *RTR*.

(e) Conclusions

(1) A cDNA encoding a novel member of the nuclear receptor superfamily was isolated. The deduced aa sequence of the new receptor, referred to as *RTR*, exhibited the highest identity (32%) with members of the *RXR* family.

(2) *RTR* is predominantly expressed in the testis and at very low levels in lung, brain, kidney and liver.

(3) *RTR* mRNA was most highly expressed in round spermatids, suggesting a role for *RTR* in the regulation of gene expression during the post-meiotic phase of spermatogenesis.

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