Characterization of the Response Element and DNA Binding Properties of the Nuclear Orphan Receptor Germ Cell Nuclear Factor/Retinoid Receptor-related Testis-associated Receptor*

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Recently, we have reported the cloning of the germ cell-specific, nuclear orphan receptor germ cell nuclear factor (GCNF)/RTR. In this study, we characterize the RTR response elements by an electrophoretic mobility shift assay/polymerase chain reaction-based, DNA binding site selection strategy. RTR binds with the greatest affinity to response elements containing TCA(AG/G/T)TCA

Recently, we reported the cloning of the novel nuclear orphan receptor RTR from a mouse testis cDNA library (27). This receptor has also been cloned from rat and has been named germ cell nuclear factor (GCNF); Ref. 28. This orphan receptor is highly expressed in the testis and in particular in round spermatids (27). These observations suggest that GCNF/RTR functions as an important transcriptional factor in the regulation of gene expression during a very specific stage of spermatogenesis.

In this study, we have analyzed the characteristics of the RTR response element (RTRE) more precisely by a DNA binding site selection strategy that is based on a combination of polymerase chain reaction (PCR) and electrophoretic mobility shift assay (EMSA) and by mutation analysis. Based on the consensus RTRE sequence obtained, we located a putative RTR response element (P2-RE) in the 5’ promoter-flanking region of the mouse protamine 2 gene, which is induced during the same stage of spermatogenesis as RTR. The ability of RTR-Ab2 to cause a supershift of an RTR-RTRE complex with nuclear extracts from different tissues correlated with the tissue- and development-specific expression of RTR.

Transfection of RTR in CV-1 cells was unable to cause RTR-dependent transactivation of a CAT reporter gene; however, an RTR-VP16 fusion protein could induce transactivation through several RTREs, including P2-RE.

The nuclear receptor superfamily constitutes a class of ligand-dependent transcription factors that regulate gene expression during many biological processes, including development, cellular proliferation, and differentiation (1–5). This superfamily includes receptors for steroid hormones, retinoids, and vitamin D, and an increasing number of orphan receptors for which the ligand has not yet been identified (6–9). Members of this superfamily share a common modular structure consisting of four major domains (6, 10–13). The DNA-binding domain is the most conserved among the nuclear receptors and is composed of two “zinc finger” motifs that play a role in DNA recognition and protein-protein interactions (10, 14). The ligand-binding domain at the C terminus is involved in several functions including ligand binding, receptor dimerization, nuclear translocation, and transcriptional activation (11, 13–16). The functions of the N-terminal region and the hinge domain that separates the DNA-binding and ligand-binding domains are still poorly understood. Recent studies have indicated that in certain instances the amino-terminal domain contains a transactivation function (17) and that the hinge domain contain sites that interact with co-repressors (18).

Nuclear receptors control the transcription of target genes by binding to DNA sequences referred to as hormone response elements (14, 16). Most members of this superfamily bind, as homodimers or heterodimers, to cis-acting DNA sequences that contain two half-site core motifs of RGGTCA configured in a direct repeat, a palindrome, or an inverted palindrome separated by spacers of different lengths (2, 7, 14, 16, 19, 20). A number of orphan receptors, including members of the ROR/RZR family (21–23), NGF1-B/Nur77 (24), and FTZ-F1/SF-1 (24–26) bind as monomers to hormone response elements consisting of a single core motif, RGGTCA, preceded by an AT-rich sequence.

Recently, we reported the cloning of the novel nuclear orphan receptor RTR from a mouse testis cDNA library (27). This receptor has also been cloned from rat and has been named germ cell nuclear factor (GCNF); Ref. 28. This orphan receptor is highly expressed in the testis and in particular in round spermatids (27). These observations suggest that GCNF/RTR functions as an important transcriptional factor in the regulation of gene expression during a very specific stage of spermatogenesis.

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1 The abbreviations used are: GCNF, germ cell nuclear factor; RTRE, RTR response element; conRTRE, consensus RTRE; PCR, polymerase chain reaction; EMSA, electrophoretic mobility shift assay; CAT, chloramphenicol acetyltransferase; bp, base pair(s); DR0 and DR1, direct repeat 0 and 1, respectively; P0 and P2, palindrome 0 and palindrome 2, respectively; RTR, retinoid receptor-related testis-associated receptor.
Response Element of the Nuclear Orphan Receptor GCNF/RTR

EXPERIMENTAL PROCEDURES

Plasmids—The plasmid pBSK-RTR containing the full-length mouse RTR cDNA in pBluescript II SK(+) (Stratagene) has been described (27). The construction of pZeosSV-RTR was made by inserting the Xho I-Kpn I fragment into the Spei-Kpn I site of the pZeos SV vector (Invitrogen). The plasmid pGEMZ-RTR, which was used for in vitro translation of RTR, was created by inserting the Xho I-Kpn I fragment of pBSK-RTR containing the full-length RTR into the expression vector pGEMZ (Promega). The CAT reporter gene constructs were created by inserting three copies of the consensus RTR binding site (conRTRE) (TGCGGAATTCCTGACAGGATCCACAAGGATCCAGTAA) into the HindIII and BamHI sites of pBLCAT5 (31) to create the reporter plasmids of p63RTRE-CAT, (1/2-RTRE)-CAT, and (conRTRE)-CAT. The pSG5-VP16-RTR chimeric expression plasmid that encodes a fusion protein consisting of the VP16 activation domain and the full-length coding region of the RTR was created as follows. pGEMZ-RTR was cut first with EcoRI and Xho I enzymes to obtain the full-length RTR fragment, which was then inserted into the BspHI and Xho I sites of pBluescript II SK to create pBSK-RTR-2. pSG5-VP16-RTR was obtained by inserting the Kpn I-Xho I fragment of pBSK-RTR into the same sites of pSG5-VP16. The pSG5-VP16-RTR chimeric expression plasmid that encodes a fusion protein consisting of the VP16 activation domain and the full-length coding region of the RTR was inserted into the BspHI and Xho I sites of pBluescript II SK to create pBSK-RTR-2. pSG5-VP16-RTR was obtained by inserting the Kpn I-Xho I fragment of pBSK-RTR into the same sites of pSG5-VP16. The pSG5-VP16-RTR plasmid was obtained from Dr. J. Lehmann (Glaxo-Welsh-Lab). The pBSK-RTR containing the full-length RTR into the expression vector pStuI fragment was inserted into the same sites of pSG5-VP16. The Xho I-Bgl II sites of pBSK-RTR were used to create a single copy of conRTRE into the pBSK-RTR-2. The expression construct pZeoSV-RTR was made by inserting the N7GTCGACAAGCTTCTAGAGCA-32 primer into the expression vector pZeoS (Stratagene). Approximately 0.2–0.5 ng (50,000 cpm) of the probe was used in EMSA with 5% nondenaturing polyacrylamide gels (National Diagnostics) containing 10 mM HEPES (pH 7.9), 50 mM KCl, 2.5 mM MgCl2, 1 mM DTT, and 10% glycerol. To prevent nonspecific binding, 1 μg of poly(dI-dC), 1 μg of salmon sperm DNA, and 0.5 μg of nonspecific oligonucleotide were included in the reaction buffer. The programmed lysates/nuclear extracts were first incubated with reaction buffer for 10 min at room temperature and then in the presence of the radiolabeled probe or without competitor for 30 min. As a control, probes were incubated with the same amount of unprogrammed lysate. For supershift assays, 1 μl of RTR-Ab2 (1:200 final dilution) was preincubated with the in vitro-translated RTR for 30 min at room temperature prior to the addition of radiolabeled probe. The RTR-nucleotide complexes were separated on 5% nondenaturing polyacrylamide gels (National Diagnostics) containing 0.5 × TBE.

Selection of RTR Binding Sites—To select for the DNA binding sites of RTR, a similar procedure was used as reported previously (21). A mixture of 70-bp DNA fragments was obtained by PCR using a random oligomer 5'-CGCCGGATCCTGACAGGAGN6-3' as a template and 5'-CCGGATCTGGCTGCTGAG-3' (primer 1) and 6'-GGCTTCTAGAAGCCCTGCG-3' (primer 2) as the forward and reverse primers, respectively. In a separate reaction, the oligomer 5'-CCGGATCTGGCTGCTGAGN6-3' was used as template and 5'-GGCTTCTAGAAGCCCTGCG-3' as forward primer and 5'-CGCCGGATCCTGACAGGAGN6-3' as reverse primer. Three cycles under the following conditions: 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C for each cycle. The double-stranded mixed DNA fragments generated were purified and incubated with in vitro synthesized RTR, and complexes corresponding to the RTR-RTR complex were excised, and the DNA was eluted in TE buffer. Recovered DNA was amplified by PCR for 15 cycles and used for EMSA analysis with RTR under the conditions described above. This procedure was repeated four times. In the fifth round, PCR products were cloned into TA vector (Invitrogen). The inserts from individual white colonies were amplified and used as competitors in EMSA. DNA that competed in EMSA effectively was subjected to sequence analysis. The sequences of 37 independent clones were analyzed.

Transactivation Assay—CV-1 cells were plated in 6-well dishes containing minimal essential medium and 10% fetal bovine serum. The next day, cells were transfected in Opti-MEM (Life Technologies, Inc.) with the expression vector pSG5-VP16-RTR or pZeosSV-RTR and pBL-CT-CAT reporter DNA under the control of various response elements using lipofectamine (Life Technologies) as described (19, 23). The plasmid β-actin-LUC was used as an internal control to correct for differences in transfection efficiency. The reporter constructs were used as (conRTRE)0-CAT, (1/2-RTRE)-CAT, (P2-RTRE)-CAT, and (conRTRE)-CAT. Cells were collected 48 h after transfection, and CAT activity was determined with an enzyme-linked immunosorbent assay kit (Boehringer Mannheim) following the manufacturer’s instructions.

RESULTS

Identification of the Consensus DNA Binding Sequence for RTR—The amino acid sequence in the C-terminal region of the first zinc finger of nuclear receptors is key in classifying their DNA binding specificity (11, 12, 14). In RTR, this sequence is CEGCGKG, suggesting that RTR, like the retinoid, thyroid hormone, vitamin D, and peroxisome proliferator activating receptors, recognizes response elements containing the consensus core motif AGGTCA (2, 14, 16). On this basis, we screened a series of well characterized natural and synthetic response elements that contain the consensus core motif RGGTCA configured either as a direct repeat, palindrome, or inverted palindrome spaced by different numbers of nucleotides for RTR binding. These results showed that RTR was able to bind to a DR0 sequence (not shown).

To determine in an unbiased manner the consensus sequence of the response element that binds RTR with high affinity (RTR), we used a DNA-binding site selection strategy that is based on a combination of PCR and EMSA. A mixture of 70-bp oligonucleotides containing 30 random nucleotides flanked by two 20-bp primers was used as template (see “Experimental Procedures”; Fig. 1A). After four rounds of selection with in vitro synthesized RTR protein, a strong radiolabeled band consisting of RTR-oligonucleotide complexes was observed in EMSA. The PCR products generated after the fourth selection were cloned into the TA vector, and the sequence of 37 independent clones was analyzed as described under “Experimental Procedures” (Fig. 1A). From these sequences the percentage of A, G, T, and C at each position was calculated, and the RTRE consensus sequence was derived (Fig. 1B). These data demonstrated that RTR bound most effectively to a DR0 consisting of a direct repeat of the core motif AGGTTC/CTCA. In addition, these results showed that a DR0 preceded by TCA (−9 to −7 position) was preferred for high affinity binding, indicating the 5′-flanking region rather than the 3′-flanking region is important for optimal binding of RTR. The −10 position did not reveal a strong requirement for a particular nucleotide. In a second DNA binding site selection experiment, a mixture of 85-bp oligomers consisting of 5'-CGCCGGATCCTGACAGGAGN6-3' as used as template. This experiment yielded the consensus sequence NTTCAGCGTCAGGCTCAATCATCAGTCATCATCATAAT (conRTRE) (Fig. 2A) and analyzed its binding to RTR in EMSAs. As shown in Fig. 2B, in vitro translated RTR formed a complex with conRTRE, while increasing concentrations of the unlabeled conRTRE competed effectively for the binding. Since conRTRE consists of two overlapping TCAAG-
GTCA motifs, we next determined whether RTR was able to bind to a single core motif (M3-RTRE) consisting of the conRTRE in which the upstream half-site was destroyed by mutating three different nucleotides (Fig. 2A). Although this single core motif RTRE did compete with 32P-conRTRE for RTR binding, it was not as effective as the conRTRE (Fig. 2B). These observations suggest that RTR can bind well to an RTRE containing one core motif but does so with a lower affinity than to an RTRE consisting of a DR0. A DR1-RTRE, identical to conRTRE except that the two core motifs are separated by one nucleotide and a P0-RTRE containing a palindromic repeat of the core motif (Fig. 2A), also did not compete as well for binding as the conRTRE (Fig. 2B). It is likely that the binding of RTR to DR1-RTRE and P0-RTRE takes place through interaction with the single motif TCAAGGTCA present in these elements.

The apparent lower affinity of M3-RTRE, DR1-RTRE, and P0-RTRE for RTR is in agreement with the results obtained in Fig. 1, demonstrating that a DR0 is most optimal for RTR binding. In addition, these results suggest that RTR very likely binds to DR0 as a homodimer and as a monomer to the single motif TCAAGGTCA.

The hypothesis that RTR binds as a homodimer to conRTRE was further investigated by analyzing the binding of the full-length RTR and RTR406, a truncated form of RTR. EMSA with either RTR or RTR406 yielded one single shifted band that migrated at different positions. We then performed EMSA using a mixture of RTR and RTR406. As can be observed in Fig. 3, EMSA analysis of the binding of RTR and RTR406 to 32P-conRTRE yielded three shifted bands that would be consistent with the formation of an RTR homodimer, an RTR-RTR406 heterodimer, and an RTR406 homodimer. When EMSA was carried out with 32P-M3-RTRE, which binds RTR or RTR406 as a monomer, only two shifted bands were observed. These findings support the interpretation that RTR binds to conRTRE as a homodimer.

Mutational Analysis of RTRE—As shown in Fig. 2B, RTR is able to bind to a single core motif (M3-RTRE) consisting of the conRTRE in which the upstream half-site was destroyed by mutating three different nucleotides (Fig. 2A). Although this single core motif RTRE did compete with 32P-conRTRE for RTR binding, it was not as effective as the conRTRE (Fig. 2B). These observations suggest that RTR can bind well to an RTRE containing one core motif but does so with a lower affinity than to an RTRE consisting of a DR0. A DR1-RTRE, identical to conRTRE except that the two core motifs are separated by one nucleotide and a P0-RTRE containing a palindromic repeat of the core motif (Fig. 2A), also did not compete as well for binding as the conRTRE (Fig. 2B). It is likely that the binding of RTR to DR1-RTRE and P0-RTRE takes place through interaction with the single motif TCAAGGTCA present in these elements. The apparent lower affinity of M3-RTRE, DR1-RTRE, and P0-RTRE for RTR is in agreement with the results obtained in Fig.
RTRE in the regulatory region of the mouse protamine 2 gene between nucleotides −213 and −228 (30) (Fig. 6A). This RTRE, referred to as P2-RE, consists of a DR0 and is almost identical to the conRTRE (Fig. 6A). Northern analysis using poly(A)+ RNA isolated from mouse round spermatids confirmed that the RTRE and protamine 2 genes are highly expressed at this stage of spermatogenesis (not shown).

The putative P2-RE was synthesized and analyzed for RTR binding in EMSA. As shown in Fig. 6B, P2-RE could compete with 32P-conRTRE for RTR binding almost as well as the unlabeled conRTRE itself. We then performed EMSA with 32P-P2-RE in the absence or presence of a 5-, 25-, and 100-fold excess of unlabeled P2-RE or P2-mRE, which is mutated at two positions (Fig. 6A). These experiments demonstrated that 32P-P2-RE forms a complex with RTR (Fig. 6C, lane 2) and that unlabeled P2-RE competed effectively with this binding (lanes 3–5), while P2-mRE had a greatly reduced ability to compete with 32P-P2-RE for RTR binding (Fig. 6C, lanes 6–8). These results show that the P2-RE present in the promoter regulatory region of the protamine 2 gene can function as a high affinity binding site for RTR and as such could play a role in the regulation of the expression of this gene.

Specific RTRE Binding of RTR in Testis Nuclear Extracts—Since RTR is highly expressed in testis, we examined whether proteins in nuclear extracts from testis were able to bind to the P2-RE. When testis nuclear extracts were incubated with 32P-P2-RE and analyzed by EMSA, multiple DNA-protein complexes were observed (Fig. 7). This is not surprising, since a number of other nuclear receptors, including SF-1/FTZ-F1 (24–26), are expressed in the testis and able to bind to the same response element. For example, SF-1/FTZ-F1 has been reported to bind TCAAGGTCA (24), which is contained in the P2-RE. We used the RTR-Ab2 antiserum to determine if any of these DNA-protein complexes contained the RTR receptor and were supershifted by the antiserum. As shown in Fig. 7A, the RTR-Ab2 caused a supershift of one of the DNA-protein complexes (lane 2). To determine the specificity of the interaction of nuclear proteins with 32P-P2-RE, we analyzed nuclear extracts from mouse liver, kidney, and brain that do not express detectable levels of RTR (27, 28) in a supershift assay. In contrast to nuclear extracts from testis, no supershift was observed with nuclear extracts from brain (lanes 3 and 4), kidney (lanes 5 and 6), and liver (lanes 7 and 8) even at longer exposures (not shown). These findings show that the presence of RTR demonstrated by the RTR-Ab2-induced supershift in EMSA correlates well with the tissue-specific expression pattern of RTR.

The expression of RTR is developmentally regulated. RTR is induced when germ cells differentiate into spermatids, a process that in mice is initiated at day 22 after birth. We therefore compared the binding of proteins of nuclear extracts isolated from testes of juvenile (17-day-old) and adult (3-month-old) mice in a supershift assay (Fig. 7B). In contrast to the nuclear extracts from testes of adult mice, no supershift was observed with RTR-Ab2 using nuclear extracts from testes of juvenile mice. These results are in agreement with the observed development-dependent expression of RTR.

The specificity of the supershift was further examined with preimmune serum and competition with unlabeled P2-RE (Fig. 7C). No supershift was noted with the preimmune serum (lane 3). The DNA-protein interactions and supershift were effectively displaced by a 100-fold excess of unlabeled P2-RE, which competed for the binding of almost all the bound protein and abolished the supershift (lanes 4 and 5).

2 Z. H. Yan, A. Medvedev, T. Hirose, H. Gotoh, and A. M. Jetten, unpublished observations.

3 Y.-L. Zhang, D. O’Brien, and A. M. Jetten, unpublished observations.
Transcriptional Activation through RTRE and RTR—To examine the transactivation activity of RTR, an RTR expression plasmid and (conRTRE)3-CAT reporter DNA were cotransfected into CV-1 cells and assayed for CAT activity. No increase in CAT reporter gene activity could be measured compared with cells transfected with (conRTRE)3-CAT reporter DNA alone (Fig. 8A). One of the reasons for the lack of transcriptional activity may be the absence of the proper ligand required for the activation of RTR. To study the interaction of RTR with RTRE in cells, we examined the ability of VP16-RTR, which encodes a fusion protein consisting of the activating domain of VP16 and the full coding region of RTR, to cause transactivation of CAT through (conRTRE)3. As shown in Fig. 8A, pSG5-VP16-RTR enhanced CAT activity about 5-fold, while no activation was observed in cells cotransfected with pSG5-VP16. We then analyzed the transactivation through several other response elements. Transactivation through (P2-RE)3 was just as effective as that through (conRTRE)3, while transactivation through (1/2-RTRE)3 and (conRTRE)1 was greatly diminished (Fig. 8B). The latter may be due to the possibility that their orientation is not the most optimal for promoting interactions with the protein TFIID complex at the TATA-box. These observations support the hypothesis that binding of RTR to RTREs, including conRTRE and P2-RE, can mediate changes in transcriptional activation of target genes.

DISCUSSION

Initial studies have indicated that GCNF/RTR can bind effectively to a response element consisting of a direct repeat of sequences of 1/2-RTRE and mutated 1/2-RTREs. Arrows indicate the site of mutation. B–D, EMSA analysis of in vitro translated RTR using 32P-1/2-RTRE as a probe and unlabeled 1/2-RTRE and mutated 1/2-RTREs as competitors. Unlabeled oligonucleotides were used at 1-5, 100-fold excess (lanes 2, 3, and 4, and 5, etc., respectively). Lane 1, unprogrammed lysate was used. Lane 2, EMSA without competitors.
the core motif RGGTCA (28). In this study, we characterized in an unbiased manner the consensus sequence of the RTRE by an EMSA/PCR-based strategy that selects for oligonucleotides with highest affinity for RTR from a pool of degenerate oligonucleotides (primer 1-N30-primer 2). The conRTRE, TCAAGGTTCA, is necessary for optimal binding of RTR. The observed differences in the affinity of RTR to conRTRE and 1/2-RTRE are consistent with the hypothesis that RTR binds as a homodimer to conRTRE and as a monomer to 1/2-RTRE. EMSA analysis using the full-length RTR and a truncated form of RTR supports this concept. As expected for dimer formation, this EMSA with 32P-conRTRE yielded three shifted bands representing the homodimers (RTR)₂ and (RTR₄₀₆)₂ and the heterodimer RTR-RTR₄₀₆. These results support the preliminary observations on the GCNF/RTR homodimerization (28).

Several other members of the steroid hormone superfamily have been reported to bind as monomers to single core motifs (21, 24–26). Members of the ROR/RZR family have been shown to bind to a single core motif flanked at the 5′-end by a 6-bp-long A/T-rich sequence (21, 23). The sequence of the consensus half-site RTRE is identical to that reported for the SF-1/FTZ-F1 receptor (24–26). Interestingly, comparison of the sequences in the T- and A-box of RTR with those of SF-1/FTZ (FTZ-F1 box) shows the presence of a highly homologous region in which 8 of 11 amino acids are identical (26, 27). Since the FTZ-F1 box has been implicated in the recognition of the SF-1/FTZ response elements, this similarity is consistent with observations showing that RTR and SF-1 bind to very similar response elements.

To determine the ability of RTR to transactivate a CAT reporter gene that is under the regulation of an RTRE, CV-1 cells were transfected with an RTR expression plasmid and (RTRE)₃-CAT. No transactivation of the CAT reporter gene was observed. However, the fusion protein RTR-VP16, consisting of the full-length RTR fused to the 80-amino acid-long activating domain of VP16, was able to cause RTRE-dependent transactivation of CAT. These results demonstrate that the fusion protein was able to bind to the RTRE through RTR and to cause transactivation of the reporter via the activation domain of VP16. The lack of transactivation by RTR could be attributed to several factors. First, the activation of RTR may require the presence of its ligand, which has not yet been identified. Alternatively, the presence of a ligand in the serum could repress the transactivation activity of RTR. We have found that neither the addition of lipid extracts isolated from testes nor the presence of delipidized serum had any effect on the ability of RTR to cause RTRE-dependent transactivation. Second, transactivation by nuclear receptors is mediated...
FIG. 8. Transcriptional activation by RTR through RTREs. A. Ability of RTR and RTR-VP16 fusion protein to induce (RTRE),-dependent transactivation of the CAT reporter gene. CV-1 cells were cotransfected with (conRTRE),-CAT (solid bars) or pBLCA75 (open bars) and expression vectors encoding RTR, VP16-RTR, or VP16 using lipofectamine. β-Actin-LUC DNA was cotransfected and served as an internal control. The relative CAT activity was calculated and plotted. B, transactivation of the CAT reporter by RTR-VP16 through various response elements. Experiments were carried out as described above with tk-CAT under the control of the following response elements: (conRTRE),, (1/2-RTRE),, (P2-RE), or (conRTRE),. z indicates co-transfection with or without pSG-VP16-RTR. Results shown are representative of three independent experiments.

through interactions with other nuclear proteins that function either as repressors or as co-activators (18, 37). Some of these interactions may exhibit a high specificity. It is therefore possible that RTR needs a co-activator that is highly testis-specific and absent in CV-1 cells. Another possibility is that RTR functions as a negative transcriptional regulator.

Previous studies reported that the orphan receptor GCNF/RTR is predominantly expressed in the testis and induced during a specific stage of spermatogenesis (27, 28). The highest level of GCNF/RTR mRNA was detected in late round spermatids (27),2 suggesting that GCNF/RTR regulates gene expression during maturation of round spermatids to elongated spermatids. This stage of differentiation is accompanied by an induction in the expression of many genes, including protamine 1 and 2, c-abl, transition nuclear proteins 1 and 2, c-pim-1, hsp-70.1 and histone 2b (29, 36). To identify candidate target genes that are regulated by RTR, the reported promoter regions of several of these genes were examined for the presence of RTREs. The 5′ promoter-flanking region of the mouse protamine 2 gene (30) was found to contain the sequence AGAAGT-TCAAGGTTCAT, referred to as P2-RE, between nucleotides −213 and −228. This sequence is highly homologous to the conRTRE. We demonstrate by EMSA analysis that RTR is able to bind to this element almost as effectively as to the conRTRE. EMSA analysis of nuclear extracts isolated from testis, brain, liver, and kidney demonstrated that the RTR-specific antisera was able to cause supershift of an RTR-DNA complex only with extracts from testis. We have demonstrated3 that the expression of RTR is initiated at day 17 after birth at a time that mouse germ cells differentiate into spermatocytes and that it is highly expressed at day 22 when spermatids appear. The RTR-specific antisera does not cause a supershift with nuclear extracts from testes of juvenile (17-day-old) mice in contrast to nuclear extracts from testes of adult (3-month-old) mice. These findings indicate that the observed supershift by the RTR-specific antibody correlates well with the tissue-specific and development-dependent expression of RTR. Comparison of this sequence between the mouse and rat protamine 2 gene shows that the mouse gene contains two core motifs (P2-RE) but that the rat gene contains only a single core motif. It is interesting to note that protamine 2 levels in rat testis have been reported to be only 5% of that in mouse (38). One could speculate that this may be related at least in part to the differences in the affinity of conRTRE and 1/2-RTRE for RTR.

In summary, in this study we provide evidence consistent with the conclusion that the orphan receptor GCNF/RTR is able to bind as a homodimer to a DR0 and as a monomer to a 1/2-RTRE. The sequence preceding the core motif is important for determining the affinity of GCNF/RTR to RTREs and consists of the consensus TCA. GCNF/RTR is unable to cause transactivation of a reporter gene through the conRTRE in CV-1 cells. This may be due to either the lack of its ligand or a proper co-activator. Alternatively, GCNF/RTR may function as a negative regulator of transcription. An RTRE was identified in the 5′ promoter-flanking region of the protamine 2 gene that is induced during the same stage of spermatogenesis as GCNF/ RTR. This gene may belong to a subset of genes that are regulated by GCNF/RTR. Studies are in progress to obtain further evidence in support of this hypothesis.

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REFERENCES
1. De Luca, L. M. (1999) FASEB J. 5, 2924–2933
2. Giguere, V. (1994) Endocr. Rev. 15, 61–79
3. Kangas, P., Mark, M., and Chambon, P. (1995) Cell 83, 859–869
4. Qui, Y., Tsai, S. Y., and Tsai, M.-J. (1993) Trends Endocrinol. Metab. 5, 234–239
5. Tastone, P., Hu, E., and Spiegelman, B. M. (1994) Cell 79, 1147–1156
6. Evans, R. M. (1988) Science 240, 889–895
7. Hirose, T., Fujimoto, W., Yamaai, T., Kim, K. H., Matsuura, H., and Jettten, A. M. (1994) Mol. Endocrinol. 8, 1667–1680
8. Lautest, V., Hanni, C., Coll, J., Catzeflis, F., and Stehelin, D. (1992) EMBO J. 11, 1003–1013
9. Ribeiro, R. C. J., Kuscher, P. J., and Baxter, J. D. (1995) Annu. Rev. Med. 46, 443–453
10. Carlstedt-Duke, J., Stromstedt, P.-E., Wrangle, O., Bergman, T., Gustafsson, J.-A., and Jornvall, H. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 4437–4440
11. Kawasaki, S., Leen, J. A., White, R., and Parker, M. G. (1999) Cell 90, 853–962
12. Freedman, L. P. (1992) Endocrinol. Rev. 13, 129–145
13. Tastone, P., Lora, L., Fromental, C., Scheer, E., and Chamoun, P. (1990) Cell 62, 1177–1187
14. Glass, C. K. (1994) Endocr. Rev. 15, 391–407
15. Gronemeyer, H. (1991) Annu. Rev. Genet. 25, 89–123
16. Truss, M., and Beato, M. (1993) Endocr. Rev. 14, 459–479
17. Nagai, S., Saunders, M., Kastner, P., Durand, B., Nakshatri, H., and Chamoun, P. (1992) Cell 70, 1007–1019
18. Horlén, A. J., Naar, A. M., Hinezil, T., Torchia, J., Glass, B., Kurokawa, R., Ryan, A., Kamei, Y., Nosterstrom, M., Glass, C. K., and Rosenfeld, M. G. (1995) Nature 377, 397–404
19. Hirose, T., Apfel, R., Pfahl, M., and Jettten, A. M. (1995) Biochem. Biophys. Res. Commun. 211, 83–91
20. Mangelsdorf, D. J., and Evans, R. M. (1995) Cell 83, 841–850
21. Giguere, V., Iino, M., Flock, G., Ong, E., Evans, R. M., and Otsukataki, G. (1994) Genes & Dev. 8, 538–553
22. Hirose, T., Smith, R. J., and Jettten, A. M. (1994) Biochem. Biophys. Res.
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