Isolation and Functional Analysis of the Mouse RXRγ1 Gene Promoter in Anterior Pituitary Cells*

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The retinoid X receptor (RXR) isoform RXRγ has limited tissue expression, including brain, skeletal muscle, and anterior pituitary gland. Within the anterior pituitary gland, RXRγ expression is limited primarily to the thyrotropes. In this report, we have isolated ~3 kb of 5'-flanking DNA of the mouse RXRγ1 gene. We have identified the major transcription start site in the thyrotrope-derived TtT-97 cells. Transient transfection studies show that a 1.4-kb promoter fragment has full promoter activity in TtT-97 cells. This promoter has much less activity in thyrotrope-derived aTSH cells, pituitary-derived GH3 somatomammotropes, and non-pituitary CV-1 cells. None of these cell lines has detectable RXRγ1 mRNA. A previous report has identified a non-consensus direct repeat (DR-1) element in the RXRγ2 gene promoter region that mediates stimulation of promoter activity by 9-cis-retinoic acid (9-cis-RA). Inspection of the RXRγ1 promoter region revealed a non-consensus DR-1 element at ~232 bp from the transcription start site. Interestingly, RXRγ1 promoter activity was suppressed 50% by 9-cis-RA in the TtT-97 thyrotropes. Further experiments in non-pituitary cells showed that suppression of RXRγ1 promoter activity was RXR-dependent. Mutagenesis of the DR-1 element abrogated suppression of promoter activity by 9-cis-RA, suggesting that this negative regulation requires both RXR and this specific DR-1 element. In summary, we have isolated the mouse RXRγ1 gene promoter region and identified the major start site in thyrotropes. Promoter activity is uniquely suppressed by 9-cis-RA through a DR-1 element. Isolation and characterization of the mouse RXRγ1 promoter region provides a tool for further investigation focusing on thyrotrope-specific gene expression as well as negative regulation of genes by retinoic acid.

Tissue and cell-specific expression of many genes occurs at the level of gene transcription. Within the anterior pituitary gland, transcription factors including thyroid hormone receptor (TRβ2), Pit-1, GATA-2, ETS-1, and steroidogenic factor (SF-1) have expression limited to certain cell types, leading to specific cell phenotypes and hormone production (1–7). The retinoid X receptor (RXR)1 has three distinct isoforms encoded on separate genes. The RXRa and RXRβ isoforms are widely distributed throughout the developing embryo and in the adult, while the RXRγ isoform has restricted tissue distribution, which includes a high level of expression in the pituitary gland early in development (8). RXRγ has two isoforms, RXRγ1 and RXRγ2, which are generated from the same gene and differ at the N terminus (9). RXRγ1 and RXRγ2 gene expression is believed to be controlled by two separate regulatory regions. The RXRγ2 coding region is entirely contained within RXRγ1 which has an additional 123 amino acids on the N-terminal end (9). Our group and others (9, 10) have shown that the RXRγ1 subtype is expressed in the anterior pituitary and has expression that is restricted to thyrotropes. RXRγ1 is not required for thyrotrope development or basal expression of the TSHβ subunit promoter within the thyrotropes, but does appear to be required for vitamin A-mediated suppression of TSHβ promoter activity within thyrotropes seen both in vitro and in vivo (10, 11). The human RXRγ2 gene 5'-flanking region has been identified and contains a retinoid-responsive element, which confers positive regulation of promoter activity by retinoic acid (12). In this report, we have isolated the RXRγ1 promoter and begun to characterize its regulation in thyrotropes. An understanding of RXRγ1 promoter function in thyrotropes and other cell types will provide insight into mechanisms governing thyrotrope-specific gene expression and phenotype, as well as the generalized mechanism of cell-specific gene expression.

MATERIALS AND METHODS

cDNA and Genomic Library Screening—A cDNA λ phage library was constructed from mouse thyrotrope tumor (TtT-97) poly(A)+ RNA as described previously (13). The library was screened with a nick-translated 32P-labeled NotI cDNA fragment of the mouse RXRγ1 coding region (plasmid kindly provided by R. Evans). This was performed to obtain 5'-untranslated sequence of mouse RXRγ1. To obtain a large fragment of the 5'-flanking region of the RXRγ1 gene, a λ EMBL-3 SphiT7 mouse genomic library (CLONTECH Laboratories, Palo Alto, CA) was screened with a nick-translated 32P-labeled PCR product of the 5'-untranslated region of mouse RXRγ1.

Rapid Amplification of cDNA Ends (RACE)—5'-RACE was carried out using the CLONTECH Marathon cDNA Amplification kit (CLONTECH, Laboratories). TtT-97 poly(A)+ RNA was treated with reverse transcriptase using a 52-bp oligonucleotide with a poly(dT) end.

1 The abbreviations used are: RXR, retinoid X receptor; RACE, rapid amplification of cDNA ends; RT, reverse transcriptase; DR, direct repeat; 9-cis-RA, 9-cis-retinoic acid.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF525492.

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Following second strand synthesis, double-stranded cDNA was blunt-ended with T4 DNA polymerase I and ligated to an adapter primer supplied with the Marathon kit. Adapter ligated cDNAs were amplified by PCR using an antisense oligonucleotide corresponding to the translational start (complement of ATG in bold) of RXR/H9253 (5′/H11032-CCA TAC ATGTTGGCTGCTCAGTT-3′) and a sense oligonucleotide within the 5′ adapter (AP-1). PCR was carried out as follows: 94°C for 1 min, 30 cycles of denaturation at 94°C for 30 s, annealing at 65°C for 30 s, and extension at 68°C for 3 min, followed by extension at 72°C for 7 min using TaKara LA Taq polymerase (Roche Molecular Biochemicals).

Amplified products were size-separated on a 1.5% agarose gel, transferred to a Nytran membrane, and hybridized with a 20-bp oligonucleotide corresponding to a region just upstream of the translational start of RXR/H9253 to verify the size and specificity of the amplification. PCR products were excised the appropriate section of the gel, purified, and subcloned into the pCR2.1 vector (Invitrogen) for sequencing.

RNase Protection—A 431-bp NsiI/HindIII fragment of the RXR/H9253 5′-flanking region, purified from the 3.1-kb XhoI/HindIII 5′-flanking region in pGL2-basic, was subcloned into pGEM3zf (digested with PstI/HindIII). The resultant plasmid was linearized with BamHI, and a radiolabeled antisense RNA probe was generated using SP6 RNA polymerase and [α-32P]dUTP (800 Ci/mmol). A positive control RNA (unlabeled sense strand) was generated using a product from 5′-RACE subcloned into pGEM7zf and linearized with NcoI. RNA was generated using SP6 RNA polymerase, and the product was treated with RQ DNase I to eliminate contaminating DNA. The resultant product, when hybridized with the 431-bp radiolabeled antisense probe and digested with RNase, should generate a 177-bp protected product. The protection assay was carried out as outlined in the Ribonuclease Protection Assay Kit (Ambion). Briefly, 5 μg of TtT-97, TSH, or GH3 poly(A) RNA was precipitated with 3×10^6 cpm of antisense riboprobe. The pellets were resuspended, and hybridization of the probe and RNA was carried out at 42°C overnight. The annealed products were treated with a 1:5 dilution of RNase (Ambion) for 30 min at 37°C, precipitated, and the digested products were size-separated on a 5% denaturing polyacrylamide gel and exposed to radiographic film.

Reverse Transcription-PCR of RXRγ1 Untranslated Region—5 μg of TtT-97 poly(A)+ RNA was treated with avian myeloblastosis virus reverse transcriptase (Promega) and random hexamer oligonucleotides. Each DNA product from 0.5 μg of poly(A)+ RNA was subsequently used
for each PCR reaction with a common 3′ antisense oligonucleotide (5′-GGGATTCCGAGTGAGTGAAC-3′) corresponding to sequence in exon 2 of the RXRγ1 coding region and different 5′ sense oligonucleotides corresponding to regions upstream and downstream of the transcription start site identified by RNase protection analysis (+33/+50 (lane A), -146/+130 (lane B), -241/+227 (lane C), -437/-420 (lane D), Fig. 3). After 35 cycles (94°C for 1 min, 50°C for 1 min, 72°C for 1 min), products were size separated on an agarose gel, transferred to nitrocellulose, and hybridized with a radiolabeled oligonucleotide corresponding to a common region in all potential products (+64/+84). The 3.1-kb XhoI/NcoI DNA fragment of the RXRγ1 gene (Fig. 1A) was used as a positive control for each of the 5′ oligonucleotides with the common 3′ oligonucleotide (+64/+84, Fig. 1B).

Transfection Plasmids—The 3.1-kb XhoI/NcoI fragment of the RXRγ1 gene (−9290 to +207) was cloned into the pGL2-basic plasmid (Promega) at the Smal site by blunt ligation. A pGL1.4 reporter plasmid was generated by blunt ligation of the 1.4-kb SacI/NcoI fragment of the RXRγ1 gene (−1139 to +207) into the pGL2-basic plasmid at the Smal site. A SacI/PvuII fragment of the RXRγ1 gene (−1142 to +194) was cloned into the Smal site of the pα3luciferase (pα3luc) plasmid in both orientations by blunt ligation. A pGL556 reporter plasmid containing the −483 to +73 region of the RXRγ1 gene was generated by PCR and subcloned by blunt ligation into the pGL2-basic plasmid at the Smal site. A pGL556/R1mut reporter plasmid was generated in a similar fashion, except the DR-1 element was replaced with an XhoI site by ligation of two separate PCR products. An RXRα expression plasmid was generated by ligation of the coding region of mRXRα into the pW1 human β-actin promoter plasmid, a modified pGEM9zf− (Promega) vector with an actin promoter (14). All plasmid constructs were verified for ligation fidelity and orientation by sequencing.

Transient Transfection Studies—Transient transfection assays have been previously described (10). Briefly, 20 μg of the different RXRγ1 reporter fragments in pA3luc or pGL2 and 1 μg pCMV-β-galactosidase DNA as an internal control were transfected by electroporation into 7–10 million TtT-97 cells, 3 million αTSH, lane 2; GH3, lane 3; positive control, lane 4; undigested probe, lane 5.

RESULTS

Cloning of the 5′-Flanking Region of RXRγ1—A TtT-97 thyrotrope cDNA library was screened with a radiolabeled probe corresponding to the coding region of RXRγ1 to obtain additional 5′-untranslated sequence present in thyrotropes. A clone was obtained containing the entire coding sequence as well as both 5′- and 3′-untranslated sequence. The 5′-untranslated sequence corresponded to a sequence previously reported from a murine skeletal muscle cDNA library (9). To define the 5′ extent of the RXRγ1 mRNA in thyrotropes, 5′-RACE was performed on TtT-97 poly(A)+ RNA. PCR was performed on the cDNA population with a specific adapter on the 5′ end using an antisense oligonucleotide corresponding to sequence in exon 2 of RXRγ1 and a sense oligonucleotide corresponding to se-

Fig. 2. RNase protection analysis of TtT-97, αTSH, and GH3 RNA. 5 μg of each poly(A)+ RNA was subject to hybridization with the 431-bp radiolabeled riboprobe, followed by RNase digestion. TtT-97, lane 1; αTSH, lane 2; GH3, lane 3; positive control, lane 4; undigested probe, lane 5.

Fig. 3. RT-PCR of 5′-flanking region of RXRγ1 RNA from TtT-97 thyrotropes. A, 0.5 μg of reverse-transcribed poly(A)+ RNA was subject to PCR using a common 3′ antisense oligonucleotide corresponding to sequence in exon 2 of RXRγ1 and four different sense oligonucleotides surrounding the transcription start site: +33/+50 (lane A), −146/+130 (lane B), −241/+227 (lane C), −437/−420 (lane D). Separated products were hybridized with a radiolabeled oligonucleotide corresponding to a common region in all products. B, the 3.1-kb XhoI/NcoI fragment of the RXRγ1 gene (Fig. 1A) was used as a positive control for each of the 5′ oligonucleotides with the common 3′ oligonucleotide (+64/+84, Fig. 1B), generating smaller products than the RT-PCR products.

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Results are expressed as -fold promoter activity over luciferase reporter lacking a specific promoter (pA3Luc). Mean values and S.E. for eight separate experiments are shown.

Functional Analysis of the 5’-Flanking Region of RXRγ1—Gene transfer of the XhoI/NotI (3.1 kb) and SacI/NotI (1.4 kb) 5’-flanking fragments of RXRγ1 in pGL2basic was carried out by electroporation in TtT-97 thyrotropes. Both fragments had 350–450-fold higher luciferase activity compared with the promoterless pGL2basic vector (data not shown), suggesting that the SacI/NotI fragment contained all of the sequences necessary for full promoter activity.

To verify the 1.4-kb RXRγ1 fragment as a promoter region in thyrotropes, a −1133 to +207 (SacI/PvuII) fragment of the RXRγ1 5’-flanking region was subcloned into pA3Luc in both orientations. Gene transfer by transient transfection was carried out in TtT-97 thyrotropes, which showed promoter activity relative to the promoterless vector (Fig. 4). Reverse orientation was transfected into 7–10 million TtT-97 cells. Results are shown in Fig. 5. The largest major protected band was 208 bp using TtT-97 RNA (arrow, lane 1). No protected bands were detected using αTSH or GH3 RNA (lanes 2 and 3). Lane 4 shows the positive control RNA, which generated the predicted 177-bp protected product. Lane 5 shows the untreated 431-bp riboprobe. The transcription start site (+1) was mapped to a position 3 bp upstream of the largest 5’-RACE product, which results in 458 bp of 5’-untranslated sequence. A TAATA element is located at −36 bp relative to the transcription start site (Fig. 1B).

To confirm that no RXRγ RNA transcripts were derived from sequences upstream of the start site in thyrotropes, RT-PCR was performed using randomly primed TtT-97 mRNA. A common antisense oligonucleotide corresponding to sequence in exon 2 was used to generate PCR products across an exon-intron boundary to avoid genomic DNA contamination. Sense oligonucleotides were synthesized corresponding to +33/+50 (A), −146/−130 (B), −241/−227 (C), and −437/−420 (D). PCR products were size-separated and transferred to nitrocellulose for Southern blot analysis. Fig. 3A shows a strong signal using oligonucleotide A, while a faint signal was generated using oligonucleotide B. Oligonucleotides C and D generated no products. PCR of a DNA plasmid control revealed relatively similar amounts of PCR products using each of the sense oligonucleotides (Fig. 3B), suggesting that the major transcription start is between −146 and +33, but a minority of larger transcripts are generated, as can also be seen in the RNase protection analysis (Fig. 2).

Fig. 4. mRXRγ1 promoter activity in TtT-97 thyrotropes. Transient transfections were carried out as described under “Materials and Methods.” 20 μg of a luciferase reporter plasmid containing the SacI/PvuII fragment of the RXRγ1 promoter either in forward or reverse orientation was transfected into 7–10 million TtT-97 cells. Results are expressed as -fold promoter activity over luciferase reporter lacking a specific promoter (pA3Luc). Mean values and S.E. for eight separate experiments are shown.

Fig. 5. mRXRγ1 promoter activity in different pituitary-derived and non-pituitary cell types. Transient transfections were carried out as described under “Materials and Methods.” 20 μg of a luciferase reporter plasmid containing the SacI/PvuII fragment of the RXRγ1 promoter either in forward, or reverse orientation was transfected into 7–10 million TtT-97 cells, 3 million αTSH cells, 5 million GH3 cells, or 0.8 million CV-1 cells. Results are expressed as -fold promoter activity in the forward orientation over promoter activity in the reverse orientation. Mean values and S.E. for at least six separate experiments are shown.

Fig. 6. Quantitative RT-PCR analysis of RNA from mouse tissue and TtT-97 thyrotropes. Total RNA was extracted from individual mouse pituitaries and subject to quantitative RT-PCR analysis as described under “Materials and Methods.” Results are from at least four separate mouse tissue samples. Measurements of skeletal muscle are from a single mouse. Error bars correspond to S.E. mRNA levels of RXRγ isotypes are expressed in pg/ng rRNA.
Adipose or thyroid tissue. RXR

Transient transfections were carried out as described under "Materials and Methods." 20 μg of a luciferase reporter plasmid containing the SacI/PvuII fragment of the RXRγ1 promoter was transfected into 7–10 million TtT-97 cells. Cells were then incubated in media for 16 h containing vehicle alone (ethanol) or increasing amounts of 9-cis-retinoic acid. Results are expressed as percent promoter activity as compared with no 9-cis-retinoic acid. Mean values and S.E. for four separate transfections are shown.

αTSH, GH3, and CV-1 cells, which either lack or have very low levels of RXRγ1 mRNA. Promoter activity was higher than the promoterless vector in all cell types tested; however, relative luciferase activity (forward compared with reverse) was greater in the TtT-97 thyrotropes compared with these other cell types (Fig. 5), suggesting cell-specific activity of the RXRγ1 promoter.

Expression of RXRγ1 and RXRγ2 mRNA in Different Murine Tissues and TtT-97 Thyrotropes—RXRγ1 promoter activity and mRNA expression are low in GH3 and αTSH cells, but RXRγ2 mRNA is detectable in these tissues (10). One hypothesis is that RXRγ2 down-regulates RXRγ1 expression in tissues and cell lines. To test this, we measured RXRγ1 and RXRγ2 mRNA expression in TtT-97 thyrotropes and a variety of mouse tissues. Fig. 6 shows that RXRγ1 and RXRγ2 are both highly expressed in the TtT-97 thyrotropes, suggesting that endogenous RXRγ2 does not inhibit the expression of RXRγ1. RXRγ1 is also expressed in skeletal muscle and brain, which confirms previous data using Northern blot analysis (9). These data also show that RXRγ1 is expressed in mouse pituitary, but not adipose or thyroid tissue. RXRγ2 is expressed in skeletal muscle, heart, and liver, which also confirms previous studies (9).

Regulation of RXRγ1 Promoter Activity—Activity of the RXRγ2 promoter is stimulated by 9-cis-RA through an imperfect DR-1 site (12). Sequence examination of the RXRγ1 promoter revealed a putative DR-1 element at position −232 (Fig. 1B). We therefore examined the effects of 9-cis-RA on the RXRγ1 promoter in thyrotropes. Fig. 7 shows that 9-cis-RA suppressed promoter activity in a dose-dependent manner in thyrotropes, suggesting opposite regulation of the two RXRγ gene promoters by retinoic acid. To examine the effect of retinoic acid on the RXRγ1 promoter in non-pituitary cells, similar experiments were carried out in mouse embryonal carcinoma cells (P19). 9-cis-RA also suppressed RXRγ1 promoter activity in these cells in an RXR-dependent manner as shown by the addition of increasing amounts of an RXRγ expression vector (Fig. 8A). An RXRγ1 promoter fragment with a mutation in the DR-1 element was generated by PCR as described under "Materials and Methods." A promoter fragment with the wild-type DR-1 element retained suppression of activity with 9-cis-RA (Fig. 8B), while mutagenesis of DR-1 in this promoter construct lost suppression of activity with 9-cis-RA.

Discussion

In this report, we have isolated the mouse RXRγ1 5′-flanking region and begun characterization of promoter activity in anterior pituitary cells. RXRa and RXRβ are widely expressed throughout development and in the adult, while RXRγ has a more limited tissue distribution. In development, RXRγ is expressed primarily in the brain, developing skeletal muscle, and anterior pituitary gland (8). Within the anterior pituitary, RXRγ mRNA and protein are limited to the thyrotropes in mice, rats, and humans (9, 10, 18–20). Studies from our group and others (10, 17, 21) have shown that vitamin A and retinoid derivatives suppress production of TSH and activity of the TSHβ promoter both in vitro and in vivo. The in vitro activity of retinoids appears to require RXRγ.

Thyrotropes express a number of genes with limited tissue distribution. The TSHβ subunit is expressed only in the thyro-
tropes, with the exception of a few reports showing limited expression in the enterocytes and pars intermedia (22). Mechanisms governing this limited expression appear to include gene activation by two transcription factors, Pit-1 and GATA-2, both of which also have limited tissue distribution (4). Interestingly, two other pituitary-restricted hormone genes, growth hormone and prolactin, also require Pit-1 for gene expression, but appear to use different partners interacting with a composite element for unique cell-type expression. The growth hormone promoter contains a binding site for the transcription factor Zn-15, which is flanked by two Pit-1 binding sites (23, 24). In contrast, the prolactin promoter contains a composite Pit-1/ETS-1 binding site believed to direct unique expression of this gene in lactotropes (5). Inspection of the RXRα promoter region does not reveal any consensus Pit-1/GATA-2 elements. This would suggest that expression of this gene in thyrotropes is through a nonconsensus composite Pit-1/GATA-2 elements, or expression of RXRα in thyrotropes is through a different mechanism than TSHβ subunit expression.

Two subtypes of RXRγ (γ1 and γ2) are generated from the RXRγ gene locus. These two subtypes appear to be under control of separate regulatory regions. The RXRγ2 protein is 123 amino acids shorter than RXRγ1 on the N-terminal end, but the remaining 340 amino acids, including the DNA binding and ligand binding domains, are identical. RXRγ2 appears to be generated from a separate promoter and contains a unique 5′ exon and 5′-untranslated region (9). The human RXRγ2 promoter has been isolated, but cell-specific expression of this promoter has not been examined (12). We have previously shown that RXRγ1 mRNA is highly expressed in mouse pituitaries, while RXRγ2 is expressed at low levels (11). We show here that the mouse RXRγ1 promoter exhibits high activity in thyrotrope-derived TtT-97 cells, which express RXRγ1 mRNA, but relatively low activity in pituitary-derived cell types (αTSH, GH3), which lack detectable RXRγ1 mRNA (10). These data suggest that the regulation of RXRα1 gene expression is influenced, at least in part, by tissue-specific regulation in the −1142 to +194 region of the RXRα1 promoter.

Analysis of the RXRγ2 human gene promoter reveals an imperfect retinoid X response element (GGTGGAAAGGTCT) immediately upstream of the transcription start site (12). The authors further show that this element is required for retinoid acid stimulation of promoter activity, which is believed to mediate RXR-dependent transcriptional autoactivation. This DR-1 element behaves like a classic RXRE in the RXRγ2 promoter. Examination of the mouse RXRγ1 promoter reveals a similar imperfect DR-1 element (CTTTCACAGATCA) at position −220 to −232. In contrast to the RXRγ2 promoter, our studies show that RXRγ1 promoter activity is down-regulated by retinoic acid in the thyrotrope-derived TtT-97 cells. One could argue that this suppression of promoter activity is thyrrotrop-specific, but we also observed this effect in the non-pituitary P19 cells, suggesting a generalized mechanism of negative regulation of this promoter/gene by retinoic acid. Regulation of the DR-1 element blocked negative regulation of RXRγ1 promoter activity by 9-cis-RA confirming this DR-1 element as necessary for negative regulation by retinoic acid.

The mouse RXRβ gene has been isolated and the promoter region has been studied (25–27). Like RXRγ, there appears to be two different isotopes, RXRβ1 and RXRβ2, which have unique regulatory regions. Examination of a limited region of the RXRβ1 promoter did not reveal a classical DR-1 element. Transient transfection studies using the RXRβ1 promoter in GH3 cells showed no regulatory response of promoter activity by treatment with 9-cis-RA (26). Both RXRγ1 and RXRγ2 promoter regions contain a DR-1 element, and both promoter activities are regulated by 9-cis-RA, albeit in different directions. These data would suggest that RXRγ1 not only has unique expression in a limited number of cell types, but unique regulation by retinoic acid in comparison to other retinoid X receptors.

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