Identification of a cDNA Encoding a Retinoid X Receptor Homologue from Schistosoma mansoni

EVIDENCE FOR A ROLE IN FEMALE-SPECIFIC GENE EXPRESSION

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Schistosoma mansoni, a multicellular eukaryotic blood fluke, is a major cause of morbidity worldwide in humans. The study of female parasite growth, development, and gene regulation is important because the eggs produced are responsible for the pathogenesis observed in schistosomiasis. p14, an eggshell precursor gene expressed only in sexually mature females in response to a male stimulus, is a model for female-specific gene regulation. The upstream region of the p14 gene shares sequences present in insect genes known to be regulated in a sex-, temporal-, and tissue-specific manner by members of the steroid receptor superfamily. Herein, we report the identification and characterization of a cDNA that encodes the S. mansoni (Sm) RXR homologue. Sequence analysis predicts and Western blot analysis confirms the synthesis of a 74-kDa protein, the largest member of the RXR family reported to date. We show by electrophoretic mobility shift assay analysis that SmRXR binds to cis-elements of the p14 gene including a direct repeat that follows the “3-4-5” rule of binding elements recognized by members of the steroid receptor superfamily. Furthermore, we demonstrate that SmRXR can act as a transcription activator in the yeast one-hybrid system. Through quantitative reverse transcriptase-polymerase chain reaction, we show that the SmRXR gene is constitutively expressed and thus must play multiple roles throughout the schistosome life cycle.

Schistosomiasis is a major public health problem afflicting 300 million people in 76 countries (1). Egg production is not only responsible for dissemination of the parasite but also for pathology of the disease in humans. Therefore, studies of female schistosome reproductive development and egg production may define targets useful in disease control. Interestingly, female reproductive development is regulated intimately by a stimulus from the male parasite (2). For many years it has been known that female schistosomes from single sex infections are stunted in size and sexually immature (2). This observation indicates that schistosomes have an intriguing developmental system requiring signaling from the male schistosome leading to direct or indirect activation of a number of female-specific genes (2–6). One gene, p14, encodes an eggshell precursor expressed only in vitelline cells of mature females in response to male stimulus (3, 7, 8). The region upstream of transcription initiation of p14 contains elements similar to those found in Drosophila and silkmoths that are known to regulate chorion gene expression in a sex-, tissue-, and temporal-specific manner (2, 7, 9, 10).

Ultraspiracle protein, a member of the RZR subfamily of the steroid receptor superfamily, binds to the s15 chorion gene of Drosophila recognizing an imperfect palindrome containing the core sequence TCACGT (11, 12). Members of the steroid receptor superfamily are likely to play a role in female-specific gene regulation of schistosomes. A similar sequence is present as part of an imperfect palindrome in the upstream region of p14 (2) (Fig. 1). Furthermore, a direct repeat that follows the “3-4-5” rule of steroid response elements is present in the p14 upstream region (13, 14). This latter sequence consists of two identical half-sites, AACTATCA, spaced by five nucleotides (Fig. 1). Presence of these cis-elements in the p14 gene led to a search for schistosome homologues of steroid hormone receptors. Herein, we report the identification and characterization of Schistosoma mansoni RXR cDNA (SmRXR), and we provide evidence for a possible role in regulation of female-specific gene expression.

EXPERIMENTAL PROCEDURES

Identification of SmRXR cDNA—Degenerate oligonucleotides representing the conserved regions of RXR genes with the amino acid sequences, CEGCKGFF (aa 288–285) and GMKKEAVQEE (aa 335–344), were synthesized for use in PCR (Fig. 2). Using a cDNA pool derived from schistosome worm pair mRNA as a template and the RXR gene-specific primers, a 170-base pair fragment was amplified. This PCR product was cloned into TOPO TA vector (Invitrogen), sequenced, and shown to be related to RXRs by NCBI Blast search analysis. The DNA fragment homologous to RXRs was radiolabeled with [α-32P]dCTP by random priming and used as a probe to screen a female adult worm ZAP cDNA library. Seven positive clones were identified, the phagemids were excised, and then the cDNA inserts were shown to be identical in sequence. Amino acid comparison of SmRXR to Drosophila USP and human RXRs was performed using the Pile-Up program from the Wisconsin Package version 9. Phylogeny trees were constructed using the PAUP Search and PAUP display program from the Wisconsin Package version 9. The following parameters were used in construction of trees to confirm accuracy of the study: heuristic with parsimony, 1. The abbreviations used are: RXR, retinoid X receptor; Sm, Schistosoma mansoni; EMSA, electrophoretic mobility shift assays; aa, amino acids; USP, ultrapisacle protein; GST, glutathione S-transferase; Gal4-AD, Gal4 activation domain; SD, synthetic dropout; AT, 3-amino-1,2,4-triazole; mRXRg, mouse RXRg; hRXRg, human RXRg; RAR, retinoic acid receptor; RT-PCR, reverse transcriptase-polymerase chain reaction; bp, base pair.
were added to each reaction. After incubation at 37 °C for 1 h, 0.5 µl of reaction mix was used as template for PCR. SmRXR-specific oligonucleotides were synthesized for PCR: 5′ AACAGATCTCCTAATTTAAAC- CATA-COH (aa 434–441) as forward primer and 5′ GCAGTATGTC- AAAGCTAATCAGG-OH (aa 581–588) as reverse primer. α-Tubulin- specific primers (16) were used in each reaction as a constitutively expressed gene control. To remain in the linear range of PCR, 28 cycles were performed. The PCR products were size-separated on a 1.5% agarose gel, visualized by ethidium bromide staining, and quantified by the Molecular Analyst program, PC version 1.5 (Bio-Rad). The amount of SmRXR PCR product was compared within each reaction to α-tubulin product and then this value was compared among the developmental stages.

Construction of Yeast One-hybrid Target Reporter Strains—The Matchmaker One-hybrid System protocol (CLONTECH) was followed with a few exceptions. The target-reporter construct pHi-si-154, was made by inserting a 154-base pair DNA fragment, delineating nucleotides −220 to −60 of the p14 gene (Fig. 1), repeated 3 times upstream of the His3 promoter in the pHIS-i-1 vector. Target-reporter construct, plasmids were then transfected into yeast cells by electroporation (17). Yeast reporter strains were analyzed by Southern blot to ensure that proper integration of the target-reporter construct had occurred.

Assay of SmRXR Activity in Vivo, Yeast One-hybrid Assay—The competent reporter yeast strains were transformed with either pACT-2 activation domain vector (CLONTECH) containing the SmRXR gene downstream of the Gal4-AD (pAC-RXR), pACT-2 vector, pADH1 expression vector containing the SmRXR (pADH-RXR), or pADH1 vector. The pADH1 vector is a 2-µm expression vector with a multi-cloning site upstream of an ADH1 promoter. Control transformations without DNA were also performed. In a sterile 15-ml conical tube, 10 µg of plasmid DNA, 200 µg of sheared salmon sperm DNA, and 330 µl of competent reporter yeast strain yeast cells were added. After mixing the DNA and yeast, 2 ml of LiAc/polyethylene glycol solution (10 mM Tris, pH 7.5, 1 mM EDTA, pH 7.5, 0.1% LiAc, and 40% polyethylene glycol) were added, and the suspension was vortexed. The transformation suspension was incubated while shaking at 200 rpm at 30 °C for 30 min. After incubation, 230 µl of methanol was added and mixed by inverting the tube gently. Cells were incubated for 15 min at 42 °C and then placed on ice for 2 min. Cells were centrifuged at 10,000 × g for 10 min, and the supernatant was aspirated. The pellet was resuspended in 2.3 ml of TE buffer. From the control tubes that contained reporter strains lacking DNA, 10 µl of cells were diluted 1:100 in TE and 200 µl per plate were spread onto SD/−his media containing 0, 15, 30, 45, and 60 mM 3-amino-1,2,4-triazole (AT, a HIS3 competitor). From each tube containing yeast cells, 100 µl of yeast culture was spotted onto SD/−leu test transformation efficiency. All plasmids used contained the selection marker leu2 gene. From each of these tubes, 200 µl per plate were spread onto SD/−his−leu−trp plates. Yeast transformed with pGal4 were spread onto SD/−leu−trp plates. Yeast transformed with pGal4 were spread on SD/−leu−trp plates. After 1 week incubation at 30 °C, colonies were assayed for β-galactosidase activity using the CLONTECH protocol.

In Vitro Transcription-Translation of SmRXR—SmRXR cDNA was inserted into the pCITE vector (Novagen) forming pCITE-RXR and transcribed and translated using the Single Tube Protein System (Novagen) according to the manufacturer's instructions. Western blot analysis was performed after electrophoresis followed by autoradiography and then used immediately.

Analysis of SmRXR Activity in Vitro, EMSA—A 250-bp sequence of the p14 upstream region, nucleotides −300 to −50 (Fig. 1), was radiolabeled.
labeled by PCR amplification of the fragment using [32P]dCTP. Every 20-μl reaction contained ZnSO4 buffer (4 mM Tris, 80 mM NaCl, 0.5 mM ZnSO4, 5% glycerol, 0.5 mM dithiothreitol, and 1 mM EDTA), 5 μg of poly(dI-dC), 12% glycerol, and 5 fmol of radiolabeled p14 template (18). Protein-DNA binding reactions contained 1 μl of transcription-translation lysate. In cold template competition reactions, unlabeled p14 template (250-bp fragment) was added in 10 and 100 3 molar excess of the radiolabeled p14 probe. In competition reactions with nonspecific DNA, a 250-bp fragment cut from pET32a DNA (Novagen), was added in 10, 100, and 250 3 molar excess for competition reactions: 5'-TTGACATTTTTAACTATCACGCTCAACTATCAT-T-OH (direct repeat), 5'-TTTAATAATGATGCACTTAGTGAGGCACA-CTCTTC-OH (imperfect palindrome), and 5'-TTCTAACGGTAGAATAAAATAGTGAGTAATTCGTG-OH (Fig. 1). Binding reactions were incubated at 23 °C for 30 min and then loaded onto a 16-cm 4% acrylamide gel (6.75 mM Tris, pH 8, 1 mM EDTA, 3.3 mM sodium acetate, 0.05% bisacylamide). Electrophoresis was performed with a 6.75 mM Tris, pH 8, 1 mM EDTA and 3.3 mM sodium acetate running buffer at 4 °C.

**Fig. 2. Schematic of the functional domains of RXR subfamily members (top).** Comparison of the amino acid sequences of SmRXR, dUSP, and human RXRa (bottom). Amino acids of SmRXR (accession number AF094759) and dUSP (accession number P20153) that share identity to RXRa (accession number P19793) in the highly conserved functional domains are shown in gray. The solid box denotes the amino acids of the DNA binding domain. Arrows indicate the two zinc fingers of the DNA binding domain. The conserved amino acids of the hinge region are shown in dark gray. The amino acids selected for the synthesis of the oligonucleotides used in PCR to clone the 170-bp SmRXR fragment are underlined. The amino acids of the dimerization and ligand binding domain are boxed in dashed lines. SmRXR sub-domains, ti and tc, are located at the amino- and carboxyl terminus of the dimerization and ligand binding domain, amino acids 457–512 and 656–676, respectively. Solid boxes within the dimerization and ligand binding domain denote the heptad repeats. The difference in size of SmRXR from other RXR family members occurs at both the amino- and carboxyl-terminal ends of SmRXR by approximately 130–170 amino acids and 50 amino acids, respectively.
RESULTS

Identification of a Schistosome RXR Homologue cDNA—A 170-base pair DNA fragment that showed homology to RXRs was amplified using degenerate oligonucleotides representing conserved regions of RXR genes as primers and adult schistosome cDNA as template in PCR (Fig. 2). The sequence of the 5′ primer used for PCR encodes for an amino acid sequence found in the second zinc finger of all members of retinoid receptors, RARs and RXRs (19–22). The sequence of the 3′ primer encodes for an amino acid sequence of the hinge region that is identical in all identified RXRs and is not found in other members of the steroid receptor superfamily. After screening 400,000 plaques of a female adult worm ZAP cDNA library with the RXR homologous DNA fragment, seven positive clones were obtained. Restriction digest and sequencing demonstrated that all seven clones were identical. The nucleotide sequence of SmRXR translates into a protein of 743 amino acids with an approximate mass of 74 kDa. SmRXR is the largest reported RXR to date. Sizes of RXRs range from 410 amino acids for mRXRβ to 508 amino acids for dUSP (11, 23, 24). Comparison of the deduced amino acid sequence of SmRXR to other RXRs reveals that this protein exhibits significant homology to the DNA binding domain and the dimerization domain of other members of the RXR family (Fig. 2). For example, comparison of the amino acids of the DNA binding domain of SmRXR (aa 271–336) to those amino acids in the same domain of hRXRα (aa 135–200) and dUSP (aa 104–169) shows identities of 69 and 68%, respectively (11, 22, 24, 25). The first zinc finger within the DNA binding domain of SmRXR has only four amino acid changes from that of hRXRα (25). The second zinc finger exhibits lower homology. Within the dimerization and ligand binding domain are heptad repeats (23, 25, 27, 35). The amino acid at positions 1, 5, and 8 containing hydrophobic amino acids at the amino and carboxyl ends of the first heptad repeat form a dimerization motif (21, 23, 24, 25). Adjacent to the heptad repeats at the amino and carboxyl ends of the dimerization and ligand binding domain are two sub-domains, τ1 and τ2, respectively, known to be important for ligand-dependent transactivation in other members of the steroid hormone receptor superfamily (21, 26–22). The τ2 sub-domain, thought to function in hormone-relieved repression, has 20–45% conservation among steroid receptors of vertebrates and arthropods (21, 33, 34). For example, there is 38–40% conservation of the τ2 domains of vertebrate RARs and RXRs (21, 23, 25). Steroid hormone receptors of vertebrates within each subfamily, such as RXR, have at least 98% identity in the τ1 domains (21–23). Homology of the τ1 domain of dUSP to that of vertebrate RXR is 50% (11, 21–25). The τ1 domain of SmRXR (aa 457–512) shares 13% identity to the τ1 domain of hRXRα (aa 277–324) and 19% identity to the τ1 domain of dUSP (aa 282–338) (11, 21–25). The τ2 sub-domain, a small amphipathic helical structure located at the carboxyl end of the dimerization and ligand binding domain, is conserved only among steroid hormone receptors that share the same ligand (22, 23, 25–32). For example, the τ2 sub-domains of mRXRβ, hRXRβ, and hRXRα, which bind 9-cis-retinoic acid, are identical to one another except for a single amino acid change of the last carboxyl terminus amino acid (23, 25, 27, 35). The τ2 sub-domains of RARs, which bind all-trans-retinoic acid, are also identical to one another but are dissimilar to the τ2 sub-domains of the vertebrate RXRs (22, 23, 25–27, 36–41). The τ2 domain of dUSP is not homologous to vertebrate RXR τ2 domains and does not bind to 9-cis-retinoic acid (24). Nineteen-cis-retinoic acid is not likely to be the ligand of SmRXR due to the variant sequence of the τ2 domain.

Immunological Identification of SmRXR in Different Worm Stages—Using an affinity purified antibody produced to a truncated form of recombinant GST-SmRXR, native SmRXR was identified in adult worm pair, mature male, mature female, and 3 h schistosomule extracts (Fig. 3). Native SmRXR has an approximate molecular mass of 74 kDa as observed in the Western blot (panel B). For a control, adult worm pair extract was probed with preimmune rabbit serum (panel C). There are irrelevant bands that appear on the Western blot, but none the 74-kDa size. These bands are not present in panel B because the serum was affinity purified.

Developmental Expression of the SmRXR Gene—RT-PCR was performed on total RNA isolated from various developmental stages to evaluate developmental expression of the SmRXR gene. In order to control for the expression of multiple schistosome RXR homologues, primers to non-conserved regions of SmRXR were used in the RT-PCR reactions. α-Tubulin-specific primers that represent a constitutive gene were added to each reaction. A 465-bp SmRXR cDNA fragment and a 500-bp α-tubulin product were amplified (Fig. 4). By comparing the amount of SmRXR PCR product to that of α-tubulin within each reaction and then comparing this value among the different life stages, we demonstrate that SmRXR is constitutively expressed throughout development (Fig. 4).

SmRXR Binds to the p14 Gene Upstream cis-Elements and Activates Transcription—To determine if SmRXR binds to cis-elements of the p14 upstream region and drives transcription...
of a female-specific gene, we employed the yeast one-hybrid system. The reporter yeast strains, YTR-154, YTR-EL1, and YTR-pHis, were transformed with pAC-RXR and pAC-2. YTR-154 transformed with salmon sperm DNA alone only grew on SD/−his media. This is expected because the His3 promoter is leaky. Therefore, growth of YTR-154 transformed with pAC-RXR on SD/−his−leu media with even low concentrations of aminotriazole would be a result of SmRXR-AD fusion protein binding to cis-elements of p14 and driving transcription of the HIS3 gene. The transformed YTR-154 cells were spread on SD/−his−leu plates with increasing amounts of AT. After 7 days of incubation, two classes of colonies grew that ranged from 0.25 to 0.5 mm in size and from 1.5 to 2.5 mm in size (data not shown). Colonies of both sizes were picked and streaked on SD/−his−leu plates containing up to 60 mM aminotriazole. The smaller colonies did not grow. The larger colonies grew on SD/−his−leu containing 60 mM AT (Fig. 5A). YTR-154 cells transformed with pAC-2 grew as small colonies (0.25–0.5 mm) in the original spreads. These colonies were picked and streaked on SD/−his−leu containing up to 60 mM aminotriazole. On the restreak plates it only took 3 days for YTR-154 growth to appear on SD/−his−leu containing 60 mM AT, whereas it took 5–6 days for growth of YTR-EL1 to appear on plates containing 60 mM AT (Fig. 5B). Growth of YTR-EL1 appeared on day 4 on the plates with 45 mM AT. Fig. 5B shows growth of five individual colonies of YTR-EL1 on 60 mM AT. The negative control included in this experiment was YTR-EL1 transformed with pAC-2 alone. Again there were small background colonies on the original spread that did not grow on the restreak (Fig. 5B). Therefore SmRXR-AD binds to the sequence including the direct repeat and enhances the transcription of the HIS3 gene.

Because RXR forms heterodimers in many species (42–50), we attempted to employ the yeast two-hybrid system to obtain schistosome clones that express proteins that interact with SmRXR. As a control, we transformed Y190 yeast cells with pAS-RXR, the bait plasmid containing the GAL4 DNA binding domain. SmRXR alone activated β-galactosidase expression (data not shown). This result indicated that SmRXR by itself may activate gene transcription, perhaps as a homodimer, or by interacting with yeast proteins. In order to determine if SmRXR by itself, or perhaps with the help of a yeast protein, could drive transcription when recognizing cis-elements of p14, SmRXR was cloned into an expression vector that lacked the Ga4 activation domain and DNA binding domain (pADH-RXR). When YTR-154 was transformed with pADH-RXR, the cells as in the case of transformation with pAC-RXR (above) grew on SD/−his−leu media. There were very small colonies, 0.25–0.05 mm in size, and larger colonies, 1.5–2.5 mm in size. Again the smaller colonies did not grow after being restreaked on SD/−his−leu plates containing AT, but the larger colonies grew on SD/−his−leu containing 60 mM AT (Fig. 5C). Growth of these colonies appeared at day 3 on the restreak plates containing 60 mM AT. As in the above experiment, YTR-154 transformed with salmon sperm alone grew only on SD/−his
media without AT. Therefore, SmRXR is binding to cis-elements of the p14 upstream region and driving transcription of the HIS3 gene without the Gal4 activation domain. Controls of this experiment included transforming YTR-154 with pADH1. Again small colonies grew on all the plates of the original spread but did not grow on SD/-his/-leu media containing aminotriazole after the restreak (Fig. 5C). Similar to the above experiment YTR-pHis, transformed with both pADH-RXR and pADH1, grew on all plates of the original streak but not after the restreak (data not shown).

YTR-EL1 cells were also transformed with pADH-RXR. Yeast colonies grew on SD/-his/-leu containing 60 mM AT (Fig. 5D). Growth of these colonies on media containing 60 mM AT was slower than YTR-154 transformed with pADH-RXR and YTR-EL1 transformed with pAC-RXR. Growth of YTR-EL1 transformed with pADH-RXR on the restreak plates containing 60 mM AT appeared on days 6 and 7 depending on the clone. Small colonies grew on plates that contained YTR-EL1 transformed with pADH1, but these colonies did not grow when restreaked on SD/-his/-leu containing AT. Indeed, SmRXR binds to the sequence containing the direct repeat (Fig. 1) and drives transcription of HIS3 (Fig. 5D).

**EMSA Analysis of SmRXR-DNA Interactions**—EMSA were performed to study the *in vitro* interaction between p14 DNA and SmRXR. A 250-bp sequence, nucleotides -300 to -50, of the p14 upstream region (Fig. 1) was used as a probe in gel shift assays to determine whether or not SmRXR, synthesized by *in vitro* coupled transcription/translation, binds to RXR recognition sites. Shown in lane 1 of Fig. 6 is the probe without lysate from the transcription-translation mix. In lane 2, the binding reaction contains lysate from a transcription-translation reaction with pCITE vector used as template. There are a few irrelevant shifts of the probe caused by contents of the lysate binding to the p14 probe. In lane 3 it is apparent that SmRXR causes unique shifts of the radiolabeled p14 fragment. These shifts are denoted by the two arrows (Fig. 6). When cold p14 DNA is added in 100 × molar amount (lanes 4 and 5), the upper shift is competed, whereas the lower shift identified by the arrow is competed to a much lesser extent. Nonspecific DNA (lanes 6–8) does not interfere with the changes in electrophoretic mobility. Therefore, the shifts observed in lane 3 are specific for SmRXR-p14 interaction. The direct repeat competes the shifts to a great extent (lanes 9–11). At 250 ×, the competitor completely competes the two shifts denoted by both arrows.

**Fig. 5. Demonstration of SmRXR activity expressed in yeast.** Panel A, individual YTR-154 colonies transformed with pAC-RXR (1–5) and pAC-2 (6) were streaked on SD/-his/-leu plates containing 60 mM AT. Panel B, individual YTR-EL1 colonies transformed with pAC-RXR (1–5) and pAC-2 (6) were restreaked on SD/-his/-leu plates containing 60 mM AT. Panel C, restreaks of individual YTR-154 transformed with pADH1-RXR (1–5) and pADH1 (6) on SD/-his/-leu containing 60 mM AT. Panel D, YTR-EL1 transformed with pADH-RXR (1–5) and pADH1 (6) on selective media with 60 mM AT. Above each plate is a cartoon showing the target element inserted upstream of the His3 reporter gene in the yeast strains. Whether or not SmRXR, expressed in the yeast strains, is fused to the Gal4 activation domain is also depicted in the schematics.
The DNA fragment that includes the sequence GTAGAATATCA, competes the upper shift slightly at 250× molar amounts (lane 14). The sequence that includes the imperfect palindrome (lanes 15–17) competes the upper shift at 100× and at 250× competes both shifts. In vitro studies demonstrate that the direct repeat sequence of the p14 gene is a preferential binding site for SmRXR.

**DISCUSSION**

Schistosomes are the only trematodes with separate sexes. This fact has driven the evolution of an interesting biological interplay between the sexes, such that the male through an unknown stimulus is able to regulate female-specific gene expression (2). A number of female-specific genes have been isolated, but as of yet no factors directly involved in the regulation of female-specific genes have been identified. In this paper we report the identification of SmRXR cDNA and provide evidence for its involvement in female-specific gene expression.

Members of the RXR subfamily in other species are important in the regulation of sex-, tissue-, and temporal- specific genes. For example, USP of *Drosophila* is employed in the regulation of chorion genes, embryogenesis, larval development, and pupation (11, 12, 45). This pleiotropy of function is representative of RXR subfamily members. RXRs are known to form heterodimers with various partners that are essential for the regulation of a number of genes involved in homeostasis and development (45–50). Examples of RXR partners include the edcysone receptor of *Drosophila*, vitamin D receptor, thyroid hormone receptor, and retinoic acid receptors (RAR) (42–50). When RXR forms heterodimers, such as RXR/RAR and USP/edcysone receptor, the conformation of its binding partner changes allowing a greater affinity not only for the DNA response element but also for the ligand (43–51). RT-PCR results demonstrated that SmRXR mRNA is constitutively expressed throughout schistosome development, not only in mature females as are the female-specific genes (2, 3, 7, 9, 10). Furthermore, Western blot analysis showed that the 74-kDa SmRXR protein is present in both sexes, mature and immature. This demonstrates that SmRXR is not female-specific and suggests that, like other members of the RXR subfamily, SmRXR is important in the regulation of a number of genes. Future studies will entail identifying SmRXR-binding partners involved in the regulation of female-specific genes and defining the putative role(s) SmRXR plays in the regulation of other genes.

To date, a genetic system to study the functions of schistosome genes in *vivo* does not exist. Therefore, evidence for the involvement of SmRXR in female-specific gene regulation was obtained by the yeast one-hybrid system and EMSA analysis. In these studies, we showed that SmRXR binds to cis-elements of the p14 gene and drives transcription of a reporter gene in yeast. Members of the steroid receptor superfamily commonly bind to cis-elements that consist of two half-sites spaced by 3, 4, or 5 nucleotides (13, 14). EMSA analysis demonstrates that the preferential binding site for SmRXR, in vitro, is a sequence containing the half-site AACTATCA spaced by five nucleotides (Fig. 1). In the upstream region of s15, a *Drosophila* chorion gene, the sequence TCACGT is an essential part of an imperfect palindrome known to be a USP response element (12). Interestingly, a similar sequence, TCACGCT, occupies the 3′ end of the first half-site and part of the spacer region of the direct repeat in the p14 gene upstream region (Fig. 1). To define the region of the p14 gene involved in SmRXR binding interaction and trans-activation, the direct repeat sequence was used as a target element in the one-hybrid assay. SmRXR most likely acts as a homodimer to drive transcription of this chimeric reporter gene. It is unlikely that SmRXR interacts with a yeast protein. RXRs have been shown to form heterodimers with only members of the steroid receptor family, and to date, steroid receptors have not been identified in yeast (42–50).

An evolutionary study was performed to better understand the function of SmRXR. Knowing the rate at which SmRXR has evolved as compared with other RXRs and RARs may answer questions about how it functions in regulating gene expression. Steroid receptors have recently been identified in early metazoans, such as cnidarians and schistosomes (52). Interestingly, in a nuclear receptor evolutionary study, the DNA binding domain of a schistosome RXR homologue, which is not identical to the SmRXR cDNA identified in our lab, was described (52). The divergence of subfamilies of the steroid superfamily was previously determined to have occurred before the divergence of the arthropods and vertebrates (22, 53) (Fig. 7). From the Escriva et al. (52) study and the phylogenetic tree designed using full-length cDNA sequences of RXRs and RARs shown in this report (Fig. 7), the above statement can be extended to note that the divergence of subfamilies occurred before the divergence of cnidarians and platyhelminthes (52, 53). Because SmRXR is deeply rooted in evolution (Fig. 7), comparison of this gene with other steroid receptors may lead to the identification of the original ancestor of the steroid receptor family.

The trans-activating function of steroid receptors is often dependent on dimerization and ligand binding (22, 42–50). Ligand binding is thought to change the conformation of the steroid receptor into an active state (51). The ability of members of the RXR subfamily to bind specific ligands appears to have evolved after the vertebrate and arthropod lineages diverged, as of yet ligands have not been found for arthropod
RXRs (22, 45, 52, 53). The \( \tau \), sub-domain, important for ligand binding specificity, of arthropod RXR homologues and SmRXR shows minimal homology to one another and to vertebrate RXRs (22, 23, 25–22). This suggests that if these more ancient receptors have evolved to bind ligands, they do not bind the same ligand. However, arthropod RXRs, like their vertebrate homologues, do form dimers with other proteins. For example, dUSP dimerizes with the eddyson receptor (42–44). Upon sequence analysis of SmRXR, it is evident that the heptad repeats that form dimerization interfaces on one side of an \( \alpha \)-helix exist in this protein (21). This suggests that, like other steroid receptors, SmRXR forms dimers that function in trans-activation (42–50). The fact that SmRXR and dUSP putatively do not bind ligands, yet have the ability to form dimers, suggests that \( \tau \) and the dimerization interface have evolved at different rates (22, 23, 25–32, 45, 51, 52). Comparing the function of early metazoan, arthropod, and vertebrate RXRs will lead to a greater understanding of the importance of these two sub-domains in RXR facilitated trans-activation.

This report provides the first evidence that RXRs play a role in gene regulation in primitive eukaryotic organisms such as schistosomes. Not only does this study add to an increasing amount of information on the evolution of steroid receptors but also has initiated the study of steroid receptors involved in a very intriguing interplay between the separate sexes of schistosomes and the regulation of female-specific genes. The study of steroid receptors, such as SmRXR, involved in female-specific gene regulation may lead to pharmacological compounds that prevent female maturation and egg production, thereby controlling schistosomiasis.

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