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Nuclear hormone receptors in parasitic helminths

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Abstract

Nuclear receptors (NRs) belong to a large protein superfamily that are important transcriptional modulators in metazoans. Parasitic helminths include parasitic worms from the Lophotrochozoa (Platyhelminths) and Ecdysozoa (Nematoda). NRs in parasitic helminths diverged into two different evolutionary lineages. NRs in parasitic Platyhelminths have orthologues in Deuterostomes, in arthropods or both with a feature of extensive gene loss and gene duplication within different gene groups. NRs in parasitic Nematoda follow the nematode evolutionary lineage with a feature of multiple duplication of SupNRs and gene loss.

Keywords

Ecdysozoa; Helminths; Lophotrochozoa; Nematoda; Nuclear receptors; Platyhelminths

Introduction

Nuclear receptors (NRs) belong to a large protein superfamily that are important transcriptional modulators in metazoans. They regulate homeostasis, differentiation, metamorphosis and reproduction (Gronemeyer & Laudet 1995; Laudet & Gronemeyer 2001). Since the first recognized NR (estrogen receptor, ER) in the 1950s (Jensen & Jordan 2003) and the first isolated NR (human Glucocorticoid receptor, GR) in 1985 (Govindan et al. 1985), numerous NRs have been identified and isolated in various animals from Porifera (sponges) to mammals. NRs share a common tertiary structure, the typical NR contains a N-terminal A/B domain, a DNA binding domain (DBD), a hinge and a C-terminal ligand binding domain (LBD) (A/B-DBD-hinge-LBD). The A/B domain is highly variable and contains a ligand independent activation function (AF1). The DBD (also known as the C domain) is the most conserved region which contains two zinc finger motifs conferring sequence-specific DNA recognition to the regulatory region of the target gene. The conserved sequence of the first zinc finger (CI) which is Cys-X2-Cys-X13-Cys-X2-Cys

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contains a motif called the P-box (Lee et al. 1993; Luisi et al. 1991) which is responsible for binding to the target gene. The conserved sequence of the second zinc finger (CII) is described as Cys-X5-Cys-X9-Cys-X2-Cys with a motif called the D-box (Luisi et al. 1991) which is involved in dimerization. As more data has and continues to become available, the information indicates that the number of the amino acids in D-box is variable. Therefore, we suggest that the conserved sequence of the second zinc finger should be described as Cys-**Xx**-Cys-X9-Cys-X2-Cys (Fig. 1). The hinge region (also called the D domain) is a poorly conserved sequence that functions as a flexible region between the DBD and LBD. The LBD contains the ligand binding pocket (LBP) in most NRs and is involved in transcriptional activation of target genes via binding of ligand and co-activators. Some NRs with no known ligand are called orphan receptors (Gronemeyer & Laudet 1995; Laudet & Gronemeyer 2001). Atypical NRs are found in some animals, NRs with a DBD but no LBD (NR0A) are found in arthropods and nematodes, members without a DBD but with a LBD (NR0B) are present in some vertebrates. Recently, NRs with two DBDs and a single LBD (2DBD-NRs) (A/B-DBDDBD-hinge-LBD) were identified in the trematode *Schistosoma mansoni* (Wu et al. 2006) and also in other invertebrates (Wu et al. 2007b) (Fig. 2). NRs are divided into six subfamilies (NR1-NR6) based on phylogenetic analysis of the conserved sequences with an extra subfamily NR0 (its members either contain only a DBD (NR0A) or only a LBD (NR0B)) (Committee 1999). The new group of NRs with two DBDs and a single LBD (Wu et al. 2007b) has yet to be included in this classification system (Committee 1999).

Helminths constitute a phylogenetic group including parasitic worms from two Phyla: Platyhelminths (flatworms) and Nematoda (roundworms). Parasitic Platyhelminths include trematodes (flukes), such as *Schistosoma mansoni* (blood fluke), *Clonorchis sinensis* (liver fluke), *Paragonimus westermani* (lung fluke) and *Fasciolopsis buski* (intestinal fluke), and Cestoda (tapeworms), such as *Echinococcus granulosus* (hydatid tape-worm) and *Diphyllobothrium latum* (fish or broad tapeworm). Nuclear receptors in Platyhelminths were first identified from *S. mansoni* (Escriva et al. 1997). Parasites in the Nematoda include some important human parasites such as *Onchocerca volvulus*, *Brugia malayi*, *Ascaris lumbricoides* and *Trichinella spiralis*. In recent years, the complete genome sequence from diverse animals has revealed the entire NR complement in organisms in Deuterostomia and Protostomia including the helminths *Schistosoma mansoni* and *S. japonicum* (Platyhelminths) and *Brugia malayi* and *Meloidogyne incognita* (Nematoda).

1. Nuclear receptors in parasitic Platyhelminths

At present, NRs in parasitic Platyhelminths are only known in the trematodes *Schistosoma mansoni* and *S. japonicum* whose genomes have been sequenced (Berriman et al. 2009b; Liu et al. 2009).

A. NRs in *S. mansoni*—*S. mansoni* is a trematode which has evolved separate sexes and has a complex life cycle that involves mammalian and snail hosts. It is one of the causative agents of schistosomiasis. The complete NR complement in *S. mansoni* contains 21 members as reported by (Wu et al. 2006)) and was verified by *S. mansoni* genome sequence (Berriman et al. 2009a). Full length or partial cDNAs of all 21 NRs have been isolated (De Mendonca et al. 2002; de Mendonca et al. 2000; Freebern et al. 1999a; Freebern et al. 1999b; Hu et al. 2006b; Hu et al. 2006c; Lu et al. 2006b; Wu & Loverde 2008; Wu et al. 2007a; Wu et al. 2006; Wu et al. 2007b; Wu et al. 2007c; Wu et al. 2008). *S. mansoni* NRs include six members in subfamily 1 (NR1), nine members in subfamily 2 (NR2), one member in subfamily 4 (NR4), two members in subfamily 5 (NR5) and three members with two DBDs and a single LBD (2DBD-NRs). No NRs were found in subfamily 3 (NR3) or subfamily 6 (NR6) (Wu et al. 2006). Phylogenetic analysis demonstrates that almost all of

the *S. mansoni* NRs have orthologues either in Deuterostomes, in arthropods or both, except the three 2DBD-NRs and a divergent member NR1 member (SmNR1) (Figure 3a, 3b and Table 1). Among these 21 receptors, full length cDNA of 14 members have been isolated and studied.

SmTR α (AY395038) and SmTR β (AY395039) (NR1A): Two homologues of the vertebrate thyroid hormone receptors (THR, TR) were identified (Bertrand et al. 2004; Verjovski-Almeida et al. 2003; Wu et al. 2007a; Wu et al. 2006). Phylogenetic analysis demonstrates that these two copies are from a schistosoma-specific gene duplication (Wu et al. 2007a). *S. mansoni* was the first known protostome exhibiting TR homologues (Bertrand et al. 2004; Verjovski-Almeida et al. 2003; Wu et al. 2007a; Wu et al. 2006). Since then TR orthologues have also been found in *S. japonicum* and the free living platyhelminth, *Schmidtea mediterranea*, in the mollusc *Lottia gigantea* and the arthropod *Daphnia pulex* (Wu et al. 2007a). GST pull-down experiments show that both SmTR α and SmTR β can form a heterodimer with one of the *S. mansoni* homologues of retinoid X receptor (SmRXR1). This suggests that non-chordate TRs are capable of forming a heterodimer with RXR like chordate TRs (Wu et al. 2007a). Electrophoretic mobility shift assays (EMSAs) show that SmTR α can bind, either as a monomer or as a homodimer, to a conserved DNA half-site AGGTCA, and to direct repeats of AGGTCA spaced with 0-5 nucleic acids (DR0-DR5) and palindrome repeat of AGGTCA without spaced nucleic acids (Pal0), but SmTR β shows weak binding to the same oligonucleotides as compared to SmTR α (Wu et al. 2007a). Although both SmTRs could form a heterodimer with SmRXR1, SmTR α /SmRXR1 or SmTR β /SmRXR1 did not bind to the tested DNA elements, this may due to the flanking or spacing of nucleotides between the hormone receptor elements (HRE). With regards to whether SmTRs can bind to a T3/T4 ligand, sequence alignment of the LBD of SmTRs which is less conserved compared to vertebrate orthologues, suggest perhaps not. However, both SmTRs do exhibit a conserved consensus motif I and consensus motif II and a putative AF2 activation domain core (AF2-AD) (Wu et al. 2007a). Thus experimental studies are needed to answer the question of whether invertebrate TRs can bind to a cognate ligand.

SmE78 (AY395058) (NR1E): An orthologue of *Drosophila* ecdysone-induced protein 78 (E78) was identified in *S. mansoni* (Wu et al. 2006; Wu et al. 2008). *Drosophila* E78 plays a role directly related to ecdysone signaling. SmE78 is the first NR1E orthologue known outside of the Ecdysozoa. Quantitative real-time reverse-transcriptase polymerase chain reaction (qRT-PCR) shows that *SmE78* was expressed throughout schistosome development with the highest levels of expression in miracidia and the egg stages (Wu et al. 2008). Previous studies demonstrated that *S. mansoni* could synthesize the steroid hormone ecdysone (Nirde et al. 1983) and that Ecdysterone was effective in stimulating host location activities in schistosome miracidia (Shiff & Dossaji 1991). Whether SmE78 is involved in transduction of an ecdysone signal in *S. mansoni* remains to be demonstrated.

SmHR96 α (SmCAR) (AY663841, AY688258) (NR1J): SmHR96 α (SmCAR) is a homologue of *Drosophila* hormone receptor 96 (DHR96) which is a close member of vertebrate NRs in NR1I group (CAR/VDR/PXR) (Hu et al. 2006b; Wu et al. 2006). Yeast two-hybrid analysis, pull-down and co-immunoprecipitation assays demonstrated that SmCAR interacts with SmRXR1 but not SmRXR2 (Hu et al. 2006a). EMSAs demonstrate that SmCAR can bind to *hsp2* ecdysone response element in a similar manner as *Drosophila* HR96, and to schistosome *p14* female-specific gene upstream region (Hu et al. 2006b). mRNA of *SmCAR* is expressed in every stage of the *S. mansoni* life cycle with high expression levels in eggs and cercaria. SmCAR protein is found in subtegumental and parenchymal cells in both male and female worms. In addition, it is found in the ovaries, the eggs and the vitelline cells of mature female worms (Hu et al. 2006b). A second homologue of *Drosophila* NR96 (SmHR96 β) was identified in *S. mansoni* by cloning a partial cDNA

that encodes a complete DBD (AY688259) (Wu et al. 2006) and by genome sequencing (Berriman et al. 2009a).

SmNR1 (AY395037, DQ439962) (NR1 divergent member): Phylogenetic analysis shows that SmNR1 clusters within NR subfamily 1 but outside of any known groups, thus SmNR1 is a divergent member (Wu et al. 2007c). Two alternative splice variants of *SmNR1* were identified, both variants encode the same protein sequence. *SmNR1* localizes on *S. mansoni* chromosome 1 as shown by fluorescent in situ hybridization (FISH) (Wu et al. 2007c). Yeast two-hybrid assay and glutathione S-transferase pull-down assay (GST-pull down) show that SmNR1 interacts with SmRXR1. EMSAs show that the heterodimer of SmNR1/SmRXR1 can bind to DNA elements containing the half-site AGGTCA, a direct repeat of the half-site separated by 0-5 nucleotides (DR1-DR5), a palindrome repeat of the half-site without spaced nucleic acids (Pal0) (Wu et al. 2007c), and a p14-DR17 element (Fantappie et al. 2008b). Mutagenesis analysis shows that SmCBP1, a co-regulatory protein that was known to interact with SmFTZ-F1 (Bertin et al. 2006), can mediate the interactions with the LBD of SmRXR1 and SmNR1 (Fantappie et al. 2008a). Transient transfection in mammalian COS-7 cells shows that SmNR1/SmRXR1 can enhance the transcriptional activation of a DR2-dependent reporter gene (Wu et al. 2007c). qRT-PCR shows that mRNA of *SmNR1* was expressed throughout schistosome development with higher expression in eggs, sporocysts and 21-day-old worms (Wu et al. 2007c).

SmRXR1 (AF094759) and SmRXR2 (AF129816, AF158102) (NR2B): Two homologues of vertebrate RXR are present in *S. mansoni* (de Mendonca et al. 2000; Freebern et al. 1999a; Freebern et al. 1999b). Like SmTRs, these two copies of SmRXRs originated from a *Schistosoma*-specific gene duplication. RXRs play a central role in a variety of nuclear signaling pathways and modulate the activity of other nuclear receptors by forming heterodimers (Mangelsdorf et al. 1995). Yeast two-hybrid experiments and/or pull down experiments show that SmRXR1 can form a heterodimer with SmTR α and SmTR β (Wu et al. 2007a), SmHR96 α (SmCAR) (Hu et al. 2006a) and SmNR1 (Wu et al. 2007c). SmRXR1 alone can bind to cis-elements of the *S. mansoni* *p14* gene (Fantappie et al. 2008b; Freebern et al. 1999b) and weakly on a half site of AGGTCA and its repeat of DR0-DR5 and Pal0 (Wu et al. 2007c). *Smp14* is a female-specific gene whose expression is regulated by a stimulus from the male schistosome (LoVerde et al. 2004). Heterodimers of SmRXR1/SmNR1 can bind to DNA elements containing the half-site AGGTCA, DR1-DR5 and Pal0 of this element (Wu et al. 2007c) and to the p14-DR17 element (Fantappie et al. 2008b). RT-PCR shows that mRNA of SmRXR1 is constitutively expressed throughout development (Freebern et al. 1999b). The co-regulatory protein SmCBP1 is able to mediate interactions with SmRXR1 and SmNR1 (Fantappie et al. 2008a).

SmRXR2 was independently isolated by two laboratories (de Mendonca et al. 2000; Freebern et al. 1999b). Unlike SmRXR1, SmRXR2 could not form a heterodimer with SmTR α or SmTR β (Wu et al. 2007a), SmHR96 α (SmCAR) (Hu et al. 2006a) or SmNR1 (Wu et al. 2007c). SmRXR2 does not bind to the consensus direct repeat response elements nor could the native SmRXR2 transactivate the transcription of a reporter gene in mammalian cells (de Mendonca et al. 2000). However, it can bind to cis-elements present in the *S. mansoni* *p14* upstream region in a yeast reporter system (Fantappie et al. 2001). SmRXR1 and SmRXR2 fail to dimerize in a yeast two-hybrid system and in immunoprecipitation experiments (Fantappie et al. 2001). SmRXR2 mRNA is expressed at all life-cycle stages with higher levels in cercariae and miracidia, the free-living larval stages (de Mendonca et al. 2000; Freebern et al. 1999a). However, the protein expression is markedly different than that of the mRNA with the high levels in schistosomula but much lower levels in cercariae and miracidia (de Mendonca et al. 2000).

SmTR4 (AY663842, AY688263) (NR2C): EMSAs show that the DBD of SmTR4 can bind to DR3 and DR4 that contain two AGGTCA half-sites spaced by three or four nucleotides. Yeast one-hybrid assay shows that the A/B, D and F domains each exhibit an autonomous transactivation activity (Hu et al. 2006c). SmTR2/4 mRNA is expressed in every tested developmental stage of *S. mansoni* with a higher level in cercaria. Western blot analysis identified two forms of SmTR2/4 protein with molecular sizes of about 230 and 245 kDa in adult worms (Hu et al. 2006c).

SmNR4A4 (AY395064, AY688260) (NR4A): In humans and *Drosophila* NR4A members, there are four conserved phenylalanines in the LBD filling the entire volume of the ligand binding pocket (LBP) suggesting that NR4A receptors are true orphan receptors (Baker et al. 2003; Codina et al. 2004; Flaig et al. 2005). Three of these conserved phenylalanines are retained in SmNR4A, this result suggests that the ancestral NR4 in the urbilateria was already an orphan receptor (Wu & Loverde 2008). qRT-PCR shows mRNA of *SmNR4A4* is regulated throughout development with different expression levels with a higher expression in the endoparasitic stages in both the snail and mammalian hosts and the highest expression is found in the daughter sporocysts and 35-day-old worms (Wu & Loverde 2008).

SmFTZ-F1 α (AY665680, AY688256) (NR5A): Phylogenetic analysis shows that SmFTZ-F1 α belongs to NR5A group (Lu et al. 2006b; Wu et al. 2006) while the previously isolated SmFTZ-F1 (AF158103) (De Mendonca et al. 2002) is an orthologue of *Drosophila* NHR39 (DHR39) which belongs to NR5B group (Lu et al. 2006b; Wu et al. 2006). EMSAs show that SmFTZ-F1 α can bind as a monomer to a response element FFIRE which is recognized by mammalian steroidogenic factor 1 (SF-1) (Lu et al. 2006a). Yeast one-hybrid experiments show that transactivation of reporter gene expression by *SmFTZ-F1 α* is A/B domain-dependent. Semi-quantitative RT-PCR shows that *Smftz-f1 α* is constitutively expressed throughout the schistosome life cycle with the highest level in the egg stage (Lu et al. 2006b). *In situ* RT-PCR on paraffin-embedded sections shows that expression of *Smftz-f1 α* is distributed throughout the parenchyma and the epithelial cells of the intestine. Western blot analysis of a schistosome extract identified two proteins, one with a size (206 kDa) predicted by the SmFTZ-F1 α cDNA sequence and a smaller component of 120 kDa (Lu et al. 2006b).

SmFTZ-F1 (DHR39 orthologue) (NR5B): Phylogenetic analysis shows that SmFTZ-F1 (De Mendonca et al. 2002) is an orthologue of the *Drosophila* HR39 (DHR 39) (Lu et al. 2006b; Wu et al. 2006). Two alternative splice variants of SmFTZ-F1 have been identified (De Mendonca et al. 2002). EMSAs show that SmFTZ-F1 can bind to the response element for SF-1 and the DR0 of SFRE, but cannot bind DR-1 to DR-5 (De Mendonca et al. 2002). SmFTZ-F1 is able to transactivate transcription of a reporter gene in mammalian cell lines. Mammalian two-hybrid assay shows that the D, E and F domains of SmFtz-F1 are each capable of interacting with the LBD of SmRXR1, and suggests, for the first time, that a NR5 member can heterodimerize functionally with RXR (Bertin et al. 2005). Co-transfected CV-1 cells show that SmFTZ-F1 and SmRXR1 can transactivate transcription, but only SmFtz-F1 is physically bound to the promoter (De Mendonca et al. 2002). Functional studies showed that SmCBP1, a CREB-binding protein (CBP) in *S. mansoni*, could interact physically with SmFtz-F1 (Bertin et al. 2006). RT-PCR shows that *Smftz-f1* is expressed in all developmental stages with higher expression in the larval miracidia, sporocysts and cercariae, but the levels of protein expression are different from that of the mRNA with high levels in cercariae, schistosomula and male worms (De Mendonca et al. 2002).

NR with two DBDs and a single LBD (2DBD-NRs): One of the most striking results in isolating NRs from *S. mansoni* is that a novel group of NRs that each contain two DBDs and a single LBD (2DBD-NRs) were identified (Fig. 2) (Wu et al. 2006; Wu et al. 2007b).

Further analysis of available genome DNA databases shows that 2DBDNRs are present in other Protostomia such as free-living Plathelminths (*Schmidtea mediterranea* and *Dugesia japonica*), mollusks (*Lottia gigantea*) and arthropods (*Daphnia pulex*) (Wu et al. 2007b). Phylogenetic analysis of the 2DBD-NRs shows that the first DBD of 2DBD-NRs belongs to one monophyletic group and the second DBD belongs to a separate monophyletic group, this suggests that 2DBD-NRs originated from a common ancestral gene (Wu et al. 2007b). All 2DBD-NRs possess the same P-box sequence, CEACKK, in the first DBD which is not present in any other known NR (Wu et al. 2006; Wu et al. 2007b). This unique P-box of the first DBD of 2DBD-NRs may determine novel target DNA specificity.

Three 2DBD-NRs are present in *S. mansoni* (*Sm2DBD-NR α* (AY395036), *Sm2DBD-NR β* (AY688251) and *Sm2DBD-NR γ* (AY698061)). They are localized on different chromosomes as shown by FISH. This suggests that the *Sm2DBD-NRs* duplicated among different chromosomes (Wu et al. 2007b). Yeast two-hybrid experiments demonstrate that *Sm2DBD-NR α* can interact as a homodimer but it does not interact with *SmRXR1* or *SmRXR2*. GST pull-down experiments verify that the LBDs of *Sm2DBD-NR α* can form a homodimer *in vitro* (Wu et al. 2007b). The P-box motif located in the DBD is responsible for NRs acting on their target genes by recognizing and binding to specific DNA core motifs. *Sm2DBD-NR α* interacting as a homodimer implies that four P-boxes may be involved in DNA binding and a novel mechanism of DNA binding is required to allow 2DBD-NR to bind to target DNA cis-elements. qRT-PCR shows that three of the *Sm2DBDs* are developmentally regulated and thus have a role in different development stages, *Sm2DBD α* was detected in sporocysts, cercariae, schistosomules, female and male worms, *Sm2DBD β* was expressed at relatively high levels in eggs, sporocysts, cercariae and male stages, but *Sm2DBD γ* was only detected in cercariae and 28-day worms (Wu et al. 2007b).

B. NRs in *Schistosoma japonicum*—*S. japonicum* is a sister species of *S. mansoni* whose genome was recently sequenced (Liu et al. 2009). Comparison of the NR complement of *S. mansoni* and *S. japonicum* shows that the two species share the same NR members except that *S. japonicum* has one or maybe two more 2DBD-NRs than *S. mansoni* (Table 1 and note at the bottom of the table). The authors of the *S. japonicum* sequence (Liu et al. 2009) claim to have found an orthologue of the glucocorticoid receptor (GR), the ecdysone-like receptor (EcR) and the estrogen receptor-related receptor (ERR), that were missing in *S. mansoni*. We reanalyzed the DNA sequences of *S. japonicum* and our results show that *S. japonicum* ‘GR’ (Sjp_0026760, Sjp_0002350) actually is a homologue of *S. mansoni* RXR1 (*SmRXR1*, AF094759), *S. japonicum* ‘EcR’ (FSE001-P00030-D02 (SJFCE5380)) (see supplementary table 22 of (Liu et al. 2009)) is an orthologue of *SmHR96a/SmCAR* (AY663841, AY688258), and the *S. japonicum* estrogen-related receptor beta like” (ERR) (FSE001-P00011-A06, supplementary table 22 of (Liu et al. 2009)) is not a nuclear receptor. Thus the suggestion, based on the ‘discovery’ of GR and EcR in *S. japonicum*, that the schistosome can utilize host hormones as ligands to regulate parasite gene expression and schistosomes can interact with ecdysone in the same way as in the Ecdysozoa (Liu et al. 2009) needs confirmation and at this point is incorrect.

C. NRs in tapeworms—No NR data is available for tapeworms (Cestoda). However, experiments suggest that there is endogenous production of testosterone which is thought to be important for the development of the larva of *Taenia crassiceps*. Cysticerci of *T. crassiceps* can produce testosterone from steroid precursors that may be important for parasite growth and development (Romano et al. 2003). The genomes of *Echinococcus multilocularis* and *Taenia solium* are currently being sequenced (Aguilar-Diaz et al. 2006). That information will open the door for studies on cestode NRs.

2. Nuclear receptors in parasitic nematodes

Nematodes belong to the Ecdysozoa, a group characterized by the presence of a thick outer covering called the cuticle which is both tough and flexible. In nematodes, the cuticle is shed as it molts from one stage to the next, usually four times before reaching the adult stage, during its life cycle. Molting which is best characterized in insects (Aguinaldo et al. 1997) is regulated by ecdysone and involves NRs. It is likely that the role of EcR and RXR/USP in parasitic nematode molting will soon be resolved, as the genomes of two parasitic nematodes, *Brugia malayi* and *Meloidogyne incognita*, have been sequenced and reveal the complete NR complement in these two species. Functional studies on the nematode EcR and RXR/USP are just beginning to appear (Tzertzinis et al. 2010; Shea et al. 2010; Graham et al. 2010). In addition, cDNAs of other NRs have been isolated and studied in several parasitic nematodes such as in *Filaria* (*Brugia malayi*, *B. pahangi*, *Onchocerca volvulus* and *Dirofilaria immitis*) in Rhabditida (*Strongyloides stercoralis*) and in hookworms (*Ancylostoma* spp., *Ancylostoma ceylanicum*, *A. caninum* and *Necator americanus*).

A. NRs in *Filaria*

a. NRs in *Brugia malayi*: *B. malayi* is a human parasitic nematode transmitted by mosquitoes. It is one of the causative agents of lymphatic filariasis (elephantiasis) in humans (Hoerauf 2008). Twenty-seven NRs have been identified in the *B. malayi* genome (Ghedini et al. 2007), ten of them are orthologues of *Drosophila*/vertebrates NRs including an orthologue of EcR and RXR (which are not present in *Caenorhabditis elegans*), and an E75, E78, ROR, DHR96, TLL, fax-1, Coup-TF1 and a NR4A (Table 1). An orthologue of OvNHR-1 (a divergent nematode member in NR subfamily 1 (Wu et al. 2007c; Yates et al. 1995)) is also present in *B. malayi*.

***Bm-nhr-11* (EU003172) (NR1D):** *Bm-nhr-11* was shown to be an orthologue of *Drosophila* E75 (NR1D), RT-PCR shows that mRNA of *Bm-nhr-11* expresses at a low level in adult males but at a higher level in females and L4 larvae (Crossgrove et al. 2008).

***Bma-EcR* (EF362469, EF362470) (NR1H):** Two isoforms of *Bma-EcR* were identified (Tzertzinis et al. 2010). Northern blot analyses showed that *Bma-EcR* expressed in adult males, females, L1, L2 and L3 larval stages. Both isoforms of *Bma-EcR* were able to bind a canonical ecdysone response element (EcRE) when partnered with USP (orthologue of RXR in *Drosophila*). The most prominent feature in *Bma-EcR* is the absence of the conserved helix 12 residues. Transcriptional activation assay showed that *Bma-EcR* can dimerize with *Bma-RXR* and that the LBD of *Bma-EcR* was capable of transducing an ecdysteroid signal in mammalian cells (Tzertzinis et al. 2010).

***Bma-RXR* (EF362471) (NR2B):** The protein of *Bma-RXR* was shown to be expressed in adult males, females and L1 larvae (Tzertzinis et al. 2010). The expression pattern of *Bma-RXR* differs from that of the dog filarial RXR, *Di-RXR-1*, as *Di-RXR-1* is not expressed in female worms. *Bma-RXR* can dimerize with *Bma-EcR* and the LBD of *Bma-EcR* was capable of transducing an ecdysteroid signal in a mammalian cell system (Shea et al. 2004; Tzertzinis et al. 2010).

b. NRs in *Brugia pahangi*: *B. pahangi* is a sister species of *B. malayi* which infects the lymph vessels of domestic cats and wild animals. Partial cDNAs of two *B. pahangi* NRs have been isolated (Moore & Devaney 1999).

***Bp-nhr-1* (AF091046):** RT-PCR shows that mRNA of *Bp-nhr-1* is expressed at very low levels in larvae, adult and microfilariae (Moore & Devaney 1999).

Bp-nhr-2 (AF091044): RT-PCR shows that mRNA of *Bp-nhr-2* is expressed in early stages after infection, adult worms and microfilariae, and is up-regulated just before the molt between the L3 and L4 stages. No expression was tested during the molt between L4 and the adult stage. Western blot analysis shows that the protein is expressed in microfilariae and adult parasites (Moore & Devaney 1999).

c. NRs in *Onchocerca volvulus*: *O. volvulus* is a human parasite transmitted by black flies that causes Onchocerciasis (River Blindness). Two NRs have been isolated from *O. volvulus*.

OvNHR-1 (OVU19360) (NR1 divergent member): Phylogenetic analysis shows that OvNHR-1 (Yates et al. 1995) is a divergent member in NR subfamily 1 (Wu et al. 2007c) and one of its orthologues is present in *B. malayi* (Ghedini et al. 2007). mRNA expression of *OvNHR-1* was shown to be developmentally regulated with a high level of expression during early embryogenesis. OvNR-1 was demonstrated to be antigenic in *O. volvulus* infected individuals (Unnasch et al. 1988; Yates et al. 1995).

OvNHR-2 (AF170551) (NR1E): Phylogenetic analysis shows that OvNR-2 is a close member of *Drosophila* E78 (NR1E) (Unnasch et al. 1999). OvNR-2 mRNA expression was shown to be tightly regulated in the adult female stage, appearing only in intrauterine microfilariae (Unnasch et al. 1999).

Interestingly, *Onchocerca* has been shown to concentrate retinol. In this regard retinoic acid binding proteins were identified (Sani et al. 1985). As retinoids act through retinoic acid receptors (RAR) in vertebrates, the identification of a RAR from the *Brugia* genome would facilitate studies to understand the role of these molecules in *Onchocerca* biology specifically and nematode biology in general.

OvNHR-4 (Ov-RXR) (EF362472) (NR2B): A cDNA of OvNHR-4 was isolated and remains to be characterized (Tzertzinis et al. 2010).

d. NRs in *Dirofilaria immitis*: *D. immitis* (dog heartworm) is transmitted by mosquitoes and is the causative agent of dog heartworm disease.

Di-nhr-6 (EU003171) (NR1D): Di-nhr-6 was shown to be an orthologue of Bmnhr-11 and E75 (NR1D). qRT-PCR demonstrated that mRNA expression of *Di-nhr-6* is at a high level in L3 larvae and female worms, but at very low levels in microfilaria (Crossgrove et al. 2008).

Di-nhr-7 (AF367206, AF367207, AF367208) (NR1E/G): cDNAs of three isoforms of *Di-nhr-7* were isolated (Crossgrove et al. 2002). Phylogenetic analysis shows that Di-nhr-7 is an orthologue of the DHR E78 (NR1E/G), EMSAs show that Di-NHR-7 can bind to Rev-erb response elements Rev-DR2 as a dimer and Rev-DR4 as a monomer. Northern blot analysis shows that Di-nhr-7 expression is female-specific (Crossgrove et al. 2002).

DiEcR (GQ149497, GQ149498, GQ149499) (NR1H): cDNA of three isoforms of an orthologue of *Drosophila* EcR (NR1H) were isolated (Shea et al. 2010). Pull-down experiments showed that DiEcR could form a heterodimer with the Di-RXR-1 and mosquito *Aedes aegypti* USP. EMSA showed that DiEcR required USP as a partner to bind hsp27 EcRE (ATTGGACAAGTGCATTGAACCCTTGCTCT). DiEcR could activate transcription of a reporter gene in a ligand-dependent manner when utilizing a 9-cis retinoic acid-activated mouse RXR as a partner in mammalian cells (Shea et al. 2010).

Di-RXR-1 (AF438229, AF438230) (NR2B): Di-RXR-1 was the first RXR/USP orthologue (NR2B) characterized in nematodes (Shea et al. 2004). Di-RXR-1 mRNA was detected in adult females but not in males. It could dimerize with *Drosophila* EcR and transcription was activated in the presence of 20-hydroxyecdysone using *Drosophila* Schneider S2 cells in an ecdysone-dependent manner (Shea et al. 2004).

B. NRs in *Meloidogyne incognita*—*M. incognita* (root-knot nematode) is a plant nematode parasite which attacks the roots of plants. Ninety-two NRs are predicted in the *M. incognita* genome with six orthologues of *Drosophila*/vertebrate NRs including two homologues of ROR (NR1F), two homologues of DHR96 (NR1I/J/K), a TLL (NR2E) and a fax-1 (NR2E) member (Table 1). Forty-one members are found to be orthologues of nematode SupNRs, 14 of them are shown to be orthologues of *C. elegans* and/or *B. malayi* (Abad et al. 2008). No further data for *M. incognita* NRs are currently available.

C. NR in parasitic nematode threadworm and hookworms

a. Orthologues of *C. elegans* DAF12: *C. elegans* larvae can undergo arrest and form dauer larvae. The infective larvae from nematodes (eg, L3 larvae of *Strongyloides*, *Ancylostoma* and *Necator*) also undergo arrest and are strikingly similar to dauer larvae (Hotez et al. 1993). The nuclear receptor DAF-12 binds the steroid hormone dafrachonic acid to regulate dauer formation in *C. elegans*. Orthologues of *C. elegans* DAF-12 have been identified in the human parasitic threadworm *Strongyloides stercoralis*, and parasitic hookworms *Ancylostoma duodenale* (human hookworm), *A. ceylanicum* (panspecific hookworm), *A. caninum* (dog hook worm) and *Necator americanus* (human hookworm) (Strube et al. 2007; Wang et al. 2009). The full length cDNAs of the orthologue of DAF-12 were isolated in *S. stercoralis* (SsDAF-12, AF145048, BG225375) and *A. caninum* (AcDAF-12, BQ125240) (Wang et al. 2009), partial cDNAs of that were identified in *A. ceylanicum* (AceDAF-12, CB276910) and *Necator americanus* (NaDAF-12) (Strube et al. 2007; Wang et al. 2009). *In vivo* and *in vitro* experiments show that the ligand of *C. elegans* DAF-12, Δ^4 dafachronic acid (Δ^4 -DA) and Δ^7 dafachronic acid (Δ^7 -DA) (Motola et al. 2006), can activate AcDAF-12 and SsDAF-12 (Wang et al. 2009). Interestingly, dafrachonic acid treatment of *Strongyloides* L3 larvae resulted in a reduction in the infective L3 population. This coupled with the crystal structure of the SsDAF-12 LBD complexed with a coactivator LXXLL peptide and Δ^4 -DA or Δ^7 -DA showing that the architecture SsDAF-12 LBD is similar to other members of the NR superfamily, identifies a target for novel drug therapy (Wang et al. 2009).

b. *Haemonchus contortus* EcR (HcEcR) (GU250809) (NR1H): cDNA of an orthologue of *Drosophila* EcR (NR1H) was isolated in *H. contortus*, a parasitic hookworm of ruminants (Graham et al. 2010) (Graham et al. 2010). The D-E-F domains of HcEcR could not form a heterodimer with D-E-F domains of *Drosophila* USP and did not bind to [3 H] ponasterone A in an insect cell system (Graham et al. 2010). The demonstration of the ability to heterodimerize awaits the identification of an appropriate partner.

3. Evolution of NRs in helminths

Helminths form a polyphyletic group containing two different lineages in the Protostomia: Platyhelminths belong to the lophotrochozoan clade and Nematoda belong to the ecdysozoan clade. Schistosome genome projects were the first to describe genomes from the lophotrochozoan clade (Berriman et al. 2009a; Liu et al. 2009). Phylogenetic analysis of the NR complement in both *S. mansoni* and *S. japonicum* shows that most of NRs in parasitic Platyhelminths have orthologues either in Deuterostomes, in arthropods or both. For example, 17 of 21 *S. mansoni* NRs have homologues in *Drosophila* and/or chordates (Wu et al. 2006). Interestingly, one divergent *Schistosoma* member, SmNR1 (Wu et al. 2007c), and

its *S. japonicum* orthologue (SjNR1) evolved specifically in subfamily 1. This member was not found in other Protostomia (including other Platyhelminths, unpublished data), which suggests that SmNR1 and SjNR1 originated from a common NR1 ancestor after the Platyhelminths diverged from other lophotrochozoans or after trematodes diverged from other Platyhelminths (no data currently available from other trematodes). Comparison of the NR complement in *S. mansoni* and *S. japonicum* with that of insects (Bonneton et al. 2008; Cheng et al. 2008; Tan & Palli 2008; Velarde et al. 2006) shows that many of the schistosome NRs appeared by gene duplication within gene groups while almost no gene duplication occurred within gene groups in insects. Thus though schistosomes and insects have a similar number of NRs, fewer NR gene groups are present in schistosome NRs (Fig. 3a and 3b). This suggests that gene lost and duplication which is more frequent in schistosomes than insects may have been an adaptation to the parasitic life style. This conjecture awaits further data.

NRs regulate transcription by recognizing and binding to the DNA element of their target genes. The functional relationship between RXR/USP with other NRs was described as the 1-2-3-4-5 rule (Shulemovich et al. 1995; Umesono et al. 1991; Wilson et al. 1992). For example, vertebrate RXR/RAR heterodimer can bind to DR1, DR2 and DR5 but not to DR3 or DR4, RXR/VDR heterodimer can bind to DR3 but not to DR1, DR2, DR4 or DR5 (Andersson et al. 1992; Desvergne 1994; Predki et al. 1994; Schrader & Carlberg 1994; Shaffer & Gewirth 2004). *Drosophila* USP/EcR heterodimer can bind to DR0-DR5 (Wilson et al. 1992). In schistosomes, SmNR1/SmRXR1 heterodimer can bind to DR0-DR5 (Wu et al. 2007c) similar to the *Drosophila* USP/EcR heterodimer (Wilson et al. 1992). Vertebrate TR homodimer can bind to DR4 but not DR1, DR2, DR3 or DR5 (Andersson et al. 1992; Naar et al. 1991; Sap et al. 1989; Umesono et al. 1991), while SmTR homodimer can bind to DR0-DR5 (Wu et al. 2007a). These results suggest that the RXR/NR heterodimer and TR homodimer acquired the ability to bind to conserved half-site repeats before the split of the Platyhelminths and Arthropods, but has not subsequently evolved a specificity for strict spacing between half-sites as that in vertebrates (Wu et al. 2007a; Wu et al. 2007c).

Unlike NRs in ecdysozoan Arthropods (most data are from the Insecta), free living nematodes *Caenorhabditis elegans* and *C. briggsae* contain more than 250-270 NRs, most of them originated from a unique burst of HNF4 duplications (supplementary NRs, SupNRs) (Robinson-Rechavi et al. 2005). *Caenorhabditis* sp. only exhibit 12-13 conserved NRs (Bertrand et al. 2004; Maglich et al. 2001; Sluder & Maina 2001; Sluder et al. 1999; Stein et al. 2003). Among the 92 predicted NRs in the plant parasitic nematode *M. incognita*, only six NRs share homologues with *Drosophila*/vertebrates. Forty-one members are homologues of nematode SupNRs (Abad et al. 2008). Of 27 *B. malayi* NRs, less than half are orthologues of *Drosophila*/vertebrates NRs (Ghedini et al. 2007), some *B. malayi* NRs are homologues of nematode SupNRs of *C. elegans* and/or *M. incognita* (Abad et al. 2008; Ghedini et al. 2007). These data suggest that the expansion of SupNRs occurred before the split of free living *Caenorhabditis*, animal parasitic nematode *Brugia* and plant parasitic nematode *Meloidogyne* and then multiple duplication events occurred independently in different nematode lineages (Abad et al. 2008; Bird et al. 2009).

The above data demonstrates that the NRs in parasitic helminths diverged into two different evolutionary lineages. NRs in parasitic Platyhelminths (data only available for schistosome) have orthologues either in Deuterostomes, in arthropods or both with a feature of extensive gene loss and gene duplication within different gene groups. NRs in parasitic nematodes follow the nematode evolutionary lineage with a feature of multiple duplication of SupNRs and gene loss.

With the increase in the genomic information from helminths available to the scientific community, we can expect a concomitant increase in the number of studies that elucidate the role of nuclear receptors in host-parasite interactions. For example, the identification of DAF-12 as a NR that governs the development of the L3 infective pathway and offers a novel therapeutic target (Wang et al. 2009).

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Figure 1. Sequence alignment of the DNA binding domain of selected NRs showing the two zinc fingers and the conserved motifs

CI: the first zinc finger with a conserved motif sequence of Cys-X2-Cys-X13-Cys-X2-Cys, CII: the second zinc finger with a conserved motif sequence of Cys-Xx-Cys-X9-Cys-X2-Cys. Cys: cysteine residue, X followed with a number indicates the number of amino acids between the Cys. Xx indicates the variation in the number of amino acids in the D-box (herein shows 3-9 amino acids). Sm2DBDα1: The first DBD of Sm2DBDNrα. The dotted boxes indicate the amino acid sequences of P and D boxes.

Figure 2. The DNA binding domain (DBD) of 2DBD-NR

Circles represent amino acids. Black circles with C represent the cysteines that contact Zn^{++} . Circles without a letter represent the amino acids outside the DBD. The dotted boxes indicate the P and D boxes. The 2-DBD domain shown in the figure is that of Sm2DBD-NR α .

Figure 3. A schematic figure and an unrooted phylogenetic tree representing *S. mansoni* nuclear receptors

a Schematic figure summarizes the identification of the NRs in *S. mansoni*, and provides a comparison with NRs in *Drosophila melanogaster* and *Homo sapiens*. Each square box indicates a NR member. Dm indicates *Drosophila melanogaster*, Hm indicates *Homo sapiens* and Sm indicates *S. mansoni*. **b**. Unrooted Bayesian tree analysis of NRs in *S. mansoni*. The phylogenetic tree was constructed from peptide sequences of the DBD, peptide sequences were aligned with ClustalW (<http://www.cf.ac.uk/biosi/research/biosoft/Downloads/clustalw.html>). The data set was carried out under the proportion of invariable sites and gamma-distributed rate heterogeneity among sites with a mixed amino acid replacement model using MrBAYES v3.1.1 (Huelsenbeck & Ronquist 2001). The trees were started randomly; four simultaneous Markov chains were run for 5 million generations with the tree being sampled every 100 generations. Bayesian posterior probabilities (PPs) were calculated using a Markov chain Monte Carlo (MCMC) sampling approach implemented in MrBayes v3.1.1, with the burn-in values set at 7,500 and shown at the branch nodes. Two human NR0B members (without DBD) were not analyzed. DHR or d before the NR name indicate *Drosophila melanogaster*

NRs, h indicates human NRs and Sm indicates *S. mansoni* NRs. The dots indicate the number of *S. mansoni* NRs found in different NR groups, a star shows a divergent *S. mansoni* NR member and +++ indicate the second DBD of 2DBD-NRs.

Tables 1

Comparison of NRs from helminths and other species

group	<i>S. mansoni</i>	<i>S. japonicum</i>	<i>D. melanogaster</i>	<i>C. intestinalis</i>	<i>H. sapiens</i>	<i>C. elegans</i>	<i>B. malayi</i>	<i>M. incognita</i>
0A			Knirps KNRL Eagle					
0B					DAX1 SHP			
1A	TRa TRb	TRa TRb		TR	TRa TRb			
1B				RAR	RARa RARb RARG			
1C				PPAR	PPARa PPARb PPARG			
1D			E75	Reverb	Reverba Reverbb	Nhr-85	BmNHR11	
1E	E78	E78	E78			sex-1	BmNHR7	
1G								
1F			DHR3	ROR	RORa RORb RORG	HR3	BmNHR13	Minc10028 Minc03383
1H			EcR	LXR FXR	LXRa LXRb FXRa		BmNHR3	
1I					VDR			

group	<i>S. mansoni</i>	<i>S. japonicum</i>	<i>D. melanogaster</i>	<i>C. intestinalis</i>	<i>H. sapiens</i>	<i>C. elegans</i>	<i>B. malayi</i>	<i>M. incognita</i>
					PXR CAR			
1J+K	HR96a (SmCAR) HR96b	HR96a HR96b	DHR96	NR1K1 NR1K2		DAF-12 NHR-8 NHR-48	BmNHR17	Minc18589 Minc13296
NR1-un classified	SmNR1	SjNR1						
2A	HNF4	HNF4	HNF4	HNF4	HNF4a HNF4g	supnrsa	supnrs	supnrs
2B	RXR1 RXR2	RXR1 RXR2	USP	RXR	RXRa RXRb RXRg		BmNHR4	
2C/D	TR2/4	TR2/4	DHR78	TR2/4	TR2 TR4	NHR-41		
2E	TLL PNR DSF	TLL PNR DSF	TLL PNR DSF FAX-1		TLL PNR	NHR-67	BmNHR15	Minc12751
2F	Coup-TFI Coup-TFII	Coup-TFI Coup-TFII	SVP	COUP-TF	COUP-TFa COUP-TFb EAR2	unc-55	BmNHR25	Minc02801
3A					ERa ERb			
3B			ERR	ERR	ERRa ERRb ERRg			
3C					GR			

group	<i>S. mansoni</i>	<i>S. japonicum</i>	<i>D. melanogaster</i>	<i>C. intestinalis</i>	<i>H. sapiens</i>	<i>C. elegans</i>	<i>B. malayi</i>	<i>M. incognita</i>
					MR PR AR			
4A	NR4A5	NR4A5	DHR38	NR4A5	NGFIB NURR1 NOR1	CNR8	BmNHR9	
5A	FTZ-F1-a	FTZ-F1-a	FTZ-F1	NR5A6	SF1 LRH1	FTZ-F1		
5B	FTZ-F1	FTZ-F1b	DHR39					
6A			GRF	GCNF	GCNF	GCNF		
2DBD	2DBD-NRa 2DBD-NRb 2DBD-NRg 2DBD-NR (4th) 2DBD-NR-like	2DBD-NRa 2DBD-NRb 2DBD-NRg 2DBD-NR (4th) 2DBD-NR-like						
total	21	23-24*	21	17	48	13 + supurs		

* There is one 2DBD-like member where the sequences are not in the same config. As we could not determine if these sequences belong to same NR or to different NRs, we list the number of Sj NR as 23-24. (If this 2DBD-like member is a true 2DBD-NR, there will be 23 members. If this 2DBD-like member is a not 2DBD-NR, the two different DBD will each represent a different NR, so there will be 24 members in *S. japonicum*.)