Int. J. Dev. Biol. 46: 687-692 (2002)

Evolution and developmental expression of nuclear receptor genes in the ascidian *Herdmania*

CHRISTINE DEVINE, VERONICA F. HINMAN and BERNARD M. DEGNAN*

Department of Zoology and Entomology, University of Queensland, Brisbane, Australia

ABSTRACT Nuclear receptors are a superfamily of metazoan transcription factors that have been shown to be involved in a wide range of developmental and physiological processes. A PCR-based survey of genomic DNA and developmental cDNAs from the ascidian *Herdmania* identifies eight members of this multigene family. Sequence comparisons and phylogenetic analyses reveal that these ascidian nuclear receptors are representative of five of the six previously defined nuclear receptor subfamilies and are apparent homologues of retinoic acid [NR1B], retinoid X [NR2B], peroxisome proliferator-activated [NR1C], estrogen related [NR3B], neuron-derived orphan (NOR) [NR4A3], nuclear orphan [NR4A], TR2 orphan [NR2C1] and COUP orphan [NR2F3] receptors. Phylogenetic analyses that include the ascidian genes produce topologically distinct trees that suggest a redefinition of some nuclear receptor subfamilies. These trees also suggest that extensive gene duplication occurred after the vertebrates split from invertebrate chordates. These ascidian nuclear receptor genes are expressed differentially during embryogenesis and metamorphosis.

KEY WORDS: *retinoic acid receptor, urochordate, metamorphosis, chordate gene evolution*

Introduction

The nuclear receptor superfamily is represented by at least 70 distinct members, all of which have the ability to bind selectively to DNA (Gronemeyer and Laudet, 1995; Sluder *et al.*, 1999). In addition to the DNA-binding domain (DBD), nuclear receptors consist of a ligand-binding domain (LBD), which has the ability to bind specific lipophilic hormones. As such, these receptors provide a direct link between lipophilic signalling molecules and the transcriptional response (Mangelsdorf *et al.,* 1995). In addition, an increasing number of orphan receptors have been described from a range of metazoan taxa (Laudet and Adelmant, 1995; Mangelsdorf and Evans, 1995; Escriva *et al.,* 2000; Grasso *et al.,* 2001).

Nuclear receptors regulate a wide range of developmental and physiological processes in metazoans. For example, in vertebrates, the retinoid signalling pathway, mediated by the retinoic acid receptors RAR and RXR, is involved in the regulation of *Hox* gene expression and pattern formation (Mendelsohn *et al.,* 1992; Mangelsdorf *et al.,* 1994; Conlon, 1995). They may therefore be important regulators of body plan patterning and cell specification, and analysis of their function is likely to contribute to knowledge of the evolution of body plan diversity (Shimeld, 1996; Hinman and Degnan, 2001).

A phylogenetic survey of nuclear receptor genes using degenerate polymerase chain reaction (PCR) primers that anneal to the most conserved regions of the open reading frame, i.e. the DNA binding domain (DBD), suggests that nuclear receptors emerged during the earliest stages of metazoan evolution prior to the divergence of cnidarians and bilaterians (Escriva *et al.,* 1997, 2000; Laudet, 1997; Grasso *et al.,* 2001). Phylogenetic analysis of the DBD sequence divides the nuclear receptor multigene family into six subfamilies: (1) a large group comprising thyroid (TR), retinoic acid (RAR), peroxisome proliferator activated (PPAR), vitamin D (VDR) and ecdysone (EcR) receptors; (2) one containing retinoid X receptor (RXR), COUP-TF and HNF-4; (3) one clustering steroid, estrogen (ER) and estrogen related orphan (ERR) receptors; (4) the NGFIB group of orphan receptors; (5) SF1, FTZ-F1 and DHR39; and (6) the GCNF1 orphan receptor (Escriva *et al.,* 1997; Laudet, 1997). Five of the six defined nuclear receptor families are present in the genomes of a diverse range of bilaterians (Kostrouch *et al.,* 1995; Thummel, 1995; Laudet, 1997; Sluder *et al.,* 1999), suggesting that this receptor superfamily was well diversified prior to the divergence of protostomes and deuterostomes.

The nuclear receptor gene family appears to have grown from two distinct gene duplication events, the first prior to the divergence of the cnidarians and bilaterians, and the second following the divergence of the vertebrate and protochordate lineages (Escriva *et al.,* 1997, 2000). Phylogenetic analyses of a number of gene families have demonstrated that one to several rounds of gene duplication have occurred during the evolution of the vertebrates

***Address correspondence to:** Bernard Degnan. Department of Zoology and Entomology, University of Queensland, Brisbane, QLD 4072, Australia. FAX: +61-7-3365-8515. e-mail: bdegnan@zen.uq.edu.au

0214-6282/2002/$25.00

Printed in Spain
www.ijdb.ehu.es

after they diverged from the cephalochordates (eg. Holland *et al.,* 1994; Sharman and Holland, 1996). Ascidians, as representative urochordates and like cephalochordates, typically possess only one copy of a gene for which several vertebrate paralogues occur (e.g. Yuasa *et al*., 1998; Meedel *et al*., 1998; Chan *et al.,* 1990). As ascidians are the most evolutionary distant group from vertebrates to display a chordate body plan, a comparison of ascidian nuclear receptor genes with vertebrate paralogues will allow inferences of the ancestral role of these genes in chordates.

We have identified eight nuclear receptor genes from the ascidian *Herdmania*. Classification of their derived amino acid sequences indicates that the ascidian possesses representatives of five of the six previously defined nuclear receptor subfamilies (Laudet, 1997). Following the addition of ascidian sequences, phylogenetic analysis demonstrates a disruption in the accepted clustering of receptor groupings. On this basis we suggest a revision of the classification scheme for the nuclear receptor superfamily be considered. In this new scheme, eight metazoan subfamilies and chordate orthology groups are defined. We further demonstrate that these genes are differentially expressed through ascidian development and are therefore likely to have significant roles in regulating a range of developmental processes, some of which may be chordate specific.

Results

Sequence Analysis of Nuclear Receptor Genes

The degenerate primers used in this study were designed to amplify 135 bp of the DBD of a range of nuclear receptor genes, which yielded 81 bp (135 bp minus priming sites) of sequence information. PCR amplification of *Herdmania* genomic DNA yielded three bands of 135, 220 and 270 bp length, of which the smallest was the most predominant. 55 clones were sequenced from these 3 bands (30, 12 and 13 from the 130, 220 and 270 bp bands respectively). PCR amplification of cDNA derived from five developmental stages (neurula and early tailbud embryos and 4 h, 1 day and 3 day postlarvae) yielded a single 135 bp band in each stage. At least twenty clones containing an insert of the correct size were sequenced from each stage.

Sequence analysis of 169 nuclear receptor-containing clones derived from the PCR amplification of genomic DNA (55 clones) and cDNA (114 clones), identified eight unique consensus sequences. The derived amino acid sequence of each of these had a significant degree of identity to previously isolated members of the nuclear receptor superfamily (Fig. 1). All of the *Herdmania* sequences possessed highly-conserved, diagnostic amino acids present in all nuclear receptors, including two cysteine (C) residues flanking the D-box at positions 37 and 43 of the DBD, two arginine residues at positions 27 and 30 and a tyrosine residue at position 35 (Fig. 1). These partial cDNA sequences were named according to the nuclear receptor sequences to which they were most closely related; all had the prefix *Hec-* added to designate they were identified from *Herdmania curvata.* The eight genes are: *Hec-RAR* [NR1B], *Hec-RXR* [NR2B], *Hec-PPAR* [NR1C], *Hec-COUP* [NR2F3]*, Hec-ERR* [NR3B], *Hec-NOR* [NR4A3], *Hec-NR* [NR4A], and *Hec-TR2* [NR2C1] (Fig. 1; receptor names in brackets follow the naming convention put forward by the Nuclear Receptors Nomenclature Committee, 1999).

All eight ascidian nuclear receptor genes were amplified from the developmental cDNAs. *Hec-COUP, Hec-TR2* and *Hec-PPAR* were derived from the 135 bp band in genomic DNA. The 220 bp band was comprised of two members of the nuclear receptor superfamily: a nuclear receptor homologue (*Hec-NR)* and an estrogen-related receptor homologue (*Hec-ERR).* Comparisons of genomic DNA and cDNA sequences revealed the presence of an 85 bp intron present in both genes (not shown). While the size of the intron was the same in these two sequences, the splice site was not. The intron site for *Hec-NR* was located directly after the first lysine residue following the 5' primer site while the *Hec-ERR* was located at the glutamine residue (Fig. 1). All sequences obtained from the largest band (270 bp) matched most closely to the vertebrate neuron-derived orphan receptor (NOR). The splice site for the 135 bp intron was the same as in *Hec-NR* (Fig. 1).

The ascidian retinoic acid receptor (*Hec-RAR*) and retinoid X receptor (*Hec-RXR*) homologues were isolated only from cDNA, suggesting an intron may exist in the amplified portion of these genes. Using gene specific oligonucleotide primers, RAR-1 and RAR-2 (Table 1), it was possible to amplify a 329 bp fragment of *Hec-RAR* from genomic DNA, enabling the analysis of intron sequence for this region. Subsequent investigation revealed an intron in the RR-2 primer site, which was sufficient to inhibit efficient binding of this oligonucleotide primer to *Hec-RAR* genomic DNA (not shown). An intron was not mapped in *Hec-RXR*.

```
                        *     *
Hec-RXR      KRTVRKDLTYTCRDNKDCVIDKRQRNR
mRXRa        -----------------L--------   96/100
zfRXRg       -----------------M--------   96/100
zfRXRa       ---I--------------Q--------   93/100
Hec-RAR      R-S-Q-NMQ---HR--N---N-ST-S-
PmRAR        R-S-Q-NMQ---HR--N---N-ST-S-  100/100
mRARa        --SIQ-NMV---HRD-N-I-N-VT---   74/96
cRARb        --SIQ-NMV---HRD-N---N-VT---   78/96
Hec-COUP     --S--RN------G-RN-PM-QHH--Q
mCOUP        --S--RN------A-RN-P--QHH--Q   93/96
XlCOUP       --S--RN------A-RN-P--QHH—-Q   93/100
Hec-TR2      --SI--S-I-S--G-R--PVN-AH---
mTR2         --SI--N-V-S--GS-----N-HH---   74/93
Hec-NOR      ----Q-NSK-V-LA--N-PV---R---
rNOR-1       ----Q-NAK-V-LA--N-PV---R---   96/96
hNOR-1       ----Q-NAK-V-LA--N-PV---R---   96/96
Hec-ERR      ---IQGSID---PASN--E-T--R-KS
mERRa        ---IQGSIE-S-PASNE-E-T--R-KS   82/96
hERR-2       ---IQGNIE-S-PATNE-E-T--R-KS   78/96
Hec-NR       --S-Q-NAQ-V-LG--N-P---KT-TH
rNR          ----Q-NAK-V-LA--N-PV---R---   81/96
mSTOR        ----Q-NAK-V-LA--N-PV---R---   81/96
Hec-PPAR     R----MR-K-KE.CEIG-K-NVKS--K
XlPPARb      R--I-MK-E-EK.C-RS-K-Q-KN--K   62/88
rPPARd        R--I-MK-K-EK.CDRI-K-Q-KN--K   65/88
```

Fig. 1. Alignment of derived amino acid sequences of *Herdmania* nuclear receptors (partial DNA binding domain only) with closest matches in the Genbank/EMBL database. *All sequences are aligned to Hec-RXR. Dashes indicate amino acid identity with Hec-RXR. Asterisks indicate highly conserved C residues in the DNA binding domain. Dots indicate gaps in the sequence. Paired values at the end of each line indicate (from left to right) the percent amino acid identity to the cognate ascidian nuclear receptor and the percent similarity with conservative amino acid changes included (i.e. I-V-L; T-S; R-K; D-E; N-Q; F-Y-W). ch, chick; Dm, Drosophila; h, Homo sapiens; Hec, Herdmania curvata; m, mouse; Pm, Polyandrocarpa misakens (ascidian); r, rat; Xl, Xenopus laevis; zf, zebrafish; All sequences obtained from the NCBI GenBank database.*

Phylogenetic Analysis of Herdmania *Nuclear Receptors*

Phylogenetic analysis of the *Herdmania* partial nuclear receptor sequences with the DBDs of other metazoan nuclear receptors allowed the assignment of the ascidian genes to nuclear receptor subfamilies (Fig. 2). The tree was mid point-rooted due to difficulties in assigning an outgroup (Escriva *et al.,* 1997). Using the neighbour-joining method and the nuclear receptor classification scheme proposed by Laudet (1997) we assigned the eight *Herdmania* genes into five of the six previously classified receptor subfamilies (Fig. 2). The remaining sixth subfamily, GCNF1, for which no ascidian member was found, has to date only been identified from representative vertebrate species (Chen *et al.,* 1994; Dreyer and Ellinger-Ziegelbauer 1996), and thus may represent a vertebrate specific nuclear receptor subfamily.

Many of the *Herdmania* nuclear receptor gene products clustered as an outgroup to their respective vertebrate homologues (Fig. 2). For example, while the ascidian RAR homologues (Hec-RAR and PmRAR) clustered with those isolated from vertebrate representatives, they were located on a separate branch to the various vertebrate RAR subtypes (α, β and γ). This arrangement was also observed for Hec-PPAR, Hec-NOR and Hec-COUP. In contrast, Hec-RXR is nested within the vertebrate RXR paralogues.

Bootstrap analyses based on both distance measures and assuming parsimony failed to find high support for any of the deepest nodes. The receptor sequences that comprise the previously classified subfamily I (Laudet, 1997) did not cluster together. Members of the PPAR, RAR and EcR groups of nuclear receptors form three distinct groups (Fig. 2) when the ascidian sequences were included in the analyses. The branch lengths between these groups is similar in length to those between other nuclear receptor subfamilies (Fig. 2). Parsimony analysis yielded similar groupings as for distance analysis (data not shown).

Differential Expression of Nuclear Receptors during Development

RT-PCR analysis of the temporal expression of each of the *Herdmania* nuclear receptor genes during embryonic and metamorphic development was conducted using gene-specific oligonucleotide primers (Table 1). This analysis allowed only the determination of gross changes of relative abundance of the different transcripts. Nonetheless, dynamic patterns of temporal expression were observed for most genes (Fig. 3).

Nuclear receptor transcripts were not detected in the fertilized egg and 32-cell embryo. *Hec-Sox* (Degnan, 1997) was included as a positive control for cDNA synthesis as it is expressed in all stages of development (Fig. 3). *Hec-ERR, Hec-NR* and *Hec-NOR* transcripts were detected from the 64-cell stage onwards (Fig. 3). Expression of *Hec-ERR* was reduced in the neurula stage, which contrasted with the pattern observed for many of the other nuclear receptor genes, which were expressed at higher levels at this stage.

RXR, a common heterodimer partner for a number of nuclear receptors including RAR, COUP, and PPAR (Mangelsdorf and Evans, 1995), was expressed concomitantly with *Hec-TR2*, *Hec-NR, Hec-NOR, Hec-RAR* and *Hec-ERR* from the 64-cell stage through to early metamorphosis (7 h postlarva). *Hec-RXR* mRNA was not observed after the 7 h postlarval stage.

The expression of *Hec-COUP* and *Hec-PPAR* appeared to be more developmentally restricted, with *Hec-COUP* transcripts being detected chiefly in the postlarval stages of development and *Hec-PPAR* expression restricted to embryogenesis (Fig. 3).

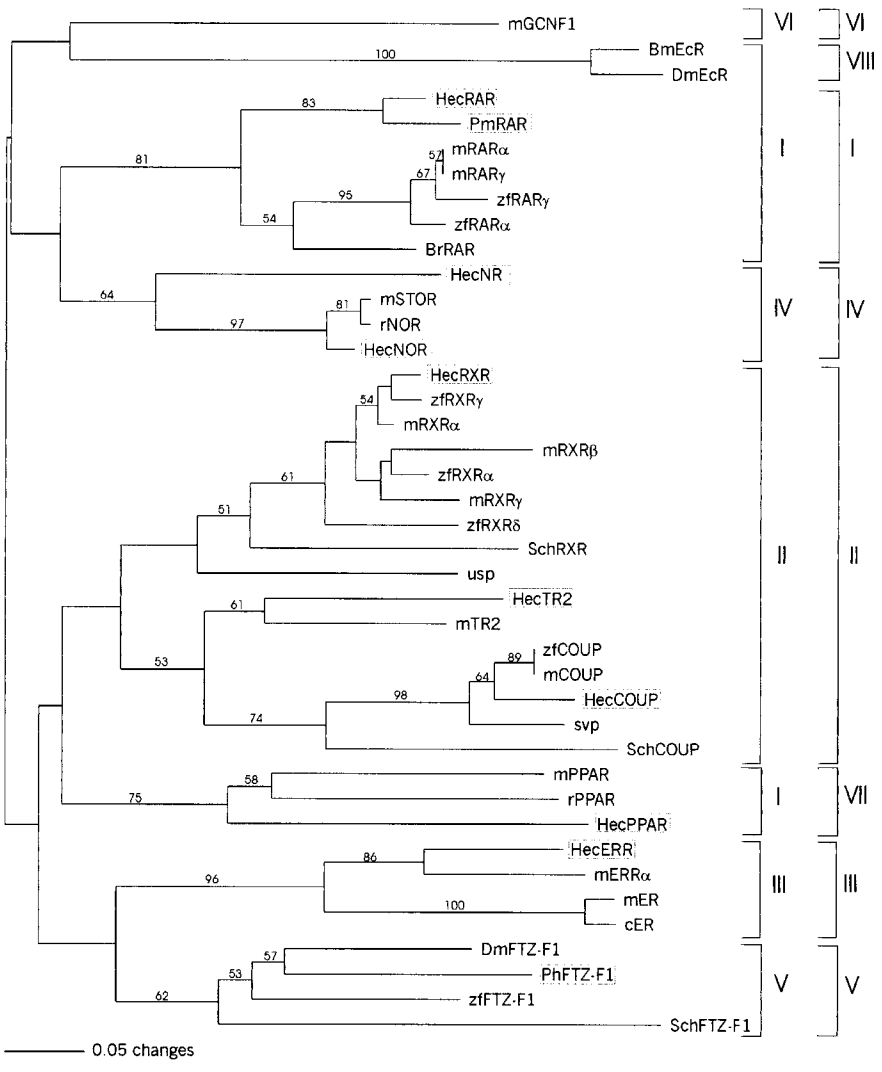

Fig. 2. Neighbour-joining tree based on the alignment of 27 derived amino acid sequences of the DNA-binding domain of nuclear receptors identified from *Herdmania* and other deuterostome and protostome representatives. *Numbers indicate the number of times a node was supported by 100 bootstrap replications. Tree is midpoint rooted. Taxa abbreviations as in Fig.1; Br, Branchiostoma lanceolatum (amphioxous); Ph, Phallusia mamillata (ascidian); Sch, Schistosoma mansoni (flatworm). Ascidian sequences are boxed. Proposed classification scheme shown to the right is that of Laudet (1997).*

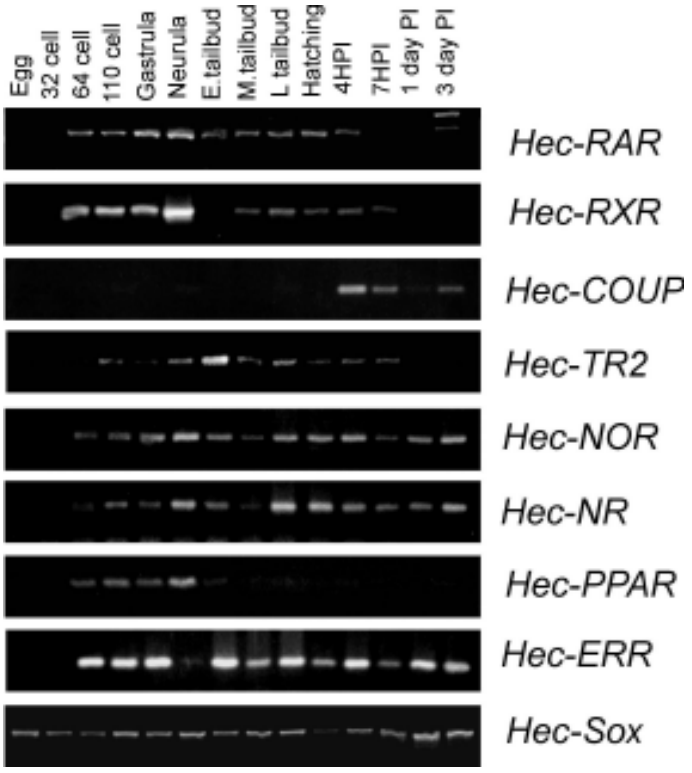

Fig. 3. RT-PCR analysis of nuclear receptor gene expression during *Herdmania* development. *Stages 32-cell to late tailbud comprise embryonic development; all stages proceeding hatching comprise postlarval development. Hec-Sox is used as a control for cDNA synthesis, being expressed maternally and throughout development.*

Discussion

Nuclear receptors have been identified as key molecules involved in a number of aspects of cellular homeostasis, differentiation, and development (Evans, 1988; Green and Chambron, 1988; Mangelsdorf *et al.,* 1995). While the current nuclear receptor classification has been used extensively as a means to evaluate nuclear receptor diversity in various animal taxa (Escriva *et al.,* 1997, 2000; Laudet, 1997), the phylogenetic basis of this scheme is derived from evolutionary trees based chiefly on vertebrate sequences. Nonetheless, it is clear that a majority of the six previously defined nuclear receptor subfamilies are present in the genomes of diploblasts, protostomes and deuterostomes (Kostrouch *et al.,* 1995; Thummel, 1995; Laudet, 1997; Sluder *et al.,* 1999; Grasso *et al.*, 2001), suggesting that this receptor superfamily had initially diversified during early metazoan evolution. Along vertebrate and nematode lineages further gene duplication and divergence occurred. Analysis of the large number of nuclear receptor genes identified from *C. elegans* (more than 200) reveals that the majority are distinct from those currently known from other phyla, suggesting massive duplication and divergence in the *C. elegans* genome (Clarke and Berg, 1998; Sluder *et al.,* 1999). In vertebrates a more universal pattern of duplication and divergence has occurred such that there are multiple paralogous genes in each subfamily (Escriva *et al.,* 1997).

Inclusion of recently identified invertebrate nuclear receptor genes (e.g. Vlahou *et al.,* 1996; Escriva *et al.,* 1997, Sluder *et al.,* 1999; Grasso *et al.*, 2001) may provide further insight into the evolution of this gene family. Ascidians, as urochordates, are the most evolutionary distinct chordate clade from the vertebrates and as such and are an ideal group to compare with vertebrates to infer how nuclear receptors have evolved along the vertebrate and other chordate lineages.

Extensive screens of both genomic and cDNAs from a range of development stages revealed eight representatives of the nuclear receptor family from the ascidian *Herdmania.* As in other evolutionary studies of the nuclear receptor superfamily (Detera-Wadleigh and Fanning, 1994; Escriva *et al.,* 1997; Laudet, 1997) we elected to restrict our phylogenetic analyses to the highly conserved DBD. This conserved region of nuclear receptors is known to possess a number of diagnostic amino acid residues, facilitating the assignment of partial genomic and cDNA fragments to the various gene subfamilies (Laudet *et al.,* 1992; Detera-Wadleigh and Fanning, 1994; Escriva *et al.,* 1997; Laudet, 1997; Sluder *et al.,* 1999). Such phylogenies have permitted the identification of six subfamilies of nuclear receptors (Escriva *et al.*, 1997, 2000; Laudet, 1997). Using the classification scheme previously defined by Laudet (1997), ascidian nuclear receptor genes (including *Herdmania* nuclear receptor homologues, *Polyandrocarpa misakiensis* RAR (Hisata *et al.,* 1998) and *Phallusia mamillata* FTZ-F1 homologues (Escriva *et al.,* 1997) can be assigned to five of the six subfamilies of receptor molecules. A member of the one subfamily that we did not detect in our survey, the GCNF1 subfamily (subfamily VI), has only been identified from vertebrate representatives (Chen *et al.,* 1994; Dreyer *et al.,* 1996).

Molecular Evolution of Chordate Nuclear Receptor Genes

From the molecular phylogenetic tree it is apparent that many of the nuclear receptor sequences identified from *Herdmania* diverged from their respective vertebrate homologues prior to the origin of the paralogous vertebrate groups. Despite the identification of numerous members of the nuclear receptor superfamily from *Herdmania*, sequence analysis demonstrates that there is little evidence for the existence of paralogous versions of nuclear receptor genes in the ascidian genome. For example the ascidian RAR homologues *Hec-RAR* and *PmRAR*) cluster with the vertebrate RARs, but are on a separate branch to the various vertebrate RAR subtypes (RARα, RARβ, and RARγ), suggesting that multiple vertebrate RAR subtypes arose after the divergence of the urochordates. Only *Hec-RXR* does not sit outside the vertebrate group. The isolation of various single-copy nuclear receptor genes from amphioxus provides further support that duplication of nuclear receptor genes occurred after the divergence of the vertebrate and protochordate lineages (Escriva *et al.,* 1997).

The phylogenetic tree presented in Fig. 2 fails to conform to the classification scheme of Laudet (1997) due to an inability to resolve subfamily I. Previous studies have been restricted by the predominance of vertebrate nuclear receptor sequences (Laudet *et al.,* 1992; Escriva *et al.,* 1997; Laudet, 1997). In this tree, the receptor sequences that comprise subfamily I did not cluster together; members of the PPAR, RAR and EcR groups of nuclear receptors forming three distinct groupings (Fig. 2)**.** The disruption of subfamily I by the inclusion of ascidian sequences led us to implement a new classification scheme that recognises each of these groupings as subfamilies in their own right (RAR, subfamily I; EcR, subfamily

VII; PPAR, subfamily VIII). This alternative classification is shown to the right of the Laudet *et al.* (1997) classification in Fig. 2.

The removal of ascidian sequences caused the remaining nuclear receptor sequences to be clustered into the three groups reminiscent of the classification scheme proposed by Laudet *et al.* (1992). Thus, it seems reasonable that the inclusion of the ascidian sequences in this analysis may be responsible for the notable differences between the nuclear receptor groups presented here and those of Laudet (1997).

Developmental Expression of Herdmania *Nuclear Receptors*

Expression of many of the *Herdmania* nuclear receptor genes is first detected at the 64-cell stage. Expression is maintained throughout development for *Hec-ERR, Hec-NR* and *Hec-NOR,* suggesting an ongoing role. In contrast, the expression of the remaining *Herdmania* nuclear receptor homologues appears to be more developmentally restricted. Expression of *Hec-RAR, Hec-RXR* and *Hec-TR2* is restricted to embryogenesis and very early metamorphosis (i.e. postlarval development), *Hec-PPAR* and *Hec-COUP* are restricted to embryogenesis and metamorphosis, respectively. Within hours of the induction of metamorphosis, there is a marked decrease in *Hec-RAR*, *Hec-RXR* and *Hec–TR2* mRNA levels and increase in *Hec-COUP* transcript abundance. During this period of development, the larval notochord, neural tube and muscle have either undergone or are undergoing programmed cell death, and juvenile endodermal and mesenchymal primordia are beginning to migrate and differentiate (Cloney, 1982; Satoh, 1994). The restricted temporal expression of these genes suggests that they may have specific roles in the establishment of either or both the larval or juvenile body plan of *Herdmania.* Our inability to detect *Hec-RXR* and *Hec-TR2* in older postlarvae does not preclude a role for these genes later in metamorphosis, as their gene products may still be present.

Materials and Methods

PCR Amplification, Cloning and Sequencing

Gametes were isolated from adult animals, embryos and postlarvae cultured in 0.2 μm filtered seawater at 25ºC as described in Degnan *et al.* (1997). Genomic DNA and total RNA from a range of developmental stages was isolated as described in Hinman and Degnan (2000).

Highly conserved regions within the DBD of nuclear receptors compiled from human, mouse, zebrafish, chick, *Xenopus*, and ascidian published sequences revealed the highly conserved motifs, CEGCKGFF and CQYCRYQKC to which fully degenerate oligonucleotide primers, RR-1 and RR-2 were designed (Table 1). RT-PCR products were generated from 1 μg total RNA isolated from neurula and early tailbud embryos, and 4 h, 1 day and 3 day postlarvae as described in Hinman and Degnan (2000). PCR products were electrophoresed on a 2% agarose gel and bands of predicted size (135 bp) for RT-PCR reactions or all bands for PCR on genomic DNA were gel purified, blunt end cloned and sequenced using ABI prism dye terminator kit on a Perkin Elmer 373A DNA Sequencer.

TABLE 1

DETAILS OF OLIGONUCLEOTIDE PRIMERS USED IN THE AMPLIFICATION OF ASCIDIAN NUCLEAR RECEPTORS

Primer	Nucleotide Sequence	Annealing Temp ºC
RR-1	5' TAYWSNTGYGARGGNTGYAARGGNTTYTT 3'	45
RR-2	5' RCAYTTYTGRTANCGRCARTAYTGRCA 3'	45
RAR-1	5' CATGCAGTATACATGTCATCGC 3'	60
RAR-2	5' CTTGTTGAGGGAGGTAGAGAGTGG 3'	60
COUP-1	5' AAGCGAAGCGTTCGACGGAATCTGACG 3'	60
COUP-2	5' CTGTGGTGCTGGTCCATCGGACAG 3'	60
ERR-1	5' GCGGACTATACAAGGAAGCATCGAC 3'	60
ERR-2	5' GTTATTTCACAATCGTTCGAAGCCGGGC 3'	60
RXR-1	5' AAGAGAACAGTCAGGAAGGACCTCACC 3'	60
RXR-2	5' CCTATTGCGCTGTCGTTTGTCTATGACGC 3'	60
STOR-1	5' GGCTTCTTCAAGCGAACGGTAC 3'	60
STOR-2	5' CTGTTGCGTCTCCGTTTATCAACTGGAC 3'	60
PPAR-1	5' GCCGAACGGTTCGTATGAGACTCAAG 3'	60
PPAR-2	5' CGACAGTACTGGCATTTGTTCCTCG 3'	60
NR-1	5' AAGCGGTCAGCCCAAAAGAATGCTC 3'	60
NR-2	5' GTTCGCGTCTTCTTGTCGATGGGGCAG 3'	60
TR2-1	5' GAGGAGTATTCGAAAGAGTCTG 3'	60
TR2-2	5' TCTGTTTCTGTGCGCCTTGTTGACAGG 3'	60

Phylogenetic Analyses

Sequences isolated from genomic DNA and cDNA screens were compared with the Genbank/EMBL database by basic BLAST similarity search. Inferred amino acid sequences for each of the eight *Herdmania* nuclear receptor homologues were aligned with their top matches from the BLAST search using Clustal X (Thompson *et al.,* 1994). Alignments were viewed and confirmed by eye using MacClade 3.06 (Madison and Madison, 1992). Trees were created using the complete data set, and also using the data set excluding the ascidian sequences. Neighbour joining trees were created in PAUP* 4.0 (Swofford, 1998) using mean character difference as the distance measure. The confidence at each node was assessed by performing 100 bootstrap replications. Maximum parsimony analyses were also performed to confirm tree topology (Swofford, 1998).

RT-PCR Analysis of Gene Expression

Sequence specific primers were designed for each of the isolated *Herdmania* nuclear receptor genes (Table 1). RT-PCR with these primers was undertaken on cDNA synthesised from RNAs from a range of developmental stages (Hinman and Degnan, 2000). *Hec-RAR, Hec-NOR, Hec-NR* and *Hec-ERR* primers were designed to amplify across introns. *Hec-Sox* was used as a control for cDNA synthesis, since it is expressed maternally and throughout development (Degnan, 1997). RT-PCR was performed on at least two separate RNA preparations to authenticate the expression patterns. Negative controls were preformed using the above conditions but omitting reverse transcriptase from the cDNA synthesis.

Acknowledgements

We thank the staff of the Heron Island Research Station for their assistance in maintenance of animals. This research was supported by an Australian Research Council grant to B.M.D.

References

CHAN, S.J., CAO, Q.-P. and STEINER, D. F. (1990). Evolution of the insulin superfamily: cloning of a hybrid insulin/insulin-like growth factor cDNA from amphioxus. *Proc. Natl. Acad. Sci. USA* 87: 9319-9323.

CHEN, J.D., COONEY, A. J., WANG, Y., LAW, S. W. and O'MALLEY, B. W. (1994). Cloning of a novel orphan receptor (GCNF) expressed during germ cell development. *Mol. Endocrinol.* 8: 1434-1444.

CLARKE, N.D. and BERG, J. M. (1998). Zinc fingers in *Caenorhabditis elegans:* finding families and probing pathways. *Science* 282: 2018-2022.

CLONEY, R.A. (1982). Ascidian larvae and the events of metamorphosis. *Amer. Zool.* 22: 817-826.

CONLON, R.A. (1995). Retinoic acid and pattern formation in vertebrates. *Trends Genet.* 11: 314-319.

DEGNAN, B.M. (1997). Molecular analysis of invertebrate development and growth: identification of developmentally-regulated genes in model and commercially important species. In *Molecular Approaches to the Study of the Ocean* (Ed. Cooksey, K. E.). Chapman and Hall, London, pp. 343-358.

DEGNAN, B. M., SOUTER, D, DEGNAN, S. M. and LONG, S. (1997). Induction of metamorphosis with potassium ions requires the development of competence and

an anterior signalling centre in the ascidian *Herdmania momus. Dev. Genes Evol.* 206: 370-376.

DETERA-WADLEIGH, S.D. and FANNING, T.G. (1994). Phylogeny of the steroid receptor superfamily. *Mol. Phylogenet. Evol.* 3: 192-205.

DREYER, C. and ELLINGER-ZIEGELBAUER, H. (1996). Retinoic acid receptors and nuclear orphan receptors in the development of *Xenopus laevis. Int. J. Dev. Biol.* 40: 255-262.

ESCRIVA, H., DELAUNAY, F. and LAUDET, V. (2000). Ligand binding and nuclear receptor evolution. *BioEssays* 22: 717-727.

ESCRIVA, H., SAFI, R., HANNI, C., LANGLOIS, M., SAUMITOU-LAPRADE, P., STEHELIN, D., CAPRON, A., PIERCE, R. and LAUDET, V. (1997). Ligand binding was acquired during the evolution of nuclear receptors. *Proc. Natl. Acad. Sci. USA* 94: 6803-6808.

EVANS, R.M. (1988). The steroid and thyroid hormone receptor superfamily. *Science* 240: 889-895.

GRASSO, L. C., HAYWARD, D. C., TRUEMAN, J. W. H., HARDIE, K. M., JANSSENS, P. A. and BALL, E. E. (2001). The evolution of nuclear receptors: evidence from the coral *Acropora. Mol. Phylogen. Evol.* 21: 93-102.

GREEN, S. and CHAMBON, P. (1988). Nuclear receptors enhance our understanding of transcriptional regulation. *Trends Genet.* 4: 309-314.

GRONEMEYER, H. and LAUDET, V. (1995). Transcription factors 3: nuclear receptors. *Protein Profile 2:* 1173-1308.

HINMAN, V. F., and DEGNAN, B. M. (2000). Retinoic acid perturbs *Otx* gene expression in the ascidian pharynx. *Dev. Genes. Evol.* 210: 129-139.

HINMAN, V. F., and DEGNAN, B. M. (2001). Homeobox genes, retinoic acid and the development and evolution of dual body plans in the ascidian *Herdmania curvata. Amer. Zool.* 41: 664-675.

HISATA, K., FUJIWARA, S., TSUCHIDA, Y., OHASHI, M. and KAWAMURA, Y. (1998). Expression and function of a retinoic acid receptor in budding ascidians. *Dev. Genes Evol.* 208: 537-546.

HOLLAND, P.W.H., GARCIA-FERNANDEZ, J., WILLIAMS, N. A. and SIDOW, A. (1994). Gene duplications and the origins of vertebrate development. *Development* Suppl. 36: 125-133.

KOSTROUCH, Z., KOSTROUCHOVA, M. and RALL, J. E. (1995). Steroid/thyroid hormone receptor genes in *Caenorhabditis elegans. Proc. Natl. Acad. Sci. USA* 92: 156-159.

LAUDET, V. (1997). Evolution of the nuclear receptor superfamily: early diversification from an ancestral orphan receptor. *J. Mol. Endocrinol.* 19: 207-226.

LAUDET, V. and ADELMANT, G. (1995). Lonesome orphans. *Curr. Biol.* 5: 124-127.

LAUDET, V., HANNI, C., COLL, J., CATZEFLIS, F. and STEHELIN, D. (1992). Evolution of the nuclear receptor gene superfamily. *EMBO J.* 11: 1003-1013.

MADDISON, W. P. and MADDISON, D. R. (1992). *MacClade: Analysis of phylogeny and character evolution. Version 3.0.* Sinauer Associates, Sunderland, Massachusetts.

MANGELSDORF, D. J. and EVANS, R. M. (1995). The RXR heterodimers and orphan receptors. *Cell* 83: 841-850.

MANGELSDORF, D. J., THUMMEL, C., BEATO, M., HERRLICH, P., SCHUTZ, G., UMESONO, K., BLUMBERG, B., KASTNER, P., MARK, M., CHAMBON, P. and EVANS, R. M. (1995). The nuclear receptor superfamily: the second decade. *Cell* 83: 835-839.

MANGELSDORF, D. J., UMESONO, K. and EVANS, R. M. (1994). The retinoid receptors. In *The Retinoids: Biology, Chemistry, and Medicine* (Eds. Sporn, M. B., Roberts, A. B. and Goodman, D. S.) Raven Press Ltd, New York, pp. 319-349.

MEEDEL T. H., FARMER, S. C. and LEE, J. J. (1997). The single MyoD family gene of *Ciona intestinalis* encoded two differentially expressed proteins: implications for the evolution of chordate muscle gene regulation. *Development* 124: 1711-1721

MENDELSOHN, C., RUBERTE, E. and CHAMBON, P. (1992). Retinoid receptors in vertebrate limb development. *Dev. Biol.* 152: 50-61.

NUCLEAR RECEPTORS NOMENCLATURE COMMITTEE. (1999). A unified nomenclature system for the nuclear receptor superfamily. *Cell* 97: 161-163.

SATOH, N. (1994). *Developmental Biology of Ascidians.* Cambridge University Press, Cambridge.

SHARMAN, A.C. and HOLLAND, P. W. H. (1996). Conservation, duplication and divergence of developmental genes during chordate evolution. *Netherlands J. Zool.* 46: 47-67.

SHIMELD, S.M. (1996). Retinoic acid, HOX genes and the anterior-posterior axis in chordates. *Bioessays* 18: 613-616.

SLUDER, A., MATHEWS, S. W., HOUGH, D., YIN, V. P. and MAINA, C. V. (1999). The nuclear receptor superfamily has undergone extensive proliferation and diversification in nematodes. *Genome Res.* 9:103-120.

SWOFFORD, D.L. (1998). *PAUP*: Phylogenetic Analysis Using Parsimony. Test Version 4.0d63.* Sinauer Associates, Sunderland, Massachusetts.

THOMPSON, J. D., HIGGINS, D. G. and GIBSON, T. J. (1994). Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 22: 4673-4680.

THUMMEL, C. S. (1995). From embryogenesis to metamorphosis: the regulation and function of *Drosophila* nuclear receptor superfamily members. *Cell* 83: 871-877.

VLAHOU, A., GONZALEZ-RIMBAU, M. and FLYTZANIS, C. N. (1996). Maternal mRNA encoding the orphan steroid receptor SpCOUP-TF is localized in sea urchin eggs. *Development* 122: 521-526.

YUASA H. J., COX, J. A. and TAKAGI, T. (1998). Diversity of the troponin C genes during chordate evolution. *J. Biochem.* 123: 1180-1190.