Cloning and characterization of two nuclear receptors from the filarial nematode *Brugia pahangi*

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Nuclear receptors (NRs) encompass a superfamily of cytoplasmic/nuclear localized receptors that on ligand binding (or by phosphorylation) directly regulate the transcription of target genes. NRs are involved in many developmental processes, including moulting in insects and dauer larva formation in *Caenorhabditis elegans*. Here we report the isolation of two genes related to NRs from the filarial nematode *Brugia pahangi*. *Bp-nhr-1* is a member of the NGF1-B subfamily of NRs and is expressed at very low levels in post-infective larval stage 3 (L3) after their transmission to the mammalian host. The second gene, *Bp-nhr-2*, is related to XR78E/F of *Drosophila*, a gene involved in the ecdysone response, over the region of its DNA-binding domain. cDNA and genomic clones have been isolated that correspond to *Bp-nhr-2*. The most striking feature of the encoded protein is that, although there is a DNA-binding domain

similar to that of other NRs, the ligand-binding domain is absent. To investigate the pattern of transcription of *Bp-nhr-2* in the filarial life cycle, semi-quantitative reverse-transcriptase-mediated PCR was performed; this analysis demonstrated that the gene is expressed in early stages after infection and in the adult and microfilariae, and is up-regulated just before the moult between L3 and L4 but is not expressed during the moult between L4 and adult. Antibodies raised against a peptide corresponding to the transactivation domain of *Bp-nhr-2* demonstrate that the protein is expressed in microfilariae and adult samples and that another cross-reactive protein is expressed in these life-cycle stages.

Key words: developmental regulation, heat-shock elements, moulting.

INTRODUCTION

Lymphatic filariasis is a mosquito-borne disease caused by infection with the nematode parasites *Wuchereria bancrofti*, *Brugia malayi* and *B. timori*. These parasites are a major cause of morbidity throughout the tropics, with over 120 million people infected and more than 1.1 billion people at risk of infection, i.e. approx. 20% of the world's population [1]. Infection of the human host is initiated by the third-stage larvae (L3), which are transmitted by the bite of an infected mosquito. The L3 migrate to the lymphatic system, where they develop through two moults to reach the adult stage. Adult worms mate and the female produces the microfilariae or first-stage larvae, which circulate in the bloodstream until ingested by a feeding mosquito. In the mosquito vector the microfilariae develop through a further two moults to the infective L3. The mechanisms controlling the development of these parasitic nematodes are poorly understood. The similarity between the developmental process in insects and nematodes, in both of which the larval stages are separated from each other by a developmentally regulated moult, has prompted several workers to propose that the underlying mechanisms controlling development might be similar [2]. It is known that in insects a steroid hormone, ecdysone, controls the moulting process by binding to the ecdysone receptor and its partner Ultraspiracle, both of which are members of the nuclear receptor (NR) superfamily [3]. The heterodimer then binds to target sequences in the 5' region of ecdysone response genes, thereby

regulating their transcription. Additionally, it is interesting to note that recent phylogenetic studies on the Nematoda and Insecta have suggested that moulting arose once in evolution and that these phyla of invertebrates should be grouped in a novel clade, the Ecdysozoa [4]. Thus it is not unreasonable to presume that the molecular events that control moulting might be conserved.

In the free-living nematode *Caenorhabditis elegans*, more than 200 genes encoding proteins related to NRs have been identified by the genome-sequencing project [5,6], many more than have been identified in any other organism so far. Sluder et al. [6] recently analysed many of the predicted NRs from *C. elegans* and provided evidence that a significant number (at least 25%) of these predicted genes were actually expressed. One of the NRs of *C. elegans*, *daf-12*, is involved in the control of dauer larva formation, an alternative developmental pathway to the L3 stage [7], and more recently has been shown to function as a heterochronic gene, different alleles of which can specify different developmental programmes [8]. A second NR from *C. elegans*, *nhr-2*, seems to function in embryonic development [9], whereas a third, *nhr-23*, has recently been shown by double-stranded RNA interference assay to be involved in the moulting process [10].

In addition to their developmental roles in insects and in *C. elegans*, NRs are involved in a wide variety of cellular processes in a variety of organisms including aspects of homeostasis, reproduction, development and differentiation [11–13]. NRs are

Abbreviations used: *Bp-nhr*, *Brugia pahangi* nuclear receptor, L1, larval stage 1, etc.; NR, nuclear receptor; HSE, heat-shock element; HSF, heat-shock factor; pfu, plaque-forming units; p.i., post-infection, RT-PCR, reverse-transcriptase-mediated PCR.

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The nucleotide sequence data reported will appear in DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession numbers AF091044 (*Bp-nhr-2* complete cDNA), AF091045 (*Bp-nhr-2* genomic) and AF091046 (*Bp-nhr-1*, genomic).

cytoplasmic/nuclear-located receptors that, on activation by a ligand (or by phosphorylation), directly regulate the transcription of responsive genes via binding to DNA response elements, present in the promoter region of such genes, either as a monomer or as homodimers or heterodimers. NRs have a modular structure consisting of six regions, A to F, the most conserved of these being regions C and E, which comprise the zinc finger DNA-binding and ligand-binding domains respectively. The DNA-binding domain is composed of two zinc finger structures named CI and CII that have complementary roles in the DNA binding process [14]. CI contains a region mediating the specificity of DNA binding, known as the P-box. The most N-terminal domain, the A domain, is known as the transactivation domain and is involved in protein-protein interactions with other factor(s) involved in transcriptional control [15].

We designed heterologous primers based on the conserved DNA-binding region of *nhr-6* (encoding protein CNR8) [16] and *daf-12* to isolate genes encoding NRs from *B. pahangi* and thus to investigate NRs in this important parasitic nematode.

MATERIALS AND METHODS

Cloning of NRs by using degenerate primers and reverse-transcriptase-mediated PCR (RT-PCR)

Degenerate primers corresponding to two separate conserved regions within the DNA-binding domain of NRs *nhr-6* and *daf-12* from *C. elegans* were used in PCR on genomic DNA prepared from *B. pahangi* by standard methods. In all, five separate primers were tested in all combinations; however, only one set resulted in a PCR product. Genomic DNA (5 ng) was used in a PCR reaction with primers CeF1 (5'-TGYGARGGITGYA-ARGGITYTTTYAAR-3', forward of the CEGCKGFFK protein sequence) and DR1 (5'-CATICCIACIGTRAARCAYT-TNCK-3', reverse of the MGVTFCKR protein sequence) at 95 °C for 5 min followed by 30 cycles at 94 °C for 1 min, 50 °C for 1 min and 72 °C for 1 min and a final extension at 72 °C for 10 min. The resulting band was excised from a 1.2% (w/v) NuSieve® GTG® gel, melted at 65 °C and ligated into pT7Blue (Novagen). Four clones were sequenced; clone pJMB1 was representative of all four and the corresponding gene was named *Bp-nhr-1* (*B. pahangi* nuclear hormone receptor 1). To clone the 5' end of this mRNA, gene-specific primers were designed and RT-PCR was performed on RNA prepared from various stages of *B. pahangi* with TriZol®, in accordance with the manufacturer's instructions (Life Technologies). Genomic DNA was removed from the RNA sample by treatment with DNase I (Life Technologies). Total RNA (2 µg) was reverse-transcribed with 200 ng of BHR1 primer (5'-AACGACAGTATTGGCAT-CGACTACG-3') by using SuperScript®II in accordance with the manufacturer's instructions (Life Technologies). Amplification by PCR was performed on 2 µl of a 1:10 dilution of first-strand cDNA by using BHR1 primer and the conserved nematode spliced leader sequence (SL1) primer (5'-gccggaattcGGTTT-AATTACCCAAGTTTGAG-3'; lower-case indicates an *EcoRI* site and extra nucleotides) at 95 °C for 5 min followed by 30 cycles at 94 °C for 1 min, 55 °C for 1 min and 72 °C for 3 min and a final extension at 72 °C for 10 min. The presence of products related to *Bp-nhr-1* was confirmed by Southern blotting and a putative positive was observed with the product derived from microfilarial mRNA. This product was separated on a 1% (w/v) NuSieve® GTG® gel; the band was excised from the gel, melted at 65 °C and ligated into pCRII (Invitrogen). This clone, pJMB20, was representative of four individual clones sequenced. This cDNA, although encoding an NR, was not the same as that contained in pJMB1 and was therefore named *Bp-nhr-2*.

Screening a microfilarial cDNA library

A total of 5×10^5 plaque-forming units (pfu) from a microfilarial cDNA library prepared by SL1/oligo(dT) PCR [17] was screened with a random-primed insert from pJMB20 (*Bp-nhr-2*). The microfilariae used in the preparation of the library had been heat-shocked to 41 °C for 2 h before RNA extraction. Eleven positive clones were isolated; each was shown to contain the same-sized insert by PCR with vector primers T3 and T7. Phagemid DNA was excised by using the helper phage R408 (Stratagene) and four clones were sequenced as above (pJMB33 was representative of all four).

Isolation of genomic clones

A total of 10^6 pfu of a *B. pahangi* genomic library prepared in lambda EMBL3 (a gift from J. Hirzmann, University of Giessen, Giessen, Germany) was screened with the insert from pJMB1 (*Bp-nhr-1*) by standard procedures, from which only one clone was isolated. λJMB1 was restriction-mapped and the 2.4 kb *HindIII/KpnI*-hybridizing band along with the upstream 500 bp *HindIII/SalI* fragment subcloned into pBluescript SK+ (Stratagene) and sequenced on both stands with a Licor automated sequencer. To isolate the genomic copy of *Bp-nhr-2*, 10^5 pfu of the same genomic library was screened with the insert from pJMB33 with the use of standard procedures. Eleven putative positives were obtained after three rounds of screening; of these, three were restriction-mapped. A 1.5 kb *XbaI* fragment (containing the 3' end of *Bp-nhr-2*) and a 5 kb *XbaI/HindIII* fragment (containing the 5' end and upstream sequences of *Bp-nhr-2*) were subcloned into pBluescript SK+ and sequenced as described above.

Semi-quantitative RT-PCR

Semi-quantitative RT-PCR was performed to assess when within the *Brugia* life cycle the *Bp-nhr-1* and *Bp-nhr-2* mRNA species were expressed [18,19]. Expression levels of each mRNA were compared with a constitutively expressed 60 S ribosomal protein gene of *B. pahangi*, *Bp-rpp-1* (accession number X91066). RNA was prepared as described above, treated with DNase I and reverse-transcribed with 200 ng of oligo(dT) by using SuperScript® II in accordance with the manufacturer's recommendations. The resultant first-strand cDNA species were stored at -20 °C until required. Amplification by PCR was performed on 2 µl of a 1:20 dilution of first-strand cDNA. For *Bp-nhr-1*, multiple rounds of PCR were necessary for detection of the cDNA product by Southern hybridization. This was performed with gene-specific primers F2 (5'-TTCGTAGTCGATGCC-AATACTGTCG-3') and R4 (5'-GCAAAAAAATTGCATTC-AATTAATTAG-3') and PCR was performed as follows: 95 °C for 5 min, followed by 30 cycles at 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min and a final extension at 72 °C for 10 min. The PCR products were precipitated and resuspended in the same volume as the original reaction and the PCR was repeated for the desired number of reactions. The cDNA product can be distinguished from the genomic product on the basis of size: 358 bp for the former and 825 bp for the latter. To check that all lanes contained approximately equal amounts of starting material, primers R1 (5'-GCATTGTTCTCAAATAGAGC-3') and NR3 (5'-GGCTCTTCCTTCTTCTTGTCCTC-3') specific to *Bp-rpp-1* were used in one round of PCR. For *Bp-nhr-2*, the number of PCR cycles was chosen empirically in preliminary reactions with each set of primers to define conditions under which reagents were not limiting. Gene-specific primers for *Bp-nhr-2*, MHRF1 (5'-GTCACCTCATTGCACGTCGAATGC-

ATC-3') and MHRR1 (5'-ATGAACTTAGTCACGGGA-CAATCC-3'), and primers R1 and NR3 as described above for *Bp-rpp-1* were used. The PCR was performed as follows: 95 °C for 5 min followed by 23 cycles at 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min and a final extension at 72 °C for 10 min. The cDNA species could be distinguished from the genomic band by size, the former being 225 bp and the latter 654 bp in *Bp-nhr-2*, and 370 and 588 bp respectively in *Bp-rpp-1*. The PCR products were separated on a 1.2% (w/v) agarose gel and transferred to Hybond-N (Amersham) by standard blotting procedures [20]. Blots were then hybridized with oligonucleotide probes, prepared by standard methods [20], designed to hybridize across the intron boundary and thus minimize hybridization to genomic PCR products (InBp-nhr-1, 5'-TGTGTATTGTGTG-AGAATTCTAC-3'; InBp-nhr-2, 5'-AAATTCTAGAATGTG-GTGCTGAG-3'; InRp, 5'-TGATGTGAAGTCGTTGATAA-3'). For *Bp-nhr-2*, the area of hybridization was cut out of the filters; radioactivity was measured by scintillation counting and compared with the counts from *Bp-rpp-1*.

Raising of antiserum and Western blot analysis

Antiserum specific to *Bp-nhr-2* was raised against a peptide within the transactivation domain, to minimize cross-reactivity with other NRs in *B. pahangi*. This peptide was chosen because of its hydrophilic nature and because it contains two strong turns in addition to being close to the N-terminus, all features that are thought to increase the antigenic potential. Peptide HHSSAAGSVSRGGSASSPHC (see Figure 2) was synthesized by Genosys and conjugated to keyhole-limpet haemocyanin; antibodies against the peptide were raised in two rabbits.

B. pahangi microfilariae and soluble extracts from adult worms were prepared by boiling worms in sample cocktail buffer for 5 min followed by centrifugation at 13 000 g for 20 min to remove insoluble material. Soluble proteins were separated on a 12.5% (w/v) SDS polyacrylamide gel and blotted on nitrocellulose paper with Tris/glycine/methanol buffer. The blots were probed with a 1:100 dilution of the antiserum raised against the *Bp-nhr-2* transactivation domain peptide or the pre-bleed from the same rabbit. All wash and incubation steps were performed with standard procedures; bound antibody was revealed with an alkaline phosphatase-conjugated secondary antibody and 5-bromo-4-chloroindol-3-yl phosphate/Nitro Blue Tetrazolium as substrate.

RESULTS

PCR amplification of *Bp-nhr-1* and *Bp-nhr-2*

PCR was performed on *B. pahangi* genomic DNA with a variety of different degenerate primers, of which only CeF1 and DR1 were successful in producing a product. These primers were based on the sequence of NRs *nhr-6* and *daf-12* from *C. elegans*. This PCR fragment encodes 419 bp of the DNA-binding domain of an NR; the corresponding gene was named *Bp-nhr-1* (*B. pahangi* nuclear hormone receptor 1). In an attempt to clone the full-length gene, RT-PCR with a *Bp-nhr-1* gene-specific primer (BHR1) and SL1 was performed on various life-cycle stages, of which only microfilarial cDNA produced a band that hybridized to *Bp-nhr-1*. Sequence analysis demonstrated that this fragment (323 bp) encoded an NR but that this receptor was distinct from *Bp-nhr-1*; it was therefore named *Bp-nhr-2* (results not shown).

Isolation of *Bp-nhr-1*

The genomic PCR fragment corresponding to *Bp-nhr-1* (pJMB1)

was used to screen a *B. pahangi* genomic EMBL3 library. After the screening of 10⁶ pfu, only one positive clone was isolated (λJM1). Comparison with the number of positives obtained while screening the same library with the insert from pJMB33 (*Bp-nhr-2*: 11 positives for 10⁵ pfu screened) suggests that this region of the genome is under-represented in the library. λJM1 was restriction-mapped and the 2.4 kb *HindIII/KpnI* hybridizing band, along with the upstream 500 bp *HindIII/SalI* fragment, was subcloned into pBluescript SK+ and sequenced on both strands. Sequence analysis of the full 2951 bp genomic clone indicated that it did not encompass the complete gene and that six introns interrupted the coding sequence.

Database searches show that *Bp-nhr-1* is most closely related to the NGFI-B/Nur77/Nurr1 gene family [21–23], of which the *C. elegans* gene *nhr-6* is a member. The pile-up in Figure 1 demonstrates the close similarity over the DNA-binding domain between the two nematode proteins and two related proteins from mammals, the nuclear receptor RNR-1, a member of the NGFI-B (nerve-growth-factor-inducible protein B) family [24], and a related human protein, TINUR (transcriptionally inducible nuclear receptor) [25]. Comparison of the *Bp-NHR-1* and CHR-6 sequences suggests that approx. 200 residues are missing from the N-terminus and 111 residues from the C-terminus of *Bp-NHR-1*. Over the DNA-binding region, *Bp-NHR-1* is 82% identical with CHR-6 of *C. elegans* and 80% identical with the mammalian nuclear receptor RNR-1. Over the available ligand-binding region, *Bp-NHR-1* is 29% identical with and 43% similar to CHR-6 and 22% identical with and 30% similar to RNR-1. Southern hybridization demonstrated that *Bp-nhr-1* is a single-copy gene (results not shown).

To assess the expression pattern of this gene and to help in identifying a corresponding cDNA clone, RT-PCR was performed with gene-specific primers F2 and R4, corresponding to *Bp-nhr-1*, and primers R1 and NR3, corresponding to a constitutively expressed 60 S ribosomal protein gene, *Bp-rpp-1*, which has been extensively characterized in this laboratory [18,19]. It was observed that *Bp-nhr-1* mRNA, when present, is expressed at very low levels (Table 1) and that multiple rounds of PCR were required for detection of the cDNA band by Southern hybridization. This experiment was repeated three times with equivalent results. Fifteen different life-cycle stages were tested, including larvae around the L3 to L4 moult [days 5 and 7 post-infection (p.i.) of the mammalian host] and L4 to adult moult (days 18 and 21 p.i.), inter-moult larvae (days 11 and 12 p.i.), sexually immature adults (day 28 p.i.), sexually mature adults (at least 3 months p.i.) and microfilariae. For each life-cycle stage tested, a signal was obtained with the constitutive gene (*Bp-rpp-1*), whereas for *Bp-nhr-1*, p.i. L3 recovered on day 3 and day 7 p.i. of the mammalian host gave the best signal, with respectively 60 or 90 rounds of PCR being required for detection of the product by hybridization. The primers used spanned an intron; cloning and sequencing of the product from days 3 and 7 p.i. L3 confirmed that the hybridizing band corresponded to the *Bp-nhr-1* cDNA (358 bp). RT-PCR on infected mosquitoes produced no evidence of expression of *Bp-nhr-1* during development in the mosquito host (days 3 and 6 p.i.). These time points represent parasites at the L1 and L2 stages of development respectively. A very weak signal (120 rounds of PCR required) was obtained with infected mosquito thoraces at day 9 p.i., at which point the worms are immature L3.

In *Drosophila* the NGFI-B counterpart DHR-38 was cloned from a larval cDNA library after treatment of larval organs with ecdysone and cycloheximide [26]. The fact that *Bp-nhr-1* is expressed at very low levels in all life-cycle stages might suggest that *Bp-nhr-1* mRNA also has a very short half-life. The low

Figure 1 Amino acid alignment of *Bp-NHR-1* with related sequences

The conceptual amino acid sequence of *Bp-NHR-1* was aligned with *C. elegans* CHR-6 (accession no. AF083224), human TINUR (accession no. I56260) [25] and rat RNR-1 (accession no. Q07917) [24] by using the ClustalW program of MacVector. The amino acid sequence of *Bp-NHR-1* was deduced from the sequence of the genomic clone; intron/exon boundaries were confirmed from the sequence of PCR fragments of the cDNA or implied by similarity to intron/exon consensus sequences [27]. Gaps were introduced to maximize the alignments. Numbers above the alignment denote *Bp-NHR-1* amino acids from the start of the open reading frame sequenced. This was aligned with residues 202–516 of RNR-1, residues 130–453 of TINUR, and residues 200–509 of CHR-6. Identical amino acids are boxed and shown in bold; similar amino acids are boxed. Residues 87–91 of *Bp-NHR-1* (CEGCK) denote the P box, residues 136–146 (VKEVRYGSLSG) the T box and residues 148–157 (RRGLPSKTK) the A box.

Table 1 Expression pattern of *Bp-nhr-1* by RT–PCR at various stages throughout the life cycle

Infected mosquitoes were analysed essentially as described in [18] at day 3 p.i. (L1), day 6 p.i. (L2) or day 9 p.i. (immature L3). Parasites were also harvested at different time points p.i. of the mammalian host, as described in [18,19]. Parasites at 24 h, 30 h, 3 days and 5 days p.i. were all p.i.L3. Day 7 parasites were in the process of the L3–L4 moult; days 11, 12, 18 and 21 were L4; day 28 parasites were sexually immature adults. Abbreviations: Ad, sexually mature adults; Mf, microfilariae obtained at least 3 months p.i. Symbols: +, gene-specific band visible by hybridization after four rounds of 30 cycles of PCR; ++, gene-specific band visible by hybridization after three rounds of 30 cycles of PCR; +++, gene-specific band visible by hybridization after two rounds of 30 cycles of PCR.

	Mosquito			Mammalian host													Ad	Mf
	Days p.i. ...	3	6	9	Time p.i. ...	24 h	30 h	3 days	5 days	7 days	11 days	12 days	18 days	21 days	28 days			
Response		–	–	+		+	+	+++	+	++	–	+	–	+	–		+	–

abundance of the message in total larval and adult RNA might also suggest that *Bp-nhr-1* expression is restricted to a limited number of cells or cell types within the larvae and/or adult

worms. Whichever is the case, the low abundance of the message has made further characterization of this gene very difficult; the rest of this paper concentrates on *Bp-nhr-2*.

Figure 2 Nucleotide sequence of *Bp-nhr-2* cDNA and upstream region

(A) Sequence of *Bp-NHR-2* cDNA clone (pJMB33). The 5' and 3' untranslated regions of the nucleotide sequence are in lower-case and the coding region is in capitals. The 22 nt SL1 sequence is in bold. The predicted amino acid sequence is shown below the nucleotide sequence and the amino acid sequence used to raise the antiserum is delineated by ↑. The DNA-binding domain is in capitals; the N-terminal (transactivation domain) and C-terminal regions are in lower-case. Cysteine residues involved in zinc fingers and the conserved methionine residue are in bold. The P- and D-boxes are labelled below the sequence. Primers used in semi-quantitative RT-PCR are in bold within the coding region. The ++ marks the 5' and 3' boundaries of the intron sequence. Singly underlined residues denote potential myristoylation sites; doubly underlined residues are potential protein kinase C phosphorylation sites; broken underlining indicates a potential N-glycosylation site. The stop codon is indicated by an asterisk. Numbers at the side denote bp. (B) Sequence of the upstream region of *Bp-nhr-2*. The ATG start codon is depicted in bold. The sequence of the 5' untranslated region covers 334 bp. Bold regions indicate half-mer sites for the *Bp-nhr-2* recognition sequence, as determined by its P-box. Double underlining indicates HSF consensus binding sites.

Isolation of *Bp-nhr-2* cDNA

The 5' region of *Bp-nhr-2* was initially cloned by RT-PCR from RNA prepared from microfilariae with a 3' primer thought to be specific to *Bp-nhr-1* and SL1 as the 5' primer. This 323 bp PCR fragment was used to screen a microfilarial cDNA library and resulted in the isolation of clone pJMB33 (Figure 2A). Initial analysis suggested that this was not a full-length clone, being only 417 bp long. More detailed analysis revealed an in-frame stop codon that was present in four separate cDNA clones after sequencing with both Licor and ABI automated sequencers. Because the library itself was prepared by PCR, the possibility of a PCR error could not be eliminated at this point. However, the isolation of a genomic clone (see below) confirmed the presence of a stop codon. The cDNA clone (pJMB33) contains the nematode SL1 sequence at its 5' end, after which there are only 3 nt separating it from the predicted start methionine. In *C. elegans*, approx. 63% of genes have between 0 and 10 nt and 41% between 0 and 5 nt between the SL1 and ATG start codon [27]. In addition, the preferred nucleotides immediately preceding the translational initiation codon are 4A residues; in *Bp-nhr-2*

there are only 3 nt preceding the ATG start codon, none of which is A (Figure 2A). After the 5' untranslated region there is an open reading frame of 339 bp (including the stop codon) encoding a polypeptide of 112 residues with a predicted molecular mass of 11.9 kDa and a pI of 9.52. Thus *Bp-nhr-2* encodes a protein with a 42-residue transactivation domain followed by a 65-residue DNA-binding domain and a further five residues before the stop codon. No consensus polyadenylation signal is seen in the 3' untranslated region of *Bp-nhr-2*; approx. 7% of genes from *C. elegans* analysed do not contain an identifiable polyadenylation signal, suggesting an alternative mechanism for 3' end formation in these genes [27].

Isolation of a genomic DNA clone corresponding to *Bp-nhr-2*

To investigate the structure of the *Bp-nhr-2* gene and confirm the position of the stop codon, a genomic λEMBL3 clone corresponding to *Bp-nhr-2* was isolated (λJMB5). A genomic Southern blot was performed that demonstrated that the *Bp-nhr-2* gene is a single copy (Figure 3). The insert derived from the cDNA clone (pJMB33) hybridizes to a single band in *Xba*I and *Bam*HI digests, neither of which is present within the probe, whereas an *Eco*RI digest resulted in hybridization with two bands. Sequence analysis of the genomic clone confirmed the presence of a single intron of 429 bp containing an *Eco*RI site within the coding region (position indicated in Figure 2A). Recently, Blumenthal and Steward [27] analysed 200 genes from *C. elegans* containing 659 introns, and demonstrated that the average intron length is 48 nt, in contrast with the much longer introns of vertebrates, plants and *Saccharomyces cerevisiae*. However, introns as long as 18 kb have been found in *C. elegans* [28] and, indeed, *nhr-6* contains introns of 9 and 1.8 kb [6].

Sequencing of the genomic clone also confirmed that the protein product of *Bp-nhr-2* does indeed lack a ligand-binding domain. The P-box (CEGCKG; Figure 2A) within the DNA-binding domain of *Bp-nhr-2* confers the specificity of the DNA-binding site and suggests that *Bp-nhr-2* binds to DNA sequences GGTC A. Two such sequences are seen in the upstream region of the *Bp-nhr-2* gene at positions -212 and -121 with respect to the start methionine (see Figure 2B). In classical cases, NRs bind as homodimers or heterodimers to their DNA recognition sequences; thus a typical DNA recognition sequence is usually made of two half-mer sites (of which GGTC A is an example) of direct or indirect repeats. However, more recently, many NRs have been shown to bind as monomers [29]. *Bp-nhr-2* is a member of the *Drosophila* XR78E/F family. Figure 4 shows a pile-up of the predicted amino acid sequence of *Bp-NHR-2* with *Drosophila* XR78E/F, a related mammalian orphan receptor, mouse TR2R1, and a recently identified NR of *C. elegans*, CHR-3 [6], illustrating the high level of conservation over the DNA-binding domain.

To gain insight into the regulation of *Bp-nhr-2*, sequences upstream of the site of SL1 addition were analysed. There are 11 heat-shock factor (HSF) consensus binding sites within the 334 bp sequenced, two of which are 100% identical with the HSF consensus binding sites (Figure 2B). The cDNA encoding the *Bp-nhr-2* gene was cloned from a library prepared from microfilariae heat-shocked at 41 °C for 2 h before RNA extraction; semi-quantitative RT-PCR has confirmed the up-regulation of the *Bp-nhr-2* gene transcript in heat-shocked microfilariae (see below).

mRNA expression profile and Western blot analysis

The expression pattern of *Bp-nhr-2* was analysed by semi-quantitative RT-PCR (Figure 5). The full analysis was performed

Figure 3 Southern blot analysis of *Bp-nhr-2*

High-molecular-mass DNA was isolated from *B. pahangi* and digested with *Bam*HI (lane 1), *Xba*I (lane 2) and *Eco*RI (lane 3). The gel was blotted and probed with the insert derived from pJMB33 (*Bp-nhr-2*) at 65 °C in 5 × SSPE [SSPE is 0.15 M NaCl/10 mM sodium phosphate (pH 7.4)/1 mM EDTA] and washed to high stringency (0.1 × SSPE plus 0.1 % SDS at 65 °C).

Figure 4 Amino acid alignment of *Bp-NHR-2*

Multiple alignment of *Bp-NHR-2* amino acid sequence with *Drosophila melanogaster* XR78E/F (accession no. U31517) [35], mouse TR2R1 (accession no. S75970) [40] and *C. elegans* CHR-3 [6]. Sequences were aligned with the ClustalW program of MacVector. Numbers above alignment denote *Drosophila* XR78E/F residues 1–127. This sequence was aligned with residues 1–113 of *Bp-NHR-2*, residues 105–224 of mouse TR2R1 and residues 1–123 of *C. elegans* CHR-3 [6]. Identical amino acids are boxed and shown in bold; similar amino acids are boxed. Gaps were introduced to maximize the alignments.

on two occasions with similar results. Transcripts were detectable at all time points tested and were up-regulated at approx. the time of the L3 to L4 moult (day 6; Figure 5). RT-PCR was also performed on larvae recovered at days 18–22 p.i., the approximate timing of the moult between L4 and adult. A signal was obtained at each of these time points with *Bp-rpp-1* primers but no signal was obtained at day 18, 19, 20, 21 or 22 with *Bp-nhr-2* primers (results not shown). Because the cDNA was cloned from a library prepared from heat-shocked microfilariae, the

Figure 5 Semi-quantitative RT-PCR of *Bp-nhr-2* mRNA abundance in different life-cycle stages of *B. pahangi*

The graph shows the ratio of expression of *Bp-nhr-2* mRNA to *Bp-rpp-1* mRNA, expressed in arbitrary units on the y-axis. RNA was isolated from parasites obtained at the following time points: 24 h and 3 days p.i. (p.i.L3), 6 days p.i. (parasites undergoing the L3–L4 moult), 12 days p.i. (L4), 28 days p.i. (sexually immature adults) and adult worms (at least 3 months p.i.) or from microfilariae incubated at 28, 37 or 41 °C before RNA extraction. First-strand cDNA was synthesized and RT-PCR was performed with gene-specific primers for *Bp-nhr-2* or for the ribosomal protein gene *Bp-rpp-1*. Primers were designed to span an intron so that amplified cDNA could be distinguished from contaminating genomic DNA. PCR products were blotted and blots were probed and quantified as described in the Materials and methods section.

expressions of *Bp-nhr-2* in microfilariae cultured at 28, 37 and 41 °C were compared. The highest abundance of *Bp-nhr-2* mRNA was observed in microfilariae cultured at 41 °C, which was consistent with the presence of HSF-binding sites in the upstream region of this gene.

Western blots were performed with an antiserum raised against a peptide (residues 8–27) from the transactivation domain of *Bp-nhr-2*. Total SDS sample cocktail extracts of microfilariae or

Figure 6 Western blot analysis of *Bp-NHR-2* expression in *B. pahangi* adults and microfilariae

Total SDS extracts of microfilariae (lanes 1 and 2) or adult worms (lanes 3 and 4) were analysed by SDS/PAGE [12.5% (w/v) gel]. Gels were blotted and probed with antibodies raised against a peptide from the transactivation domain of *Bp-NR-2* (lanes 2 and 4) or the pre-bleed (lanes 1 and 3), as described in the Materials and methods section. The positions of molecular mass markers are indicated at the right.

Table 2 Comparison of *B. malayi* NRs from the genome-sequencing project with *Bp-nhr-1* and *Bp-nhr-2*

Three nucleotide sequences encoding *nhr* genes are present in the *B. malayi* genome sequencing database. Percentage identities are shown with *Bp-nhr-1* and *Bp-nhr-2* at the nucleotide level.

<i>B. malayi</i> accession no.	Life-cycle stage	Identity <i>Bp-nhr-1</i> (%)	Identity <i>Bp-nhr-2</i> (%)
N44459*	L3	53.2	59.8
AA072514*	L3	54.4	62.8
AA675734†	Adult female	58.2	60.8

* S. A. Williams (unpublished work).

† M. L. Blaxter (unpublished work).

adult worms were analysed. Figure 6 shows a representative blot in which the antibody recognized a band of approx. 14.4 kDa in both microfilariae and adult worms. An additional component of approx. 45 kDa was detected in both life-cycle stages. Analysis of the predicted *Bp-NHR-2* protein reveals a variety of sites that could be post-translationally modified, e.g. an N-linked site, two potential phosphorylation sites and two potential myristoylation sites (see Figure 2A). Any of these possible modifications could account for the increased size of the 14.4 kDa band in comparison with that of the protein predicted from the cDNA (11.9 kDa).

Bp-nhr-1 and *Bp-nhr-2* are 72% identical with each other. During the course of this study, a number of sequences that encode fragments of NRs were deposited in the *B. malayi* genome sequencing database. These are single-pass sequences containing multiple errors from which it is difficult to determine an open reading frame accurately. A comparison of the nucleotide sequences of three of these clones with *Bp-nhr-1* and *Bp-nhr-2* is shown in Table 2; two of the *B. malayi* sequences derive from L3 libraries, whereas the third was cloned from an adult female cDNA library. Further sequences would be required for a determination of the relationship of the *B. malayi* sequences to the *B. pahangi* genes reported here.

DISCUSSION

We have isolated a partial gene encoding an NR, *Bp-nhr-1*, and a further full-length gene, *Bp-nhr-2*, encoding a protein related to the DNA-binding domain of NRs. *Bp-nhr-1* was expressed at very low levels during the *Brugia* life cycle, with the highest level of expression detected in p.i. L3 stages; however, even in day 3 p.i. L3, 60 cycles of PCR were required before a product could be detected by hybridization. This very low level of expression suggests that the mRNA might be expressed for only very limited periods or in a limited number of cells. *Bp-nhr-1* is related to the *C. elegans* gene *nhr-6*, which can be detected by RT-PCR in all life-cycle stages [6]. By sequence analysis, *Bp-nhr-1* seems to be a member of the NGFI-B/Nur 77/Nurr 1 gene family, the *Drosophila* counterpart of which, DHR-38, has been extensively characterized [30]. Recent studies have shown that DHR-38 is expressed exclusively in the epidermis in late larval, pre-pupal and pupal stages [31], where it seems to function in cuticle formation in adult flies. DHR-38 interacts with DNA either as a monomer or as a heterodimer in partnership with Ultraspiracle and therefore might modulate ecdysone signalling by competing with the ecdysone receptor for Ultraspiracle [26]. In the absence of the full open reading frame of *Bp-nhr-1*, it is difficult to predict the likely structure of the native protein.

The second NR cloned in this study, *Bp-nhr-2*, is more abundantly expressed in *B. pahangi* than in *Bp-nhr-1*, with a peak of expression at day 6 p.i., just before the moult between L3 and L4. When RT-PCR was performed on parasites at around the time of the L4 to adult moult (days 18–22), no signal was

obtained with *Bp-nhr-2*, whereas a signal was obtained with the constitutive gene *Bp-rpp-1*, indicating the competence of the cDNA template. Therefore if *Bp-nhr-2* has a positive regulatory role in moulting, it is active only at the moult between L3 and L4. An NR from *C. elegans* encoded by *nhr-23* was recently shown to be expressed in the epidermal cells, the tissue responsible for production of the new cuticle [10]. RNA interference assays demonstrated that this gene is required for proper shedding of the L1 cuticle; it was postulated that it might regulate the onset of moulting by activating a collagenolytic pathway or by directly regulating collagen gene expression, although it is not yet known whether this effect is due to a direct or to an indirect result of *nhr-23* gene expression.

Bp-nhr-2 contained no ligand-binding domain and in the truest sense is not an NR because it does not contain sequences that are able to bind ligand, nor does it contain the dimerization domain known to be involved in homodimerization or heterodimerization between most, but not all, NRs [32]. However, *Bp-nhr-2* is not unique among the NR superfamily in its structure because, in recent years, various unusual NRs have been isolated. For example, *E78*, a gene involved in the ecdysone response in *Drosophila*, has two isoforms as the result of alternative splicing; *E78A* possesses DNA and ligand-binding domains, whereas *E78B* is a truncated receptor that has no DNA-binding domain but shares the same ligand-binding domain as *E78A* [33]. *Bp-nhr-2* could regulate the expression of NR response genes by binding to DNA response elements in the promoter region and thus compete with another, as yet unidentified, NR. If this were so, the other competing NR is not presumed to be identical with *Bp-nhr-2* over its DNA-binding domain because genomic Southern analysis demonstrated *Bp-nhr-2* to be a single copy. However, because it is the P-box that specifies DNA-binding recognition sequences, and in mammalian systems there are numerous distinct NRs with varying functions that contain this same P-box, then perhaps *Bp-nhr-2* acts as a general negative control factor. In this respect it is interesting that an abundance of novel P-box sequences have been observed in the *C. elegans* NRs [6]. The ligands for most NRs remain unidentified (the so-called orphan receptors) and Sluder et al. [6] hypothesized that many of the *C. elegans* NRs might act as direct regulators of transcription without requiring ligand binding for activation and DNA binding. Like most NRs, *Bp-nhr-2* contains potential phosphorylation sites and thus could possibly be activated to a DNA-binding state by phosphorylation.

If *Bp-nhr-2* were to have a functional role in *B. pahangi*, it would be predicted to bind to recognition sequences as a monomer because it lacks the dimerization domain necessary to interact with either itself or other NRs. Expression of the ecdysone receptor is self-regulated, both positively and negatively, by varying ecdysone concentrations [34], as might be indicated for *Bp-nhr-2*; however, whether this regulation is likely to be positive or negative cannot be assessed from DNA gazing

alone. *Bp-nhr-2* shows the greatest similarity to a *Drosophila* orphan receptor, XR78E/F, which is closely related to a variety of mammalian orphan receptors. XR78E/F is thought to repress the transcriptional activity of the ecdysone receptor complex or other NRs by binding to the same DNA-binding sites [35].

Sequence analysis of 334 bp of the 5' upstream sequence revealed the presence of multiple heat-shock elements (HSEs), usually found upstream of heat-shock-protein genes. It is difficult to determine transcription start sites in *trans*-spliced genes, because SL1 addition is a relatively efficient event and therefore consensus sequences surrounding transcription start sites in nematodes have not been determined. This makes it difficult to ascertain the likelihood that these transcription factor consensus binding sites have a role in the promoter machinery of *Bp-nhr-2*, because distance between transcription factor binding and transcript start is often crucial. Functional studies involving DNA binding assays would be required to prove formally that the HSEs are functional. That these sequences are involved in the transcriptional regulation of *Bp-nhr-2* is suggested, however, by the observation that heat-shocking microfilariae to 41 °C results in an increased expression of the mRNA. Although microfilariae do not routinely experience these temperatures, fevers do occur in infected individuals. The induction of transcription factors, other than HSF, at elevated temperatures might possibly aid the survival of the parasite. Whatever the case, the presence of multiple HSEs in a non-*hsp* gene is intriguing. Recent work on *Drosophila* has shown that HSF is essential for early larval growth and oogenesis, functions that are independent of *hsp* induction [36], suggesting that HSF might regulate the expression of non-*hsp* genes involved in growth and development. Whether *Bp-nhr-2* might constitute an example of an HSF-regulated gene remains to be determined.

Previous studies on the filarial nematodes *B. pahangi* and *Dirofilaria immitis* have produced circumstantial evidence that ecdysone-like molecules have a role in various developmental processes; for example, Warbrick et al. [37] demonstrated that the application of 20-hydroxyecdysone stimulated moulting *in vitro* of *D. immitis* L3 to L4, whereas a potential inhibitor of the ecdysone pathway, azadirachtin, inhibited moulting. Similarly, Barker et al. [38] showed that incubation of the ovaries of *D. immitis* in ecdysone stimulated the meiotic reinitiation of oocytes. The cloning and functional characterization of additional NRs from filarial nematodes should enable their role in the parasite life cycle to be elucidated. Furthermore, such studies, together with the data available from the *B. malayi* genome sequencing project [39], will allow the comparison of the *Brugia* NRs with those of *C. elegans*. In this respect it will be interesting to determine whether the extensive proliferation of NRs in *C. elegans* and *C. briggsae* is restricted to that genus, or whether a similar expansion has occurred elsewhere in the nematodes.

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