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Structural and functional characterisation of the fork head transcription factor-encoding gene, *Hc-daf-16*, from the parasitic nematode *Haemonchus contortus* (Strongylida)

Min Hu[a,1], **James B. Lok**[b,*], **Najju Ranjit**[b], **Holman C. Massey Jr**[b], **Paul W. Sternberg**[c], and **Robin B. Gasser**[a,*]

James B. Lok: jlok@vet.upenn.edu; Robin B. Gasser: robinbg@unimelb.edu.au

[a] Department of Veterinary Science, The University of Melbourne, 250 Princes Highway, Werribee, Vic. 3030, Australia

[b] Department of Pathobiology, School of Veterinary Medicine, University of Pennsylvania, 3800 Spruce Street, Philadelphia, PA 19104, USA

[c] Biology Division, California Institute of Technology, Pasadena, CA 91125, USA

Abstract

Despite their phylogenetic diversity, parasitic nematodes share attributes of longevity and developmental arrest (=hypobiosis) with free-living nematodes at key points in their life cycles, particularly in larval stages responsible for establishing infection in the host. Insulin-like signalling plays crucial roles in the regulation of life span and arrest (=dauer formation) in the free-living nematode, *Caenorhabditis elegans*. Insulin-like signalling in *C. elegans* negatively regulates the fork head boxO (FoxO) transcription factor encoded by *daf-16*, which is linked to initiating a dauer-specific pattern of gene expression. Orthologues of *daf-16* have been identified in several species of parasitic nematode. Although function has been demonstrated for an orthologue from the parasitic nematode *Strongyloides stercoralis* (Rhabditida), the functional capabilities of homologues/orthologues in bursate nematodes (Strongylida) are unknown. In the present study, we used a genomic approach to determine the structures of two complete *daf-16* orthologues (designated *Hc-daf-16.1* and *Hc-daf-16.2*) and their transcripts in the parasitic nematode *Haemonchus contortus*, and assessed their function(s) using *C. elegans* as a genetic surrogate. Unlike the multiple isoforms of *Ce*-DAF-16 and *Ss*-DAF-16, which are encoded by a single gene and produced by alternative splicing, mRNAs encoding the proteins *Hc*-DAF-16.1 and *Hc*-DAF-16.2 are transcribed from separate and distinct loci. Both orthologues are transcribed in all developmental stages and both sexes of *H. contortus*, and the inferred proteins (603 and 556 amino acids) each contain a characteristic, highly conserved fork head domain. In spite of distinct differences in genomic organisation compared with orthologues in *C. elegans* and *S. stercoralis*, genetic complementation studies demonstrated here that *Hc-daf-16.2*, but not *Hc-daf-16.1*, could restore *daf-16* function to a *C. elegans* strain carrying a null mutation at this locus. These findings are consistent with previous results for *S. stercoralis* and

* Corresponding authors. Tel.: +1 215 898 7892; fax: +1 215 573 7023 (J. B. Lok), tel.: +61 3 97312000; fax: +61 3 97312366 (R.B. Gasser).
Present address: Department of Agricultural Sciences, La Trobe University, Bundoora, Vic. 3086, Australia.

demonstrate functional conservation of the *daf-16b* orthologue between key parasitic nematodes from two different taxonomic orders and *C. elegans*. We conclude from these experiments that the fork head transcription factor DAF-16 and, by inference, other insulin-like signalling elements, are conserved in *H. contortus*, a parasitic nematode of paramount economic importance. We demonstrate that functionality is sufficiently conserved in *Hc*-DAF-16.2 that it can replace *Ce*-DAF-16 in promoting dauer arrest in *C. elegans*.

Keywords

Daf-16; Fork head transcription factor; Dauer; *Haemonchus contortus*; *Caenorhabditis elegans*; Transgenesis

1. Introduction

Fork head transcription factors are a large group of DNA-binding molecules that play key roles in the regulation of gene expression during embryogenesis, cell differentiation, development and/or ageing (Kaufmann and Knochel, 1996; Kaestner et al., 2000; Galbadage and Hartman, 2008). The first fork head transcription factor (designated FKH) was discovered in the terminal regions of early embryos of *Drosophila melanogaster* (see Weigel et al., 1989). At the time of its discovery, no known functional motifs were recognised in FKH. Shortly after this report, however, a mammalian fork head transcription factor, designated HNF-3A, was described and shown to contain a 160-amino acid region which is essential for DNA-binding and is structurally distinct from the binding domain of any known transcription factor (Lai et al., 1990). Comparison of the amino acid sequences of HNF-3A and FKH revealed a high degree of sequence identity in the DNA-binding domains (Weigel and Jackle, 1990). This domain, called the fork head/HNF-3 domain, was later identified in more than 100 molecules from a range of eukaryotes excepting plants (reviewed by Lai et al., 1993; Kaufmann and Knochel, 1996; Granadino et al., 2000; Kaestner et al., 2000).

Owing to the complexities of their names and classification, a new, unified nomenclature for these proteins as fork head box (Fox) transcription factors has been introduced and reflects the phylogenetic relationships of all known chordate Fox proteins (Kaestner et al., 2000). The subfamilies (A to O) of fork head transcription factors are presently designated based on amino acid sequence differences within the fork head domain. One of these subfamilies, FoxO, is considered to be particularly important in regulating the expression of genes involved in cell-cycle control, stress response, apoptosis, DNA damage repair, cell differentiation, ageing and tumour formation (e.g., Tran et al., 2003; Accili and Arden, 2004; Huang and Tindall, 2007).

In the free-living nematode *Caenorhabditis elegans*, the functions of the FoxO encoding gene, designated *daf-16* (or *Ce-daf-16* where necessary to distinguish it from its orthologues in other species) have been studied extensively (Murphy, 2006; Braeckman and Vanfleteren, 2007). The regulation of DAF-16 represents the key output of the insulin-like growth factor pathway in *C. elegans* (see Kenyon et al., 1993; Lin et al., 1997; Ogg et al., 1997). It plays critical roles in the regulation of life span and dauer formation, characterised by stress-resistant filariform morphology and arrested development (Kenyon et al., 1993; Lin et al., 1997; Ogg et al., 1997). Under conditions favouring growth and reproduction, DAF-16 is phosphorylated by the kinases from the insulin-like growth factor pathway and is transported to the cytoplasm, allowing the continuous development of *C. elegans* larvae to the adult stage. In contrast, under dauer-inducing conditions, such as starvation and/or overcrowding, insulin signalling ceases and unphosphorylated DAF-16 remains in the nucleus, binds to its response elements in the genome and brings about a pattern of gene expression, resulting in dauer-developmental arrest and its associated changes in morphology and life span. Recently, an orthologue of *daf-16*,

originally called *fktf-1*, and now *Ss-daf-16*, was identified in the parasitic nematode *Strongyloides stercoralis* (see Massey et al., 2003. Comparison between *Ss-daf-16* and *Ce-daf-16* revealed similarities in inferred amino acid sequence and gene organisation. For example, both genes produce multiple transcripts via alternative splicing, and the highest levels of homology (79.5% identity in amino acid sequence) exists in the DNA-binding or "fork head" domain. Lower levels of sequence similarity are seen in the C-termini (31.4% identity) and N-termini (51.4% identity) of these proteins (Massey et al., 2003). Like *Ce-daf-16*, *Ss-daf-16* is expressed at similar levels throughout development (Ogg et al., 1997; Massey et al., 2003). Besides these structural similarities, the ability of *Ss-daf-16* to complement a null mutation in *daf-16* also suggests that it has similar developmental regulatory capability to its *C. elegans* orthologue (Massey et al., 2006). These findings support the hypothesis that insulin-like signalling functions in *S. stercoralis* and that *Ss*-DAF-16 plays important roles in this pathway, possibly by regulating the formation of the infective L_3 (iL_3). While some information is now available for *S. stercoralis*, nothing is known about the functions of *daf-16* orthologues in the vast majority of medically or economically important parasitic nematodes, such as those of the order Strongylida. Studying the structures and functions of *daf-16*-like transcription factors in these parasites will be important in gaining an understanding of their developmental biology, particularly as it relates to the infective process. Therefore, in the present study, we characterised the structures of the *daf-16* orthologue in *Haemonchus contortus* (the barber's pole worm of small ruminants) and the DNA complementary to its transcripts.

2. Materials and methods

2.1. Propagation of H. contortus

Merino lambs (males; 8–12 weeks of age), maintained under helminth-free conditions, were infected intraruminally with 8000 iL_3 of *H. contortus*. The patency of the infection (~24 days) was ascertained by the detection of strongylid eggs in the faeces using the McMaster flotation method (MAFF, 1977). L_1, L_2 and iL_3 were collected after 1, 3 and 7 days of incubation of faeces at 28 °C, respectively, and purified by repeated sedimentation and migration through a nylon sieve (mesh size: 20 μm). For the collection of L_4 and adults of *H. contortus*, infected lambs were euthanised with an overdose of pentobarbitone sodium (Lethobarb, Virbac Pty. Ltd.), administered i.v. 8 and 30 days p.i., respectively. Adult worms were collected from the abomasums at necropsy using fine forceps, washed extensively in chilled (4 °C) PBS, and males and females (adults) separated prior to snap-freezing in liquid nitrogen and subsequent storage at −70 °C. Animal ethics approval (AEC No. 0707528) was given by The University of Melbourne, and the care and maintenance of sheep followed this institution's guidelines.

2.2. Isolation, purification, treatment and storage of nucleic acids

Total genomic DNA was extracted from ~0.5 g of single-sex (male or female) adult worms using a small-scale SDS/proteinase K extraction procedure (Gasser et al., 1993), followed by mini-column (Wizard® Clean-Up, Promega) purification. Total RNA was extracted separately from different developmental stages (L_2, L_3, L_4 or adults) or sexes of *H. contortus* (homogenised under liquid nitrogen using a mortar and pestle) employing the TriPure isolation reagent® (Roche Molecular Biochemicals). RNA yields were estimated spectrophotometrically, and the integrity of RNA was confirmed by detecting discrete 18S and 28S rRNA bands on ethidium bromide-stained gels. Each RNA sample (~10 μg) was treated with 2 U of *DNase* I (Promega) and incubated at 37 °C for 30 min prior to heat denaturation of the enzyme (75 °C for 5 min). Both DNA and RNA samples were stored at −70 °C.

2.3. Isolation of the full-length cDNA encoding Hc-daf-16 from H. contortus

Using the degenerate oligonucleotide primers DAF-16F100: 5′-CARGTNTAYGARTGGATGGT-3′ and DAF-16R100: 5′-CCNGCNCCYT CRTTYTG-3′,

designed to a relatively conserved element (between nucleotide positions 679–698 and 805–821 with reference to the *C. elegans* gene; Accession No. NM_001026427), a portion of *Hc-daf-16* was amplified by PCR from cDNA synthesised from total RNA extracted from adults of *H. contortus*. PCR products were cloned into the pGEM®-T-Easy vector (Promega) and sequenced. Based on these sequences (Accession No. FN433208), gene-specific primers Hc-daf16/1F: 5′-CAGGTGTACGAGTGGATGGTGCAG-3′; Hc-daf16/2R: 5′-GCTGAATGTAACGAGAGATTGTGCCGAA-3′; Hc-daf16/ 3F: 5′-GTGCCGTATTTCCGAGACAAGGGGCGA-3′ and Hc-daf16/4R: 5′-TCCGGCCCCTTCGTTTTGGATACGC-3′ were designed. Using pairs of gene-specific primers and primers specific to the nematode spliced leader 1 (SL1), two partially overlapping cDNA fragments were produced separately from total RNA from adult *H. contortus* using 5′- and 3′-rapid amplification of cDNA ends (RACE) (SMART™ RACE cDNA Amplification Kit, BD Biosciences). These cDNAs were ligated into the pGEM®-T-Easy vector. *Escherichia coli* (strain JM109) (10^8 colony forming U/μg) was transformed with recombinant plasmids via heat shock and grown overnight at 37 °C on Luria Bertani (LB) plates containing 10 mg/ml ampicillin, 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and 80 μg/ml X-gal (5-bromo-4-chloro-3-indolyl-β-galactosidase). Plasmid DNA was isolated from recombinant clones and column-purified (Wizard®, Promega) from overnight cultures, and inserts sequenced in both directions using vector oligonucleotide primers (M13 and SP6), employing Big Dye Terminator v.3.1 chemistry, in an automated ABI-PRISM sequencer (Applied Biosystems). Based on the resultant sequences, selected oligonucleotide primers were designed to amplify the full-length *Hc-daf-16*.

2.4. Isolation of the full-length genomic DNA of Hc-daf-16

Four overlapping fragments representing the entire *Hc-daf-16* gene were amplified by long-range PCR (Advantage 2 PCR Polymerase Kit, BD Bioscience) from ~100 ng of total genomic DNA from a single adult female of *H. contortus*, employing the primers designed based on the cDNA sequences determined (Accession Nos. FN432341 and FN432342) including Hc-daf16/9F: 5′-ACCGGTATG GCAAATCAGCTCTCGCAGACGC-3′, Hc-daf16/11F: 5′-ACCGGTATGA GCAGCCAAGTCACTGCC-3′; Hc-daf16/2R, Hc-daf16/3F, Hc-daf16/22R: 5′-GGAGTTGGCGGCGGCAGCGGCT-3′; Hc-daf16/5F: 5′-AGCCG CTGCCGCCGCCAACTCC-3′; Hc-daf16/16R: 5′-CACGCGCTGCACACCT ACTTGAG-3′. The cycling conditions in a 2400 thermal cycler (Applied Biosystems) were: 92 °C, 2 min (initial denaturation); then 92 °C, 10 s (denaturation); 60 °C, 30 s (annealing); 68 °C, 3 min (extension) for 35 cycles, and a final extension at 68 °C for 7 min. After the optimisation of conditions, the PCR yielded a single, abundant product, which was excised from the agarose gel (1%), purified over a mini-spin column (Wizard® PCR-Preps, Promega), cloned into the vector pGEM®-T-Easy and then used as a template for automated sequencing (as described in Section 2.3), employing (separately) vector primers T7 and SP6. The sequences obtained were assembled manually. The exon/intron boundaries of the full-length *Hc-daf-16* were inferred based on the alignment of the cDNA and genomic DNA sequences of *Hc-daf-16*, following the AG-GT rule (Brethnach and Chambon, 1981).

2.5. Bioinformatic and phylogenetic analyses

Nucleotide sequences were assembled using the program EGassembler (http://egassembler.hgc.jp/) and compared with those in non-redundant databases (GenBank) using the BLAST v.2.0 (Altschul et al., 1997) suite of programs from the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/ BLAST), the Sanger Centre (www.sanger.ac.uk/Projects/Celegans/) and the Parasite Genome database (www.ebi.ac.uk/parasites/parasite_blast_server.html) as well as the genomic sequence data for *H. contortus* available at the Wellcome Trust Sanger Institute (http://www.sanger.ac.uk/Projects/H_contortus/) to confirm the identity of the genes isolated.

The conceptual translation of individual cDNAs into amino acid sequences was performed using the selection “translate”, available at http://bioinformatics.org/ sms/. Protein motifs were identified by scanning the databases PROSITE (Bairoch, 1993; www.expasy.ch/tools/scnpsit1.html) and Pfam (Bateman et al., 2000; www.sanger.ac.uk/Software/Pfam/). Amino acid sequence alignments were carried out using Clustal W (Thompson et al., 1994).

The amino acid sequences of fork head domains from 27 FoxOs representing various vertebrate and invertebrate taxa, including eight species of nematodes, were retrieved from NCBI GenBank databases and then aligned with the amino acid sequences inferred from the *Hc-daf-16.1* and *Hc-daf-16.2* transcripts from *H. contortus*. Phylogenetic analyses were conducted using maximum parsimony (MP) and neighbour-joining (NJ) methods, employing PAUP* v4.0b10 (Swofford, 1999), as described recently (Hu et al., 2005). Characters were weighted equally and treated as unordered. An heuristic search with tree bisection–reconnection (TBR) branch swapping was used to infer the shortest trees. The length, consistency index (C.I.), excluding uninformative characters, and retention index (R.I.) of the most parsimonious trees were recorded. A bootstrap analysis (using 1,000 replicates) was conducted using heuristic searches and TBR-branch swapping with the MulTrees option to determine the relative support for clades in the consensus tree.

2.6. Reverse transcription real-time PCR analysis

Total RNA (1 μg) was used to synthesise first-strand cDNA by random priming using Superscript II reverse transcriptase (Cat. No. 18064-022, Invitrogen), following the manufacturer's instructions. Reverse transcription (RT) real-time PCR was used to analyse the transcriptional profiles among different developmental stages of *H. contortus*. Isoform-specific primers Hc-daf16/9F and Hc-daf16/18R: 5′-CATGTGGGCCGTCTGATTAGGC-3′ for *Hc-daf-16.1*; primers Hc-daf16/11F and Hc-daf16/20R: 5′-GACGAAGCACTTAGAGGTAG-3′ for *Hc-daf-16.2*, designed based on cDNA sequences (cf. Accession Nos. FN432341 and FN432342), were used in PCR. For each sample, 0.5 ng of cDNA was subjected to PCR (20 μl) using the SYBR Green-ER qPCR SuperMix Universal (Cat. No. 11762-100, Invitrogen) in a Rotor-Gene 3000 thermal cycler (Corbett Life Sciences) under the following conditions: one cycle of 50 °C for 5 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 s and 72 °C for 30 s, following by melting from 70 °C to 99 °C at 1 °C increments. Each sample was tested in triplicate, using a normaliser (i.e., a 364 bp region within the β-tubulin 8–9 gene, *tub8–9*) (Geary et al., 1992) using the primers tub8–9F3: 5′-GTTGTTCCATCACCCAAGGTA-3′ and tub8–9R4: 5′-TAAGCTCAGCAACTGTCGAA-3′ as well as positive and no-template controls. The specificity and identity of individual amplicons were verified by melting-curve analysis and direct, automated sequencing using the same primers employed for PCR. Relative transcriptional differences were calculated from normalised values using a well-accepted method (Livak and Schmittgen, 2001). Statistical analysis was conducted using a one-way ANOVA; $P \leq 0.05$ was set as the criterion for significance.

2.7. Genetic stocks of C. elegans and transformation constructs

The genetic stocks of *C. elegans* used were the same as described in a previous study (Massey et al., 2006). The strains were obtained from the *Caenorhabditis* Genetics Center (CGC) (University of Minnesota) and included the wild-type N2, mutants of *daf-2* (e1370) and *daf-2* (e1370); *daf-16* (mg54). All known *daf-16* alleles that affect dauer development in *C. elegans*, the phenotype of interest in the present study, map to the region of the gene encoding the *daf-16a* isoform, and the *mg54* allele chosen for the present heterologous complementation study involves an amber stop mutation within the DNA-binding domain in this region (Ogg et al., 1997). The *mg54* allele completely suppresses the dauer-constitutive phenotype exhibited

by *daf-2(e1370)* mutants at elevated temperature, making it advantageous for the double mutant approach to *daf-16* complementation described in Section 2.8. Worms from all genetic stocks as well as transgenic lines were maintained on nematode growth medium (NGM) agar plates containing *E. coli* OP50 lawns (cf. Lewis and Fleming, 1995.

For studies of cross-species complementation, the putative rescuing plasmid vectors pMH207-16a and pMH207-16b (see Fig. 1), containing the coding regions of *Hc-daf-16.1* and *Hc-daf-16.2*, respectively, were made from vector pPV207, reported in the previous study (Massey et al., 2006). In brief, *Ss-daf-16b* was removed from vector pPV207 by restriction digestion with enzymes *Age*I (A) and *Ngo*MIV (N) at sites located on the either end of *Ss-daf-16b*. The cDNAs of *Hc-daf-16.1* and *Hc-daf-16.2*, with restriction sites A and N at either end were amplified from cloned full-length cDNAs of *Hc-daf-16.1* and *Hc-daf-16.2*, digested with the enzymes and then cloned into vector pPV207 with *Ss-daf-16* removed, creating vectors pMH207-16a and pMH207-16b (see Fig. 1). A previously synthesised "rescuing" plasmid vector, pPV200 (Massey et al., 2006), was also used in the cross-species complementation experiments.

2.8. Establishment of transgenic lines and dauer-switching assay

The method used for establishing lines of *C. elegans* transformed with parasite sequences was the same as described previously (Massey et al., 2006). In brief, *C. elegans* of the *daf-2; daf-16* double-mutant strain were transformed by microinjecting 80 ng/μl of plasmid pRF4, which contains the *rol-6* mutation (Kramer et al., 1990), and 20 ng/μl of either pPV200 (Massey et al., 2006), pMH207-16a or pMH207-16b into the gonads of young hermaphrodites. Microinjected worms were then reared in isolation, and transformants were selected from their F1 progeny based on the "roller" phenotype and re-plated. The broods of individual micro-injected worms that transmitted transgenes to the F2 generation and beyond were propagated as transgenic lines. As in a previous functional study of *Ss-daf-16* (originally designated *fktf-1*) (Massey et al., 2006), we used this double mutant approach to capitalise on the ability of particular mutations in *Ce-daf-16* to suppress dauer-constitutive phenotypes associated with mutations in *daf-2* as a "read-out" for heterologous complementation. We reasoned that any *daf-16*-like activity associated with a hypothetical parasite orthologue should reverse the genetic suppression occurring in a *daf-2; daf-16* double mutant and give a significant increase in the percentage of dauer larvae arising under culture conditions of adequate nutrition. Such an approach offers the added advantage of obviating the application of an exogenous dauer pheromone, which would be necessary to undertake the complementation assay using *daf-16* single mutants.

Expression of transgene-specific mRNA was verified in each of the transformed lines by qualitative RT-PCR (Massey et al., 2006). Worms were collected from OP50 plates and placed into PCR tubes containing 48 μl of worm lysis buffer (Williams et al., 1992). Tubes were placed at −80 °C overnight. To extract RNA from the worms, samples were thawed and 4 μl of RNAsin (New England Biolabs) added to each tube. The samples were incubated at 65 °C for 75 min and then at 95 °C for 15 min. RNA was treated with *DN*ase I (Promega; according to the instructions) and then used as a template for RT-PCR, conducted using transgene-specific primers (Table 1) and Superscript II reverse transcriptase (Invitrogen; as specified in the instructions).

Lines with verified expression of transgene-specific mRNA were used in a dauer-switching assay to ascertain the capacity of *Hc-daf-16* to complement the null mutation in *C. elegans daf-16*, carried out as described previously (Massey et al., 2006). In brief, 20–30 egg-laying transformant (roller) hermaphrodites were placed on 60 mm NGM agar plates with standard *E. coli* OP50 lawns, formed by drying 40 μl of an overnight LB broth culture. The hermaphrodites were allowed to produce eggs for 3–8 h and were then removed from the plates.

The plates were then incubated at 25 °C for 65–70 h. At the end of this incubation, individual transformants, which were identified based on the right roller phenotype, were scored as non-dauer individuals (L_4 or adult) or dauer larvae. Mean proportions of dauer and non-dauer development were calculated from at least four biological replicates of the assay. The total numbers of transformed worms observed per group in four replicates ranged from 61 in the *Hc-daf-16.1* transgenics to 173 in the *daf-2* mutants. Proportions of dauer development in transgenic lines were compared with those in the *daf-2; daf-16* parental stock by χ^2 analysis; $P \leq 0.05$ was set as the criterion for significance.

3. Results

3.1. Characterisation of the cDNA of Hc-daf-16 from H. contortus and comparison of protein homologues

The full-length cDNAs of two transcripts representing *Hc-daf-16.1* and *Hc-daf-16.2* were isolated by RACE from *H. contortus*. Both cDNAs were trans-spliced by SL1. The full-length cDNA of *Hc-daf-16.1* (Accession No. FN432341) was 2,453 bp in length, including an open reading frame (ORF) of 1,812 bp, a 5′-untranslated region (UTR) of 191 bp, and a 3′-UTR of 428 bp. The full-length cDNA of *Hc-daf-16.2* (Accession No. FN432342) was 2,247 bp in length, containing an ORF of 1,671 bp, a 5′-UTR of 126 bp and a 3′-UTR of 428 bp. The cDNAs of *Hc-daf-16.1* and *Hc-daf-16.2* encoded predicted proteins of 603 and 556 amino acids, respectively. An alignment of the two protein sequences revealed that the 447-amino acid C-terminal domains of both (positions 157–603 for *Hc*-DAF-16.1 and positions 110–556 for *Hc*-DAF-16.2) were almost identical (Table 2), except for amino acid substitutions at positions 54 and 138 at the C-terminal end. The predicted amino acid sequences of *Hc*-DAF-16.1 and *Hc*-DAF-16.2 were aligned with the DAF-16s of five other nematode species (see Fig. 2) for which full-length sequences are available in the GenBank database. The alignment showed that sequence identities at the N-terminal and C-terminal regions were low (3.3–97.5% and 21.4–99.2%, respectively), whereas the fork head domains were conserved (57–100%). The fork head DNA-binding domains of nematodes also had high similarity to human FoxO1 (see Fig. 2). Predicted kinase phosphorylation sites and 14-3-3 protein-binding sites were also identified in the alignment. The two fork head domains of *H. contortus* were identical to those proposed for *Ancylostoma caninum* and *Ancylostoma ceylanicum* (Accession Nos. ACD85815 and ACD85816, respectively; Gao et al., 2009) (Fig. 2). In addition, the C-termini of isoforms *Ss*-DAF-16A and *Ss*-DAF16B were identical over a stretch of 378 amino acids (positions 364–741 for *Ss*-DAF-16A and 189–566 for *Ss*-DAF-16B, respectively) as were those of isoforms *Ce*-DAF-16A1 and *Ce*-DAF-16B over 320 amino acids (positions 191–510 for *Ce*-DAF-16A1 and 211–530 for *Ce*-DAF-16B, respectively) (see Fig. 2 and Table 2).

The sequences of the fork head DNA-binding domains of nematode DAF-16s were also aligned with representatives of each subfamily of fork head transcription factors (A to O) from humans (cf. Kaestner et al., 2000) (Fig. 3). The alignment revealed that nematode fork head domains had similarities to human FoxO1 ranging from 5.8% to 65.8%, whereas they usually showed lower similarities (1.7–22.2%) to human fork head domains from other subfamilies. The most conserved region was in the H3 domain, which is the DNA-binding region (Clark et al., 1993). Although there is substantial amino acid sequence variation in the alignment, the two signatures of fork head domain in the PROSITE database: W(Q,K,R)(N,S)S(L,I,V)RH and (K,R)P(P,T,Q)(Y,F,L,V,Q,H)S(F,Y)xx (L,I,V,M)xxx-(x)(A,C)(I,L,M) were readily identified in the alignment for nematode fork head domains (see Fig. 3).

3.2. Relationship of the predicted proteins Hc-DAF-16.1 and Hc-DAF-16.2 with DAF-16s inferred for other species

The amino acid sequences of fork head domains of *Hc*-DAF-16.1 and *Hc*-DAF-16.2 (inferred from the *H. contortus* genes *Hc-daf-16.1* and *Hc-daf-16.2*, respectively), eight other nematode DAF-16s and 19 FoxOs from selected non-nematode species were aligned and subjected to phylogenetic analyses (Fig. 4). There was concordance between the MP and NJ trees. The fork head domains of all vertebrate FoxO1s included in the present analysis grouped together with strong bootstrap support (89–94%), as did those of all vertebrate FoxO3s (86–89%). Two fork head domains from the genus *Drosophila* clustered together with 100% bootstrap support. All FoxOs from vertebrates and invertebrate formed a group with 99% bootstrap support. The fork head domains of the isoforms *Hc*-DAF-16.1 and *Hc*-DAF-16.2 were identical and both clustered closely with those from *A. caninum* and *A. ceylanicum* (designated as *Aca*-DAF-16 and *Ace*-DAF-16, respectively) with 100% bootstrap support. All isoform A sequences formed a group with selected vertebrate and invertebrate FoxOs, with strong bootstrap support (93–94%), to the exclusion of DAF-16B isoforms of *C. elegans*, *S. stercoralis* (*Ce*-DAF-16B and *Ss*-DAF-16B) and *Brugia malayi*, which clustered together with strong bootstrap support (84–96%, Fig. 4).

3.3. Genomic structure of Hc-daf-16 and comparison with homologues in C. elegans and S. stercoralis

Two full-length *Hc-daf-16* gene sequences (from start codon to stop codon) were isolated from genomic DNA of *H. contortus*. The genomic sequences representing gene *Hc-daf-16.1* (Accession No. FN432343) and *Hc-daf-16.2* (Accession No. FN432344) were 3,106 bp and 2,965 bp in length, respectively. Both genes contained four exons of 61–1,380 bp and three introns of 326–568 bp (Fig. 5). Comparison of the two genes revealed that the sequence divergence was predominantly in the first 471 nucleotides in *Hc-daf-16.1* and first 330 nucleotides in *Hc-daf-16.2*, respectively. The sequence identity in this region was 25.9%, whereas there was 99.6% of sequence identity between the 2,635 nucleotides downstream. In contrast to the situation in *H. contortus*, where the *daf-16a* and *daf-16b* transcripts are encoded by separate genes, the multiple isoforms of *C. elegans* DAF-16 and *S. stercoralis Ss*-DAF-16 are encoded by single genes and produced by alternative splicing (Fig. 5).

3.4. Transcriptional analysis in different developmental stages by RT real-time PCR

Real-time PCR analysis showed that *Hc-daf-16.1* and *Hc-daf-16.2* were transcribed in larval and adult developmental stages as well as both sexes of *H. contortus* examined (Fig. 6). There was no significant difference in the levels of transcription among different developmental stages for either isoform ($P > 0.05$).

3.5. Results of cross-species complementation experiments

In general, diagnostic RT-PCR confirmed the presence of appropriate transgene-specific mRNA in each of the transgenic *daf-2; daf-16* lines (Fig. 7A). As expected, *Ce-daf-16a* message was detected in N2 (wild type) worms, *daf-2* mutants and in the *daf-2; daf-16* double mutants carrying the *Ce-daf-16a* transgene. Also, as expected, the *Ce-daf-16a* message was absent from *daf-2; daf-16* double mutants carrying only the *rol-6* marker. Relatively weak signals were detected in RT-PCR with *Ce-daf-16a*-specific primers and cDNAs derived from both *Hc-daf-16.1* and *Hc-daf-16.2* transgenics, which might be due to a degree of cross-hybridisation between the *C. elegans*-based primers and the *Haemonchus* cDNAs. An alignment revealing that the forward primer Ce-daf16aF is 45% similar to the corresponding *Hc-daf-16* sequence and that the reverse primer is 65% similar supports this proposal. Reactions involving *Hc-daf-16.1*- and *Hc-daf-16.2*-specific primers detected transgene-specific message only in the respective transgenic line.

In dauer-switching assays conducted on well-fed larvae at 25 °C (Fig. 7B), N2 (wild type) worms developed uniformly to non-dauer individuals, as expected. These were mainly hermaphrodites with a minority of late L_4. By contrast, 100% of *daf-2* mutants developed to dauer L_3, reflecting the expected pattern of development in worms carrying the temperature sensitive *daf-2* (e1370) allele (Kimura et al., 1997). Ninety-nine percent of double mutants carrying both the *daf-2* (e1370) and the *daf-16* (mg54) alleles developed to non-dauer individuals (primarily hermaphrodites with a few L_4), and one percent to dauer larvae. This pattern is consistent with the characterisation of mutations in *daf-16* as genetic suppressors of *daf-2* mutations and with the requirement for the DAF-16 transcription factor in the formation of normal dauers (Lin et al., 1997;Ogg et al., 1997). Given the distinct and clear-cut phenotypic differences between *daf-2* single mutants and *daf-2; daf-16* double mutants, we reasoned that the restoration of significant levels of dauer development to the double mutant worms by expression of *Hc-daf-16* isoforms would be a straightforward indication of genetic complementation. This was clearly in evidence among *daf-2; daf-16* individuals expressing the *Ce-daf-16a*-encoding transgene in plasmid pPV200 (Fig. 7B). As might be expected, this homologous-rescuing construct restored full *daf-16* function in the double mutants, causing them to revert to the uniform dauer-constitutive phenotype of the *daf-2* single mutant (χ^2 = 162, $P < 0.0001$). Significantly, *daf-2; daf-16* double mutant *C. elegans* expressing *Hc-daf-16.2* from plasmid pMH207-16b also exhibited a complete phenotypic shift from the predominantly continuous pattern of development seen in untransformed worms of this genetic background to the dauer-constitutive pattern associated with *daf-2* single mutants (χ^2 = 183, $P < 0.0001$). By contrast, *C. elegans daf-2; daf-16* double mutants expressing the *Hc-daf-16.1* isoform from plasmid pMH207-16a did not show a significant departure from the phenotype seen in untransformed *daf-2; daf-16* double mutants (χ^2 = 4: P = 0.2854). It is emphasised that, as a control for any side effects of the *rol-6* co-transformation marker, all genetic strains as well as the *daf-16* transgenics were transformed with plasmid pRF4.

4. Discussion

In the present study, we structurally and functionally characterised the *daf-16* orthologue of *H. contortus*. The full-length cDNA sequences of two isoforms (*Hc-daf-16.1* and *Hc-daf-16.2*) were isolated. Comparison of their predicted amino acid sequences revealed that they possess identical fork head domains, suggesting that they bind to the same DNA sequence in the promoter regions of their target genes. This finding is distinct from the results reported for *Ss*-DAF-16 of *S. stercoralis* and *Ce*-DAF-16 of *C. elegans*. The fork head domains from two isoforms of either transcription factor (*Ss*-DAF-16 or *Ce*-DAF-16) shared identical sequences only in the C-terminus of the domain (66 residues for *Ss*-DAF-16 and 67 residues of *Ce*-DAF-16; see Table 2). The fork head domain contains the crucial sequence-specific DNA-binding motif characteristic of fork head transcription factors (Granadino et al., 2000;Carlsson and Mahlapuu, 2002). Differences in the amino acid sequences of the isoforms A and B of *Ss*-DAF-16 and *Ce*-DAF-16 occur in the first half of the DNA-binding domain; most of these primary structural differences in these two isoforms are conserved between species (Lin et al., 1997;Ogg et al., 1997;Lee et al., 2001). This information suggests conserved differences in binding specificity of orthologous DAF-16 isoforms. In contrast to *Ss*-DAF-16 and *Ce*-DAF-16, the two isoforms of *Hc-daf-16* have identical fork head domains. Interestingly, the fork head domain sequences of two hookworm species, *A. caninum* and *A. ceylanicum*, are identical to that of *Hc-daf-16* (Figs. 2 and 3), suggesting that the fork head transcription factors of all three strongylids have similar DNA-binding characteristics and similar functions. In the present study, the sequences of the fork head domains of *H. contortus*, *A. caninum* and *A. ceylanicum* grouped with isoform A of DAF-16 from both *S. stercoralis* and *C. elegans* to the exclusion of isoform B of both genes. This finding suggests that fork head transcription factors of strongylid nematodes have a similar function to isoform A of *Ce*-DAF-16, which is responsible for the major genetic activity from the *daf-16* locus for *daf-2*-mediated dauer arrest

and longevity control (Lee et al., 2001). Further studies of the function of fork head transcription factors of strongylid nematodes (e.g., *H. contortus*) are required but could be challenging, given the limitations with maintaining live worms for longer periods in vitro.

In spite of their identical fork head domains, sequences of the two isoforms of *Hc-daf-16* diverge in the N-terminal domains, which comprise 156 amino acid residues in *Hc*-DAF-16.1 and 109 amino acid residues in *Hc*-DAF-16.2 (Table 2 and Fig. 2). Comparison of this region of *Hc*-DAF-16.1 and *Hc*-DAF-16.2 with that of DAF-16s of other nematodes revealed that N-terminal region sequences of *Aca*-DAF-16 and *Ace*-DAF-16 were more similar to those of *Hc*-DAF-16.1 than to that of *Hc*-DAF-16.2. For example, an alignment (Fig. 2) showed that amino acid residues of *Hc*-DAF-16.1 at alignment positions 62–131 were almost identical to those of *Aca*-DAF-16 and *Ace*-DAF-16 between alignment positions 45–114, except for four amino acid substitutions. Sequence comparisons also reveal numerous insertions in the N-terminal domains of most parasite DAF-16 molecules compared with *C. elegans* DAF-16A or DAF-16B and *S. stercoralis* DAF-16B. This plasticity of structure in the N-terminal domain is likely related to functional divergence of these molecules. N-terminal domains in some fork head transcription factors, such as HNF-3β protein, are required for transcriptional activation (Pani et al., 1992;Qian and Costa, 1995). The difference in this region between the two isoforms reflects their possible difference in the transcriptional activation process.

In contrast to the high amino acid sequence similarity among fork head domains of fork head transcription factors of nematodes, the structures of the genes encoding these factors are distinctly different. *Ss-daf-16* and *Ce-daf-16* have similar gene organisations, although the numbers of exons and introns are distinct (Massey et al., 2003). Both genes are trans-spliced by SL1 and have alternative splicing, producing two isoforms. The promoter for isoform A (designated α for both *C. elegans* and *S. stercoralis* in Fig. 5) of either gene is located in the 5′-UTR upstream of the gene, respectively, whereas the promoter for isoform B (designated β for both species in Fig. 5) is in the large intron of each gene. This structural similarity suggests that the two genes might utilise similar transcriptional and/or splicing mechanisms. In *H. contortus*, genes encoding two distinct isoforms were identified. Both genes have three small introns and four exons. The main difference between these isoforms (at the nucleotide level) occurs at nucleotide sequence positions 1–471 for *Hc-daf-16.1* and 1–330 for *Hc-daf-16.2*, both at the 5′-end. The rest of the gene sequences (positions 472–3,106 bp for *Hc-daf-16.1* and 331–2,965 bp for *Hc-daf-16b*) share 99% sequence identity. All introns found within and upstream of the region encoding the fork head DNA-binding domain in *Ce-daf-16* and *Ss-daf-16* are absent from the *H. contortus* genes (Fig. 5). One of these missing introns contains the β-promoter and first exon of the *daf-16b* isoforms of *C. elegans* and *S. stercoralis* (see Ogg et al., 1997; Massey et al., 2003), suggesting that the loss of this isoform from *H. contortus* occurred as a result of “intron loss”. Although the presence of *daf-16b* isoforms in the *Ancylostoma* spp. could not be excluded by Gao et al. (2009), the substantial similarity of the encoded proteins and lack of *daf-16b* expressed sequence tags (ESTs) for these species suggests that an organisation similar to that in *H. contortus* might also occur in a range of Strongylida (clade V; Blaxter, 1998. Nevertheless, *H. contortus* has two forms of the *daf-16* gene. Due to the close similarity of the amino acid sequences and identical genomic organisation of the *Hc-daf-16.1* and *Hc-daf-16.2* isoforms, the most parsimonious model for their evolution is by gene duplication after an intron loss and their subsequent divergence, particularly in the N-terminal domain. In this case, the *Hc-daf-16.2* cannot be considered orthologous to either *Ss-daf-16b* or *Ce-daf-16b*, rather both *Hc-daf-16.1* and *Hc-daf-16.2* are interpreted to be paralogous descendents of an ancestral *daf-16a* duplicated after the *daf-16b* encoding intron had already been deleted.

Full complementation of the *C. elegans daf-16* (mg54) mutation by *Hc-daf-16.2* indicates that the parasite fork head transcription factor encoded therein is capable of fulfilling the dauer

regulatory functions of DAF-16 in the *C. elegans* context. This finding is consistent with the previous observation that the *Ce-daf-16b* orthologue of another parasitic nematode, *S. stercoralis*, can also complement the *daf-16* (mg54) mutation, albeit incompletely (Massey et al., 2006). The greater efficiency of complementation by *Hc-daf-16.2* may reflect the closer phylogenetic relationship between *C. elegans* and *H. contortus* (both within clade V), compared with the relatively distant relationship of *C. elegans* and *S. stercoralis* (a member of clade IV (Blaxter et al., 1998). A similar correlation between cross-species complementation efficiency and phylogenetic relatedness is seen in the case of parasite orthologues of the heat shock protein 90 (HSP-90) encoded by *C. elegans daf-21* (Gillan et al., 2009). These authors found that the *H. contortus* orthologue *Hc-hsp-90* can partially complement a *daf-21* mutation in *C. elegans*, whereas the orthologue *Bm-hsp-90* from the filarial nematode, *B. malayi* (a member of nematode clade III (Blaxter et al., 1998) cannot.

Another similarity between the cross-species complementation studies conducted with *daf-16* orthologues in *H. contortus* and *S. stercoralis* is the ability of one isoform of the *daf-16* orthologue to complement the *daf-16* (mg54) mutation in *C. elegans* and the failure of the second isoform to do so. In contrast, the functions of the isoforms *Ce*-DAF-16A and *Ce*-DAF-16B seem to be interchangeable in *C. elegans* when placed under the control of the *daf-16*α promoter (Lee et al., 2001) as they were in the present study. Therefore, it is puzzling that the *Hc-daf-16.2* and *Ss-daf-16b* isoforms complement the *Ce-daf-16a* null mutation, whereas the *Hc-daf-16.1* and *Ss-daf-16a* isoforms do not. One explanation might lie in differences between the free-living and parasitic life cycles. These differences must reflect differences in the regulatory circuitry of these organisms, likely including the associations of transcription factors, such as DAF-16, with other molecules of the regulatory network, including CREB (Nasrin et al., 2000) and nuclear hormone receptors, such as DAF-12 (Dieterich and Sommer, 2009; Wang et al., 2009). The complementation of *Ce-daf-16* null mutations by *Hc-daf-16.2* and *Ss-daf-16b* indicates that many of the functions of these molecules are conserved, but the lack of complementation by *Hc-daf-16.1* and *Ss-daf-16a* may reflect independent divergence of these molecules, as these two taxa evolved independently to the parasitic state. In each case, these divergences have rendered these transcription factors unable to function in the genetic milieu of *C. elegans*. It would be interesting to know whether the paralogous DAF-16A molecules in *H. contortus* and *S. stercoralis* can complement each other. These experiments would necessarily focus on the N-terminal domains of these molecules, which have been under-investigated compared with the fork head and C-terminal domains.

Interest in *daf-16* and other insulin-like signal transduction intermediates in parasitic nematodes stems, in part, from parallels drawn between the biologies of dauer larvae of *C. elegans* and the iL_3 of parasitic nematodes. The role of insulin-like signalling, in general, and of *daf-16*, in particular, in regulating dauer development in *C. elegans* invites the hypothesis that similar mechanisms regulate development of parasitic L_3. Complementation of mutations in *C. elegans* by insulin-like signalling intermediates from parasites, as shown here, constitutes a line of evidence supporting this hypothesis. However, viewed strictly, such findings only prove conservation of biochemical functionality in the *C. elegans* context, and functional homology in the parasite context awaits some method of altering the expression or function of a parasite gene of interest and assessing resultant phenotypes. Such direct functional genomic methods have been challenging to develop for parasitic nematodes. However, a recent study (Castelletto et al., 2009) shows that expressing mutant forms of FKTF-1/Ss-DAF-16 designed to exert a dominant negative effect on the endogenous transcription factor in transgenic *S. stercoralis* brings about a reversal of some dauer-like characteristics in post free-living larvae of this parasite and even induces an abnormal L_3–L_4 moult in some individuals in this phase of the life cycle. Together with functional data of this kind, cross-species complementation, as

reported here, strongly supports the hypothesis of a similar developmental regulation of free-living dauer larvae and the iL_3 of parasitic nematodes by insulin-like signal transduction.

In conclusion, we have identified genes encoding two isoforms of *Hc-daf-16*, an orthologue of *C. elegans daf-16* from *H. contortus*. We also characterised the cDNA and genomic DNA structures of *Hc-daf-16.1 and Hc-daf-16.2*, ascertained their transcription in key developmental stages of *H. contortus* and demonstrated conservation of the regulatory function of dauer in *Hc-daf-16.2* using *C. elegans* as a genetic surrogate. The findings from this study provide further evidence of the functional conservation of *daf-16*, and of insulin-like signalling, generally, between key parasitic nematodes and *C. elegans*. This study has important implications for understanding the developmental processes of parasitic nematodes, particularly those of the order Strongylida. Confirmation of the role of *Hc-daf-16* in arrest and reactivation of the iL_3 stage of *H. contortus* awaits direct functional assessment in the parasite itself.

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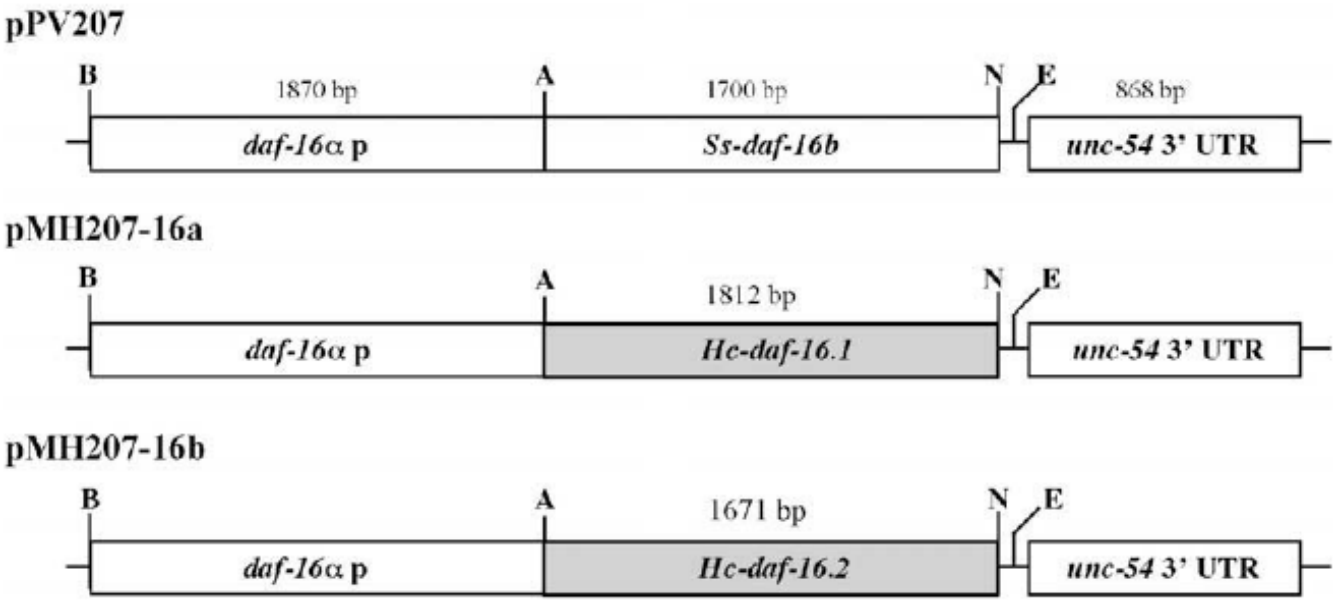

Fig. 1.

Cloning strategy for rescuing constructs. The constructs for *Hc-daf-16.1* (pMH207-16a) and *Hc-daf-16.2* (pMH207-16b), which encode two distinct isoforms of a fork head transcription factor of *Haemonchus contortus*, were made in the vector pPV207 by replacing the coding sequence *Ss-daf-16b* of *Strongyloides stercoralis* (see Massey et al., 2006). UTR, untranslated region.

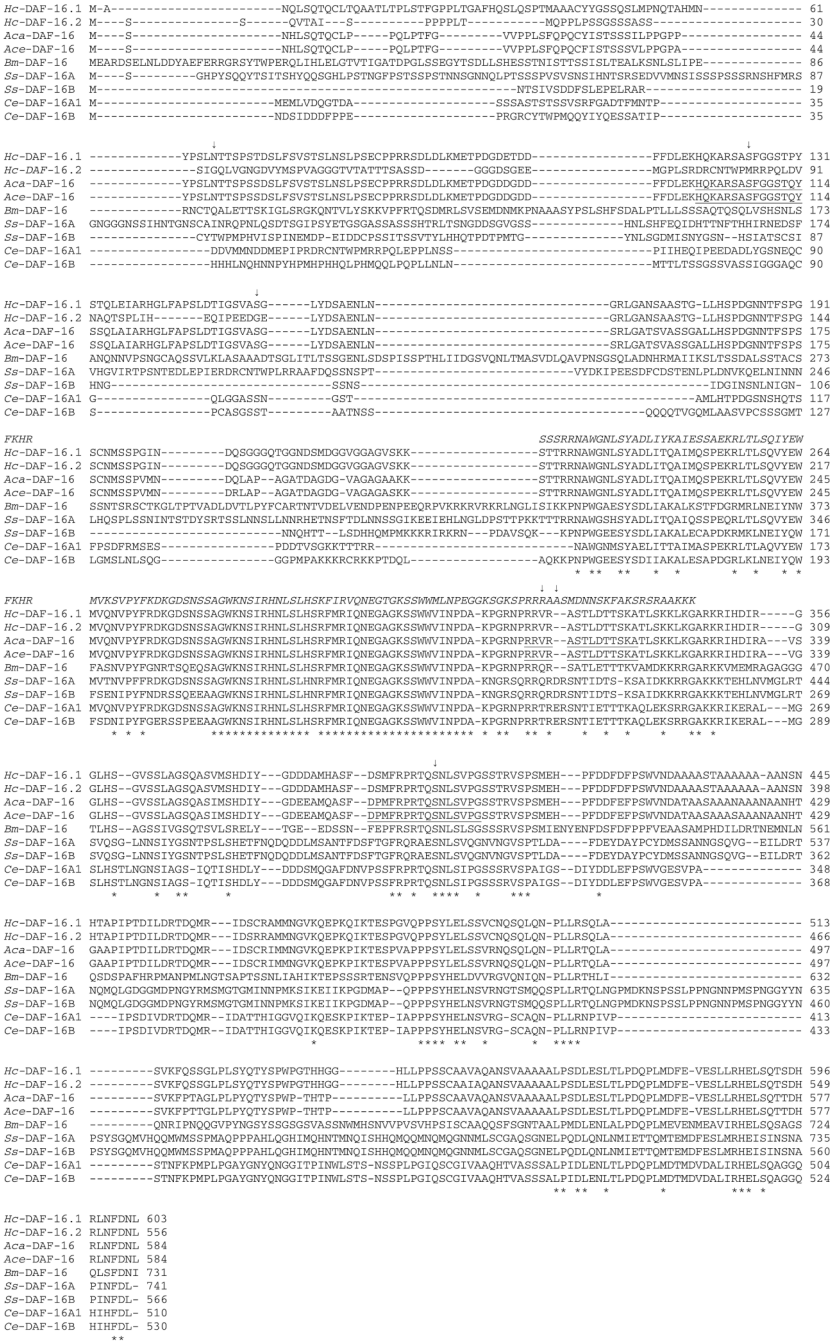

Fig. 2.

Alignment of amino acid sequences of fork head transcription factors of *Haemonchus contortus* (*Hc*-DAF-16.1 and *Hc*-DAF-16.2) and other nematode species for which full-length cDNA sequences of fork head transcription factors are available. The accession numbers of full-length sequences available from current databases are: ACD85816 (*Ancylostoma caninum Aca*-DAF-16), ACD85815 (*Ancylostoma ceylanicum Ace*-DAF-16), XP_001901487 (*Brugia malayi Bm*-DAF-16), AAQ23177 (*Strongyloides stercoralis Ss*-DAF-16A), AAQ23178 (*Strongyloides stercoralis Ss*-DAF-16B), AAB84390 (*Caenorhabditis elegans Ce*-DAF-16A1) and AAB84392 (*Caenorhabditis elegans Ce*-DAF-16B). The representative fork head domain region of FKHR (Accession No. AF032885) is displayed at the top in the

alignment. Identical amino acids are indicated by asterisks. Arrows at the top of the alignment indicate the phosphorylation sites recognised for *Ss*-DAF-16A, *Ss*-DAF-16B, *Aca*-DAF-16 and *Ace*-DAF-16. Underlined are predicted binding sites for 14-3-3 protein.

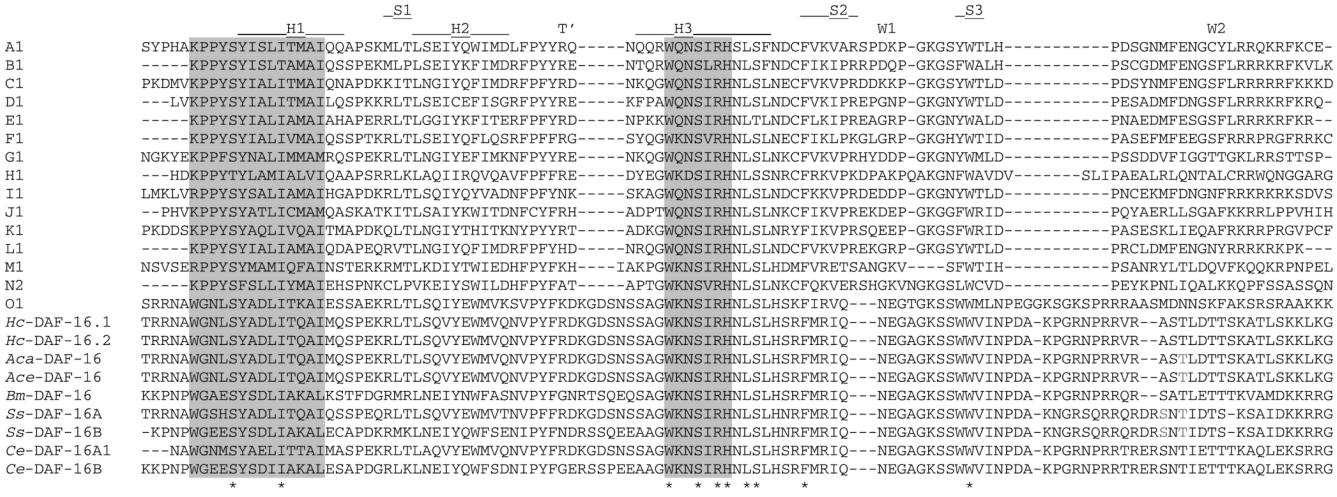

Fig. 3.

Alignment of amino acid sequences of fork head domains of human transcription factors representing each subfamily (cf. Kaestner et al., 2000) and all nematode fork head transcription factors for which full-length cDNA sequences are available in current databases. The accession numbers representing these sequences are: U39840 (A1), AF071554 (B1), U13221 (C1), U59831 (D1), U89995 (E1), AF085343 (F1), X78202 (G1), AF076292 (H1), U13224 (I1), X99349 (J1), U58196 (K1), U13225 (L1), U74612 (M1), U57029 (N2), Y11284 (O1), FN432341 (*Haemonchus contortus Hc*-DAF-16.1), FN432342 (*H. contortus Hc*-DAF-16.2), ACD85816 (*Ancylostoma caninum Aca*-DAF-16), ACD85815 (*A. ceylanicum Ace*-DAF-16), XP_001901487 (*Brugia malayi Bm*-DAF-16), AAQ23177 (*Strongyloides stercoralis Ss*-DAF-16A), AAQ23178 (*S. stercoralis Ss*-DAF-16B), AAB84390 (*Caenorhabditis elegans Ce*-DAF-16A1) and AAB84392 (*C. elegans Ce*-DAF-16B). Identical amino acids are indicated by asterisks. Structural elements of the domain (Clark et al., 1993) are shown on top of the alignment. Highlighted are the two signatures of fork head domain in the PROSITE database.

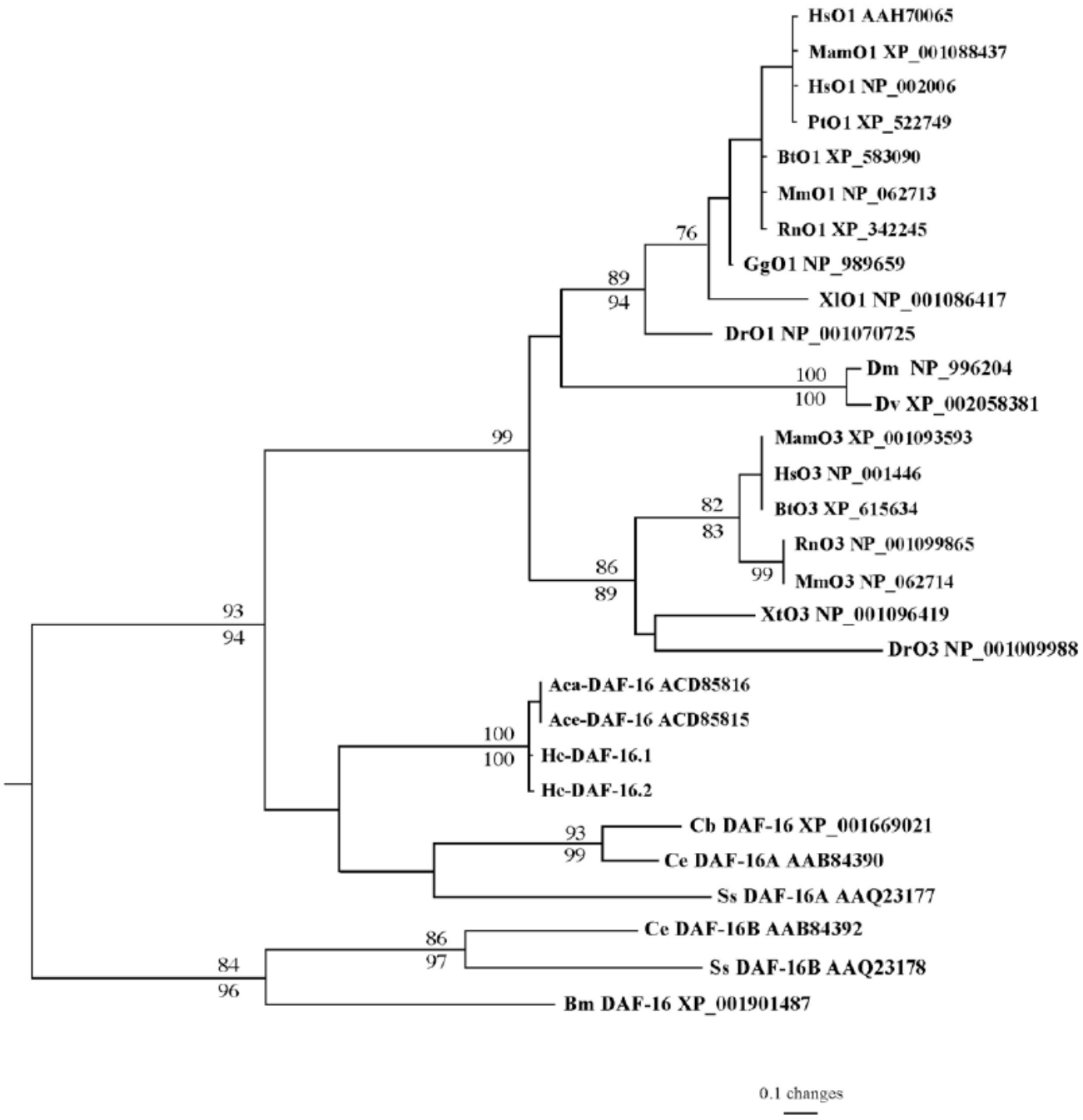

Fig. 4.

Neighbour-joining tree from amino acid sequences of fork head domains of transcription factors subfamily FoxO (O1: FoxO1; O3: FoxO3) from a range of species. Accession numbers identify individual sequences representing individual species. Abbreviation for species names are as follows: Hs: *Homo sapiens*; Mam: *Macaca mulatta*; Pt: *Pan troglodytes*; Bt: *Bos taurus*; Mm: *Mus musculus*; Rn: *Rattus norvegicus*; Gg: *Gallus gallus*; Xl: *Xenopus laevis*; Dr: *Danio rerio*; Dm: *Drosophila melanogaster*; Dv: *Drosophila virilis*; Xt: *Xenopus tropicalis*; Aca: *Ancylostoma caninum*; Ace: *Ancylostoma ceylanicum*; Hc: *Haemonchus contortus*; Cb: *Caenorhabditis briggsae*; Ce: *Caenorhabditis elegans*; Ss: *Strongyloides stercoralis*; Bm: *Brugia malayi*. The fork head domain amino acid sequence of human FoxM1 (Accession No.

U74612) was used as an outgroup. Numbers above and below the branches represent the bootstrap values obtained using the neighbour-joining and maximum parsimony methods, respectively; values >75 are indicated.

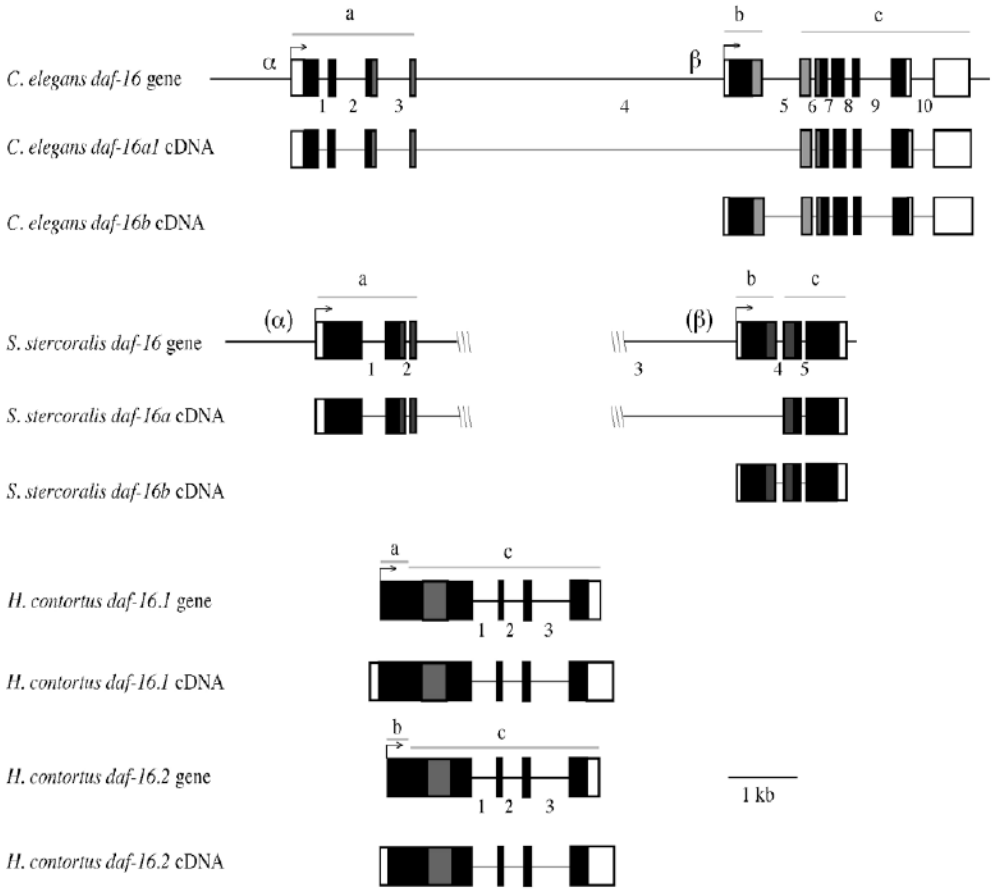

Fig. 5.

Genomic structure of *Hc-daf-16.1* and *Hc-daf-16.2*, which encode two distinct isoforms of a fork head transcription factor of *Haemonchus contortus*, compared with its *Caenorhabditis elegans* orthologue *Ce-daf-16* (Paradis and Ruvkun, 1998) and *Strongyloides stercoralis* orthologue *Ss-daf-16* (Massey et al., 2003). Narrow bars represent untranscribed sequences or introns; wide bars represent exons; white, non-coding; black, N- and C-terminal regions; grey, fork head domain. Regions specific to a-isoforms and b-isoforms are beneath bars and marked with “a” and “b”, respectively; those common to both isoforms are marked with “c”. The numerals represent the positions of introns. Arrows indicate direction of transcription and splice acceptor sites for the splice leader, SL1. For *C. elegans*, promoters for a- and b-isoforms are designated as α and β, respectively; for *S. stercoralis*, they are in parentheses at their expected locations.

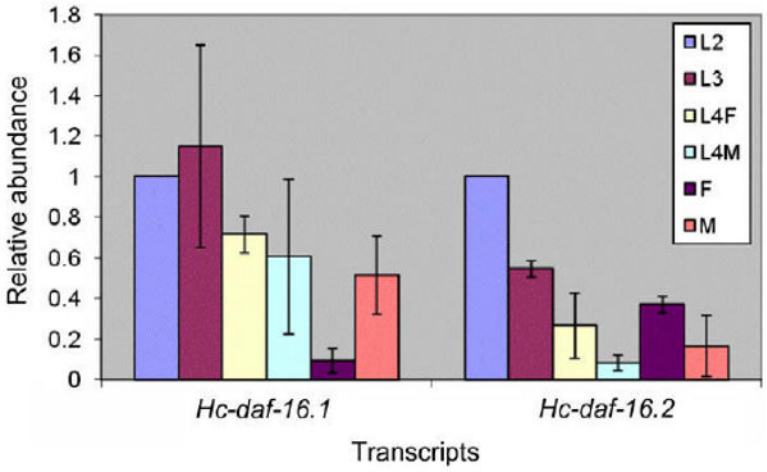

Fig. 6.

Transcriptional profile of of *Hc-daf-16.1* and *Hc-daf-16.2*, which encode two distinct isoforms of a fork head transcription factor of *Haemonchus contortus*, in different developmental stages (L_2, L_3 and L_4) and genders [females (F) and males (M)] of *H. contortus*, determined by real-time PCR analysis. Data shown are mean values (±SEM) derived from three replicates in repeat experiments. Relative transcription was calculated by normalisation of the raw data, followed by the determination of abundance relative to a calibrator. Quantification of the cDNA representing *Hc-daf-16* in each sample was normalised, using cDNA derived from transcripts of the β-tubulin gene (*tub*8–9) as an endogenous control.

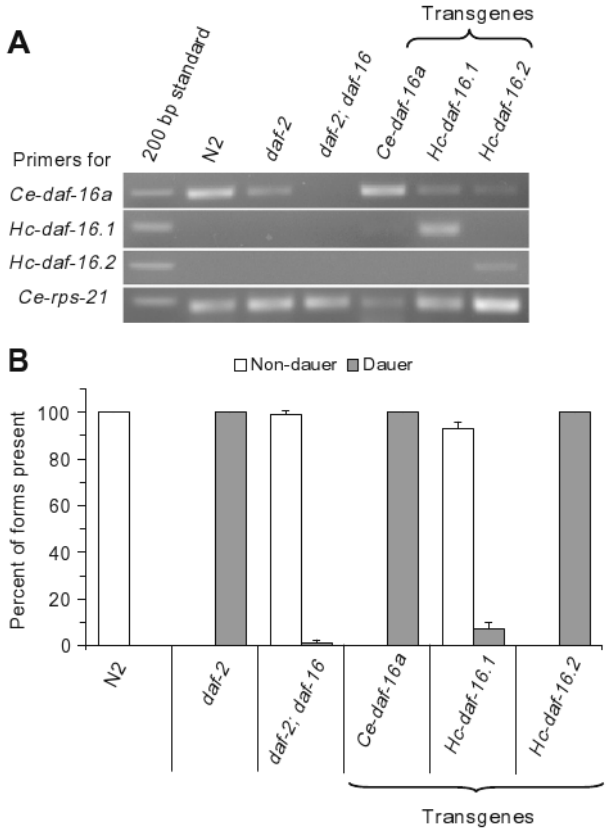

Fig. 7.

The construct for *Hc-daf-16.2*, but not *Hc-daf-16.1* of *Haemonchus contortus*, complements a loss-of-function mutation in *Ce-daf-16* of *Caenorhabditis elegans*. (A) Results of diagnostic reverse transcription (RT-PCR) on mutant and transgenic lines of *C. elegans*. Primer sets are indicated in the first column. Following the 200 bp size marker, gel lanes contain products of RT-PCR reactions with indicated primer sets from the three genetic lines assayed and from *daf-2; daf-16* double mutants transformed with transgene constructs containing the indicated coding sequences, expressed under the *daf-16α* promoter. These constructs are displayed in Fig. 1. Products derived from primers specific for the constitutively expressed small ribosomal subunit protein encoded by *Ce-rps-21* constituted the loading control. (B) Results of dauer-developmental assays on well-fed specimens from genetic lines, and *daf-2; daf-16* mutants transformed with the indicated transgenes. White and shaded bars represent non-dauer and dauer individuals, respectively. Error bars represent S.D. for results from four biological replicates. Total numbers of roller worms observed per group in four replicates were 100 for the N2 strain, 173 for *daf-2* mutants, 101 for *daf-2; daf-16* mutants, 77 for *Ce-daf-16a* transgenics, 61 in the *Hc-daf-16.1* transgenics and 103 for the *Hc-daf-16.2* transgenics. All genetic and transgenic lines contain the *rol-6* co-transformation marker. Frequencies of dauer development in *Ce-daf-16a* and *Hc-daf-16.2* transgenics differed significantly from the parental *daf-2; daf-16* stock ($P < 0.0001$), whereas that observed in the *Hc-daf-16.1* transgenics did not ($P = 0.2854$).

Table 1

Primers used in reverse transcription PCR to verify transcription from transgenes in stable lines of *Caenorhabditis elegans daf-2; daf-16* mutants transformed with rescuing plasmids in cross-species complementation studies (see text). Primers used to amplify *rps-21*-specific cDNA in loading controls are also listed.

Target transgene	Primer designation	Sequence
daf-16a	Ce-daf16aF	5′-CTCTCGATTCCTGGATCGTC-3′
	Ce-daf16aR	5′-TTAATCGGCTTCGACTCCTG-3′
Hc-daf-16.1	Hc-daf16aF	5′-ATGGCAAATCAGCTCTCGCAGACGC-3′
	Hc-daf16aR	5′-CATGTGGGCCGTCTGATTAGGC-3′
Hc-daf-16.2	Hc-daf16bF	5′-ATGAGCAGCCAAGTCACTGCC-3′
	Hc-daf16bR	5′-GACGAAGCACTTGTGGTAG-3′
rps-21	Ce-rps-21F	5′-GCTTTACGTTCCACGCAAG-3′
	Ce-rps-21R	5′-CCTTCTGGGCAAGTCTCAAGA-3′

Table 2

Numbers of amino acids at N-terminal and C-terminal regions of predicted protein sequences of fork head transcription factors of *Haemonchus contortus*, *Strongyloides stercoralis* and *Caenorhabditis elegans*.

Species	Isoform	Number of amino acids in full-length sequence			Number of amino acids in fork head domain		
		Total	N-terminal region	C-terminal region	Total	N-terminal variable region	C-terminal identical region
H. contortus	1	603	156	447	117	0	117
H. contortus	2	556	109	447	117	0	117
S. stercoralis	a	741	363	378	118	52	66
S. stercoralis	b	566	188	378	117	51	66
C. elegans	a	510	190	320	117	49	67
C. elegans	b	530	210	320	120	52	67