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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)

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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 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*.

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

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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