Published in final edited form as:

Int J Parasitol. 2011 April ; 41(5): 533–543. doi:10.1016/j.ijpara.2010.12.001.

Characterization of hookworm heat shock factor binding protein (HSB-1) during heat shock and larval activation[☆]

Joseph Krepp, Verena Gelmedin, and John M. Hawdon^{*}

Department of Microbiology, Immunology, and Tropical Medicine, the George Washington University Medical Center, 2300 I St. NW, Washington, D.C. 20037, USA

Abstract

When hookworm infective L3s infect their mammalian host, they undergo a temperature shift from that of the ambient environment to that of their endothermic host. Additionally, L3s living in the environment can be exposed to temperature extremes associated with weather fluctuations. The heat shock response (HSR) is a conserved response to heat shock and other stress that involves the expression of protective heat shock proteins (HSPs). The HSR is controlled by heat shock factor 1 (HSF-1), a conserved transcription factor that binds to a heat shock element in the promoter of HSPs, causing their expression. HSF-1 is negatively regulated in part by a HSF binding protein (HSB-1) that binds to and removes HSF-1 trimers bound to HSP gene promoters, resulting in attenuation of the HSR. Herein we describe an HSB-1 ortholog, *Ac*-HSB-1, from the hookworm *Ancylostoma caninum*. The *Ac-hsb-1* cDNA encodes a 79 amino acid protein that is 71% identical to the *Caenorhabditis elegans* HSB-1, and is predicted to share the characteristic coiled-coil structural motif comprised of two interacting alpha helices. Recombinant *Ac*-HSB-1 immunoprecipitated *Ce*-HSF-1 expressed in mammalian cells that had been heat shocked for 1 h at 42°C, but not from cells incubated at 37°C, indicating that HSB-1 only bound to the active DNA binding form of HSF-1. Expression of *Ac-hsb-1* transcripts decreased following 1 h of heat shock, but increased when L3s were incubated at 37°C for 1 h. Activation of hookworm L3s induces a five- to six-fold increase in *Ac-hsb-1* expression that peaks at 12 h, coincident with L3 feeding, but that subsequently decreases to two- to three-fold above control at 24 h. Recombinant *Ac*-HSB-1 immunoprecipitates greater amounts of 70 and 40 kDa proteins from extracts of activated L3s than from non-activated L3s. We propose that an increase in *Ac-hsb-1* levels early in activation allows feeding to resume, but that a subsequent decrease in expression permits a HSR that protects non-developing L3s at host-like temperatures. Further investigations of the HSR will clarify the role of HSB-1 and HSF-1 in hookworm infection.

Keywords

Hookworms; *Ancylostoma caninum*; Heat shock factor; Heat shock response; Activation; Transition to parasitism

[☆]Note: Nucleotide sequence data reported in this paper are available in the GenBankTM, EMBL and DDBJ databases under the accession number **HQ423167**.

^{*}Corresponding author. John M. Hawdon, Department of Microbiology and Tropical Medicine, The George Washington University Medical, 725 Ross Hall, 2300 Eye St. N.W., Washington, DC 20037, USA., Tel.: +1-202-994-2652; fax: +1-202-994-2913., mtmjmh@gwumc.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1. Introduction

Hookworm infection remains one of the most important infectious diseases globally, infecting over 700 million people (de Silva et al., 2003; Stepek et al., 2006). Heavy infection with hookworms results in debilitating and sometimes fatal iron-deficiency anemia, caused directly by blood loss to feeding adults in the intestine. Hookworm induced anemia is especially devastating in growing children, resulting in stunted physical and cognitive development, which may be permanent (Lozoff, 1989; Lozoff et al., 1991). Pregnant women, in whom hookworm infection can result in poor fetal outcomes (Bundy et al., 1995; Dreyfuss et al., 2000), and the elderly (Bethony et al., 2002) are also at high risk of morbidity due to hookworm disease. Hookworm is estimated to have caused the loss of 1.8 million disability adjusted life years (DALYs) worldwide in 2001 (WHO, 2002; Brooker et al., 2004). Failure of a recent vaccine (Hotez et al., 2010) leaves periodic, repeated deworming with anthelmintic drugs as the only method of control. However, rapid re-infection and the development of anthelmintic resistance limit the long-term effectiveness of this approach (Wolstenholme et al., 2004). Already treatment failures and possible resistance of human hookworms to common drugs have been reported (De Clercq et al., 1997; Reynoldson et al., 1997; Albonico et al., 2004; Kopp et al., 2007; Geary et al., 2010). Thus, there is a critical need to identify new vaccine and drug targets, which will require a more detailed understanding of the basic biology of these devastating parasites.

Individuals are infected with hookworms upon exposure to the developmentally arrested infective L3s in contaminated soil. Once inside the host, the L3 receives a signal that restarts the arrested developmental programs. Expression of the “parasitic gene set” (Rogers and Petronijevic, 1982; Petronijevic and Rogers, 1983) in response to the host-specific signal results in development to the L4 and finally the adult stage. Execution of the developmental program during invasion is the critical event in establishing a parasitic relationship, yet the molecular mechanisms controlling these events are poorly understood (Hawdon and Hotez, 1996).

In response to cues indicating a degrading environment, the closely related free-living nematode *Caenorhabditis elegans* enters a developmentally arrested alternative developmental stage known as a dauer (Cassada and Russell, 1975). Like the infective larva of hookworms, the dauer stage is developmentally arrested at the L3 stage, and will resume development only in an environment conducive to completion of the life cycle. Recovery from the dauer stage is analogous to resumption of development of hookworm L3 during infection, and has been proposed as a model for infection (Rogers and Sommerville, 1963; Hawdon, 1991; Hawdon and Schad, 1991a). An insulin-like signaling (ILS) pathway mediates recovery in *C. elegans* (Kimura et al., 1997; Paradis and Ruvkun, 1998), and the same pathway controls activation of hookworm L3 (Tissenbaum et al., 2000; Hawdon and Datu, 2003; Brand and Hawdon, 2004; Gao et al., 2009).

Hookworm L3s are subjected to a rapid change in temperature when they invade their endothermic host. This infection-associated temperature change is an important component of the stimulus that initiates activation of hookworm L3s (Hawdon and Schad, 1990) and development in other parasitic nematodes (Devaney and Lewis, 1993; Hunter et al., 2001; Thompson et al., 2001). The heat shock response (HSR) described in numerous organisms results in the rapid induction of a set of protective heat shock proteins (HSPs) that help maintain homeostasis and increase survival by acting as molecular chaperones (Morimoto, 1993; Feder and Hofmann, 1999; Santoro, 2000). The HSR is mediated by heat shock factor (HSF), a transcription factor that controls expression of HSPs in response to temperature increase. HSFs are a group of highly conserved transcription factors that have been described in a wide array of eukaryotic species (Wu, 1995; Santoro, 2000; Hahn et al.,

2004). Under stress, HSF1 converts from a monomeric to a trimeric DNA-binding active state in which it can induce transcription of the HSR genes (Morimoto, 1993; Satyal et al., 1998; Santoro, 2000). The HSF1 trimer is localized to the nucleus where it effects gene transcription. Its activation is controlled by a complex and highly regulated interaction with HSP-70 and HSP-90 (Morimoto, 1998; Shi et al., 1998; Nollen and Morimoto, 2002; Voellmy and Kivie, 2004).

During attenuation of the HSR in *C. elegans*, trimeric HSF-1 is negatively regulated by interaction with several HSPs and a HSF binding protein (HSB-1) (Satyal et al., 1998). HSB-1, together with HSP-70, bind to hydrophobic repeats in trimeric HSF-1 and cause conversion to the monomeric state and the loss of DNA binding and transcriptional activity. This, together with evidence that over-expression of the *C. elegans hsb-1* gene severely decreased life expectancy (Satyal et al., 1998), suggests that HSB-1 negatively regulates the HSR by its interaction with HSF1.

In addition to regulating the HSR in *C. elegans*, HSF-1 is required for normal development. Knockdown of *Ce-hsf-1* by RNA interference (RNAi) causes larvae to enter developmental arrest at the L1/L2 molt at elevated temperatures, and reductions in fertility, longevity and thermotolerance at normal temperatures (Walker et al., 2003; Morley and Morimoto, 2004). A role for HSF-1 in longevity is further supported by the increased lifespan in worms over-expressing *hsf-1* (Hsu et al., 2003). Also, *hsf-1* RNAi suppressed formation of the dauer stage in dauer-constitutive ILS mutants (Walker and Lithgow, 2003). The forkhead transcription factor DAF-16, together with HSF-1, was required for the HSR in *C. elegans*, and the increase in longevity is due to the expression of a set of small HSPs (sHSP) (Hsu et al., 2003). These data indicate an interaction between the HSR and ILS signaling in *C. elegans*.

Previously, we have demonstrated the importance of ILS in hookworm larval activation (Hawdon and Datu, 2003; Brand et al., 2005). As in *C. elegans*, hookworm DAF-16 binds to and directs transcription from a conserved DAF-16 binding element, and is regulated by nuclear exclusion following phosphorylation (Gao et al., 2009; Kiss et al., 2009). Given this high level of conservation between their life cycles, we hypothesized that *C. elegans* and hookworms share a similar HSR pathway. Furthermore, we propose that HSF-1 is required for maintenance of the developmentally arrested state in hookworm L3s, and that repression of HSF-1 is necessary for exit from developmental arrest. Currently, little is known about the role of the HSR and HSF-1 in the hookworm L3 transition to parasitism. Here we describe the identification and characterization of an HSB-1 ortholog from the hookworm *Ancylostoma caninum*, show that it binds to *Ce*-HSF-1 under heat shock conditions, and characterize its expression during activation of L3s in vitro.

2. Materials and methods

2.1. Parasites

The Baltimore strain of *A. caninum* (US National Parasite Collection no. 100655) was maintained as previously described in beagles (Schad and Page, 1982). Dogs were housed and treated according to a protocol approved by the George Washington University Institutional Care and Use Committee, USA. Infective L3s were recovered from fecal cultures by a modified Baermann technique approximately 1 week after culture and stored for up to 3 weeks in BU buffer (50 mM Na₂HPO₄, 22 mM KH₂PO₄, 70 mM NaCl, pH 6.8) (Hawdon and Schad, 1991b) at 22°C, or snap-frozen in liquid N₂ and stored at -80°C until lysates were prepared.

2.2. In vitro activation of L3s

Ancylostoma caninum L3s were activated under host-like conditions as described previously (Hawdon et al., 1999). Approximately 5000 decontaminated L3s were incubated at 37°C, 5% CO₂ for 24 h in 0.5 ml RPMI1640 tissue culture medium supplemented with 25 mM HEPES (pH 7.0) and antibiotics (RPMI-c) in individual wells of 24-well tissue culture plates. L3s were activated by the addition of 10% (v/v) of a less than 10 kDa ultrafiltrate of canine serum and 15 mM S-methyl-glutathione (GSM, Sigma Chemical, St. Louis, MO, USA) in RPMI-c. Non-activated L3s were incubated with RPMI-c medium alone. The percentage of activated L3s, as indicated by feeding, was determined as described previously (Hawdon and Schad, 1990). Following incubation, L3s were collected, snap-frozen and stored at -80°C until used.

2.3. Heat shock of L3s

Approximately 5000 axenized L3s were heat shocked in 0.5 ml RPMI-c at 42°C for 15, 30 and 60 min in individual wells of a 24-well cell culture plate. Following heat shock for 60 min, L3s were attenuated to room temperature for 15, 30 and 60 min. The same treatment was performed at 37°C followed by attenuation to 22°C. After treatment, L3s were collected, immediately snap-frozen and stored at -80°C until RNA was isolated.

2.4. RNA isolation

Frozen L3 pellets were suspended in 0.5 ml TRIzol reagent (Invitrogen, Carlsbad, CA, USA) containing zirconium oxide beads and homogenized twice for 5 min in a bullet blender (Next Advance Inc, NY, USA) at speed 8. Total RNA was isolated according to the manufacturer's instructions and was subsequently used for cDNA synthesis.

2.5. Cloning of Ac-hsb-1

An expressed sequence tag encoding an *A. caninum* HSBP-1 (pb17c01.y1) was used to design specific forward and reverse oligonucleotide primers for PCR. The *Ac-hsb-1* cDNA segments were amplified in three separate PCR reactions. The middle segment was amplified from first strand cDNA synthesized from L3s by standard methods (Hawdon and Datu, 2003). The reverse primer HSB-1-R3 (5'-TCGGAGTGTTCGATAACTTAATGCGG-3') and the forward primer HSB-1-F1 (5'-CAGCAGACGCAGGATCGATTCCAGC-3') were used in a PCR. The primers and cDNA were incubated for 30 cycles of 1 min at 94°C, 1 min at 55°C, 2 min at 72°C, followed by a final extension for 5 min at 72°C. The 3' end was isolated using the same reaction conditions from an *A. caninum* L3 directional cDNA library (Hawdon et al., 1995a) using gene-specific primer HSBP-1-F2 (5'-AGAAGAACA-TATCGGAAGTGATGGC-3') and the flanking vector sequence primer T7-PROM (5'-TAATACGACTCACTATAG-3').

The 5' end was isolated by 5' rapid amplification of 5' ends (RACE) according to the manufacturer's protocol (Life Technologies, USA). First strand cDNA was made from 4 µg of total *A. caninum* L3 RNA isolated as described above. HSBP-1-R3 was annealed to the RNA and Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) was used to produce first strand cDNA. A dCTP tail was added using TdT according to the manufacturer's instructions. PCR amplification was then performed on the dCTP-tailed cDNA using the manufacturer's Abridged Anchor forward primer and the reverse primer HSBP-R2 (5'-ATGCGCTTCGTCATGTCATCAATGCG-3'). The abridged anchor primer, HSBP-R2, and cDNA were incubated for 2 min at 94°C followed by 35 cycles of 1 min at 94°C, 1 min at 55°C, 2 min at 72°C, followed by a final extension for 5 min at 72°C. One microliter of a 1/100 dilution of the first round reaction was used as template for the second round PCR, using the manufacturer's Abridged Universal Amplification Primer and the

reverse nested primer HSBP-R1 (5'-TGTGCTGGAATCGATCCTGCGTCTGC-3') under the same conditions as the first round PCR.

A 153-bp 5' end product, 195-bp middle segment product, and a 295-bp 3' end product were cloned using a pGEM-T Easy TA cloning kit (Promega, Madison, WI, USA), and the DNA sequences determined. The DNA sequences of the three segments were combined to give the 489-bp full-length composite sequence of *Ac-hsb-1* including a 19-bp poly-d(A) tail.

2.6. *Ac-hsb-1* expression profile by reverse transcriptase (RT)-PCR

The developmental expression pattern of *Ac-hsb-1* was determined by reverse transcriptase (RT)-PCR. Total RNA was isolated from *A. caninum* eggs, L1s, L3s, and adult stages by homogenization in TRIzol Reagent as described above, and first strand cDNA was synthesized from 1 µg of total RNA using the Superscript First-Strand Synthesis System (Invitrogen) as described previously (Brand et al., 2005). Primers spanning the 5' and 3' ends of the protein coding regions were used to amplify *hsb-1* at individual life cycle stages. The forward (5'-ATGGCGTCAGACAAAGTGGACAGC-3') and reverse (5'-CGTCGAAACATGGTATGCTTAGGG-3') primers amplified a near full-length *Ac-hsb-1* cDNA of approximately 258 bp. Control primers that amplified a portion of the 3' untranslated region (UTR) of the protein kinase A catalytic subunit from all stages were used as a positive control (Hawdon et al., 1995a).

2.7. Quantitative RT-PCR

Total RNA was treated with DNase to digest genomic DNA, purified and used to synthesis cDNA using iScript reverse transcriptase and primer mix according the manufacturer's instructions (Biorad Inc, CA, USA). Relative quantitative RT-PCR (qRT-PCR) was performed using RT² Real-Time™ SYBR Green qPCR Master Mix (SABiosciences, MD, USA) and the CFX96 detection system (Biorad). A gene-specific primer set (HSB-1-RT-fw 5'-AGCATCATTCCTTCGGAGTC-3' and HSB-1-RT-rev 5'-CTGCGTCTGCTGAAGAACTC-3') was designed to amplify a 90 bp fragment of *Ac-hsb-1*. A fragment of 60S acidic ribosomal gene was amplified with the primers 60sRNA-Fw 5'-GTCGGAATCGTCG-GAAAGTA-3' and 60sRNA-Rv 5'-GTCTTGTTGCATTTCGAGCA-3' as reference gene (Trivedi and Arasu, 2005). Amplification efficiency of the target and reference gene was tested using standard curve experiments in triplicate. The ΔC_T between the target and reference gene was plotted versus the log of input template, and linear regression analysis conducted to confirm that the slope was less than 0.1 (Livak, 2001). Fold expression relative to the untreated control L3s was calculated for each sample run in triplicate by the comparative $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001). Template minus and RT minus controls were included, and melt curves were performed to ensure the presence of a pure amplicon.

2.8. Bacterial expression and purification of recombinant Ac-HSB-1(rAc-HSB-1)

The entire coding region of the *Ac-hsb-1* cDNA was amplified by PCR from *A. caninum* L3 cDNA using the forward primer HSBP-FX2 (5'-TTAGGATCCATGGCGTCA GACAAAGTGGACAGC-3') containing a 5' *Bam*HI site (underlined) and the reverse primer HSBP-RX3 (5'-GATTCTCGAGCGTCGAAACATGGTATGCTTAGGG-3') containing a 5' *Xho*I site (underlined). The PCR product was digested using *Bam*HI and *Xho*I restriction endonucleases (New England Biolabs, Beverly, MA, USA), gel purified, and directionally cloned into the bacterial expression vector pET28A (EMD Chemicals, Gibbstown, NJ, USA) that had been cleaved using the same restriction enzymes and gel purified. The construct was designed such that the resulting fusion protein would contain N-terminal hexahistidine and T7 tags, and was verified by DNA sequencing. For expression, the plasmid was transformed into *Escherichia coli* BL-21 (DE3) competent cells, expressed

and purified by standard methods (Delaney et al., 2005). Following purification, aliquots of the supernatant and cell pellet were separated on pre-cast 4–20% Tris-Glycine SDS-PAGE gradient gels (Invitrogen) and stained with Coomassie Blue. Solubilized rAc-HSB-1 protein was purified using a His Bind Purification Kit (EMD Chemicals) and eluted in 15 mL of elution buffer containing 250 mM imidazole.

2.9. Co-immunoprecipitation (co-IP) with rAc-HSB-1 and far-western detection

Co-immunoprecipitation (co-IP) was used to determine whether rHSB-1 bound to any *A. caninum* proteins during larval activation. Protein extracts were isolated from activated and non-activated *A. caninum* L3s as described previously (Kiss et al., 2009). Twelve µg of purified rAc-HSB-1 were mixed with 100 µg of soluble larval proteins and 485 µL of PBS containing 0.2% Tween 20 (PBS-T) and incubated for 10 h at 4°C on a nutating mixer. Control incubations containing larval proteins without recombinant HSB-1 and without worm proteins were established. After incubation, 2 µL of horseradish peroxidase (HRP)-conjugated T7 antibody were added, and the reactions incubated for 16 h at 4°C. Next, 100 µL of washed Protein-A agarose (50% slurry) were added and the incubated for 1.5 h at 22°C. Protein-agarose complexes were centrifuged for 30 s at 10,000 g, and the supernatants removed and retained for analysis as unbound samples. The agarose was washed with 1 ml of PBS-T for 5 min at 22°C and collected by centrifugation as above. The supernatants were discarded and the agarose-protein complexes washed two additional times. The washed agarose was re-suspended in 25 µl of 5 X SDS-PAGE sample buffer and processed for electrophoresis according to standard methods. The precipitated proteins, the control reactions, unbound samples, and recombinant HSB-1 (positive control) were separated through a pre-cast 4–20% Tris-Glycine gel. The separated proteins were transferred to a polyvinylidene difluoride (PVDF)-Plus transfer membrane (Osmonics, Inc., USA) at 25 V, 250 mA, for 1.5 h by standard methods (Delaney et al., 2005). Following transfer, the membrane was blocked using 5% non-fat dry milk in PBS-T for 16 h at 4°C. The blocked membrane was probed with 0.5 µg/mL of purified T7-tagged recombinant HSB-1 in 1% non-fat dry milk in PBS-T containing at 22°C for 1 h. The membrane was sequentially washed five times with 25 mL of PBS-T for 5 mins per wash, followed by incubation with HRP-conjugated anti-T7 (1/25,000 dilution in 1% PBS-T) for 1 h at 22°C. Following washing, immunoreactive bands were detected using the ECL Chemiluminescent Western Blotting Detection System (Amersham Biosciences) according to the manufacturer's instructions.

2.10. Cloning and mammalian expression of *C. elegans* hsf-1

As attempts to isolate the *Ac-hsf-1* cDNA proved unsuccessful, we cloned and expressed the *hsf-1* cDNA from *C. elegans* as a surrogate to investigate Ac-HSB-1 function. The *Ce-hsf-1* cDNA segment was amplified from first strand *C. elegans* (N2 strain) mixed stage cDNA by PCR. A reaction containing forward primer CEHSF-CMV-FR (5'-ATCGAATTCGCCATGCAGCCA-ACAGGGAATCAAATAC-3') containing an *EcoRI* site (underlined), reverse primer CEHSF-RFL (5'-AACCAAATTAGGATCCGATGGACTTGG-3'), 0.3 mM dNTPs, 2 mM MgSO₄, and Platinum *Taq* High Fidelity DNA Polymerase (Invitrogen, Carlsbad, CA, USA) was incubated for 35 cycles of 1 min at 94°C, 1 min at 50°C and 4 min at 72°C. After gel purification, 1 µL of a 1/200 dilution of the first round reaction was used as template for the second round PCR using the forward primer CEHSF-CMV-FR and the reverse primer CEHSF-CMV-RFL (5'-AAAACTCGAGAACCAATTAGGATCCGATGGAC-TTGG-3') containing an *XhoI* site (underlined). The PCR product was digested using *EcoRI* and *XhoI* restriction enzymes (New England Biolabs, Beverly, MA, USA), gel purified and directionally cloned into the mammalian vector pCMV-Tag4a that had been cleaved using the same restriction enzymes and gel purified. The pCMV-Tag4a/*Ac-hsf-1* construct was designed to encode the full-length *Ce-*

HSF-1 protein fused to a C-terminal FLAG tag. The ligated plasmid was transformed into DH5-alpha competent bacterial cells by standard methods as previously described (Delaney et al., 2005). The plasmids were isolated by using a QIAprep Spin Miniprep Kit according to the manufacturer's instructions (Qiagen, Valencia, CA, USA), and the construct was verified by DNA sequencing.

To express *Ce*-HSF-1, HEK 293T cells were transfected with 2 µg of pCMV-Tag4/*Ac-hsf-1* plasmid as described (Kiss et al., 2009). After 48 h incubation at 37°C, 5% CO₂, half of the transfected cells were incubated for an additional 1 h at 42°C while the other half remained at 37°C. Following incubation, media were removed and 150 µL of lysis buffer (50 mM Tris HCL pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1:200 dilution Protease Inhibitor Solution (Qiagen, Valencia, CA, USA)) were added to each well to lyse the cells. The lysed cells were vortexed for 30 s, and frozen at -80°C overnight. Samples were thawed, vortexed again and centrifuged at 22°C for 5 min at 2,300 g. The supernatants were collected and stored at -20°C until analyzed. Total protein concentrations of the lysates were determined by the bicinchoninic acid method (Micro BCA, Thermo Fisher) according to the manufacturer's instructions.

2.11. Co-IP of *Ce*-HSF1 with rAc-HSB-1

The interaction of recombinant *Ac*-HSB-1 with *Ce*-HSF-1 under host-like and heat shock conditions was evaluated by co-IP. *Ce*-HSF-1 from pCMV-Tag4/*Ac-hsf-1* transfected cells incubated at 37°C and 42°C was immunoprecipitated by the addition of 10 µg of rAc-HSB-1 to 100 µg of cell lysate. Control reactions containing mock lysate alone, non-heat shocked *Ce*-HSF-1 lysate alone, and mock lysate with rAc-HSB-1 were also established. The reactions were brought to a final volume of 500 µL using lysis buffer and were incubated at 4°C for 8 h on a nutating mixer. Following incubation, 20 µL of compact anti-FLAG M2 resin (Sigma) were prepared according to the manufacturer's instructions and added to each sample. The samples were incubated for 18 h at 4°C with rotation. Bound proteins were collected by centrifugation at 10,000 g, and the supernatants discarded. Following three washes with PBS-T, the proteins were eluted from the beads in 2 X SDS-PAGE sample buffer, separated by electrophoresis, and transferred to PVDF membranes as described in section 2.9. Immunoprecipitated *Ce*-HSF-1 was detected using anti-FLAG monoclonal primary antibody (1/10,000) and HRP-conjugated anti-mouse IgG (1/10,000), whereas *Ac*-HSB-1 was detected using HRP-conjugated anti-T7 monoclonal antibody (1/7,500). Immunoreactive bands were detected using the ECL (*Ce*-HSF-1) or ECL-Plus Chemiluminescent Western Blotting Detection System (*Ac*-HSB-1) according to the manufacturer's instructions (Amersham Biosciences).

2.12. DNA sequencing and sequence analyses

Dideoxy DNA sequencing using the ABI BigDye Terminator Cycle Sequencing Ready Reaction Kit v 3.1 was performed by the Nevada Genomics Center, USA, with the reactions run on an ABI3730 DNA Analyzer. Sequence analysis was performed using BioEdit Sequence Alignment Editor v 5.0.9 (Hall, 1999), and multiple alignments performed using CLUSTAL W at Swiss EMBnet (www.ch.embnet.org/). Homology searches were done using BLASTP (Altschul et al., 1990, 1997) at the National Center for Biotechnology Information (blast.ncbi.nlm.nih.gov/Blast.cgi) and WU-BLAST Parasite Genome Database Query at the European Bioinformatics Institute (www.ebi.ac.uk/Tools/blast2/parasites.html). The percentages of identity and similarity were determined using the BLASTp algorithm with the BLOSUM62 matrix. Secondary structure analyses were performed using the Protein Homology/analogy Recognition Engine (PHYRE version 0.2) at Imperial College London, UK (www.sbg.bio.ic.ac.uk/~phyre/index.cgi)

3. Results

3.1. Identification of *Ac-hsb-1*

An *A. caninum* expressed sequence tag (EST) similar to *Ce-hsb-1* (K08E7.2) was used to design oligonucleotide primers to amplify the full-length cDNA sequence by PCR. Three different PCR reactions were used to generate a composite sequence of *Ac-hsb-1*. The 153-bp 5' end product, amplified by 5' RACE, overlapped the first 27-bp of the 195-bp middle segment product that was amplified by PCR. A 295-bp 3' end product containing a poly(dA) tail overlapped the last 110-bp of the middle segment. The segments were combined in silico to yield a 470-bp full-length composite sequence. Primers were designed from the composite sequence to amplify the protein-coding region in a single amplicon for cloning into the bacterial expression vector pET28A.

The full-length *Ac-hsb-1* cDNA contained the 22 bp nematode spliced leader sequence 1 (Bektesh et al., 1988; Hawdon et al., 1995a) followed by four untranslated bases and the ATG codon encoding the starting methionine at nucleotide 27. The cDNA encoded a predicted 79 amino acid open reading frame which ended with a TAA stop codon at nucleotide 264. There is a 204 bp 3' untranslated region containing a canonical AATAAA polyadenylation signal 18 nucleotides upstream of the poly(dA) tail (Hajarnavis et al., 2004). The full-length *Ac-HSB-1* protein has a predicted molecular weight of 8,999 Da and an estimated pI of 4.34. The full-length sequence was deposited in GenBank (accession number **HQ423167**).

A BLASTp (Altschul et al., 1997; Schaffer et al., 2001) search of the non-redundant GenBank database using an *Ac-HSB-1* amino acid sequence revealed the highest homology with HSB proteins from three other nematodes: *Caenorhabditis briggsae* (71% identity, 85% positives over 67 amino acids), *C. elegans* (68% identity, 80% positives over 70 amino acids) and *Brugia malayi* (65% identity, 81% positives over 69 amino acids). As shown in Fig. 1, the nematode HSB-1 proteins share considerable identity with the HSB from humans (*Hs-HSB-1*, 59% identity, 79% positives over 59 amino acids), indicating a high level of conservation during evolution.

HSB proteins have a characteristic coiled-coil structural motif comprised of two interacting alpha helices, each containing heptad repeats of hydrophobic amino acids (designated **abcdefg**) (Fu et al., 2006). Typically the first and fourth residues (**a** and **d**) are on the surface at the helical interface, and interact with the corresponding residue on the opposing helix (Tai et al., 2002). The stability is determined by the interaction between the **a** and **d** residues, while the specificity depends on interactions between the **e** and **g** residues (Fu et al., 2006). The alpha helices of HSB-1 self-associate, as well as mediating the interaction with the trimerization domain of HSF-1 (Satyal et al., 1998; Tai et al., 2002). In *Hs-HSB-1*, the C-terminal heptad repeat region (HR-C) contains a perfect repeat pattern, whereas the pattern is discontinuous in the N-terminal repeat (HR-N) due to a "stutter" caused by the deletion of three residues of the heptad (Tai et al., 2002). Comparison with *Hs-HSB-1* and *Ce-HSB-1* confirms that *Ac-HSB-1* has the conserved arrangement of heptad repeats, including the missing three positions of the heptad at position 47 in HR-N (Fig. 1), and is therefore orthologous to *Ce-HSB-1*.

3.2. *Ac-hsb-1* expression during heat shock

Qualitative RT-PCR using cDNA synthesized from egg, L1, non-activated and activated L3, and adult mRNA indicated that *Ac-hsb-1* is expressed throughout the hookworm life cycle (Fig. 2). However, *Ac-hsb-1* expression levels might change at key developmental stages in response to changes in environmental temperatures. Infective L3s are free-living, and therefore may be exposed to potentially lethal high temperatures before entry into the host.

Also, the transition from the external environment to the internal environment of an endothermic host represents a potential heat shock that may involve a HSR that alters *hsb-1* expression levels. To determine whether expression levels change during heat shock above host temperatures, *A. caninum* L3s were exposed to heat shock at 42°C for 15, 30 and 60 min in individual wells of a 24-well cell culture plate, and the expression levels compared by qRT-PCR with L3s incubated at 37°C. As shown in Fig. 3, *hsb-1* transcript levels were similar in L3s exposed to 37°C and 42°C for up to 30 mins. However, at 60 min levels of *hsb-1* were up-regulated in L3s incubated at 37°C, but down-regulated in those incubated at 42°C. When the L3s were attenuated to room temperature following exposure to elevated temperature, levels of *Ac-hsb-1* in those incubated at 37°C remained high, whereas those exposed to 42°C showed a variable response, with levels remaining low at 15 min of attenuation, rising to levels of untreated L3s at 30 min at room temperature, and dropping again at 60 min of attenuation. If *Ac*-HSB-1 functions as a negative regulator of HSF-1 and the HSR as it does in *C. elegans* (Satyal et al., 1998), these results indicate that while exposure to 42°C for 60 min elicits a HSR in L3s, exposure to a host-like temperature of 37°C does not.

3.3. *Ac*-HSB-1 interacts with HSF-1 from *C. elegans* during heat shock

HSF-1 exists in the inert monomeric state at 37°C with minimal protein binding activity but forms a trimer upon heat shock (Satyal et al., 1998). The biologically active trimer can subsequently bind DNA and promote the transcription of heat shock genes, as well as bind HSB-1. To determine whether *Ac*-HSB-1 interacts with active HSF-1, 293T cells were transfected with a plasmid encoding FLAG-tagged *Ce*-HSF-1 and incubated at 37°C or 42°C for 1 h prior to collection. *Ce*-HSF-1 was used because we were unable to isolate *Ac*-HSF-1. Recombinant *Ac*-HSB-1 was incubated with the cell lysates, and anti-FLAG M2 used to IP *Ce*-HSF-1 and any interacting proteins. As shown in Fig. 4, HSF-1 precipitated *Ac*-HSB-1 after 1 h at 42°C but not at 37°C (Lane 1 versus 2). This indicates an interaction occurs between *Ac*-HSB-1 and active HSF-1 trimers in heat shocked cell cultures.

3.4. *Ac-hsb-1* expression during activation

In addition to a potential heat shock, infecting hookworm L3s are exposed to host-specific physico-chemical signals that induce the resumption of development (Hawdon and Schad, 1991a). As HSF-1 is required for normal development in *C. elegans* (Walker et al., 2003; Morley and Morimoto, 2004), we considered whether HSF-1 and the HSR were components of the activation signal encountered by L3s during invasion. Early events of activation can be mimicked in vitro by incubating L3s in host-like stimuli at 37°C, and activation detected by determining feeding in the L3s (Hawdon and Schad, 1990, 1991a). To investigate the role of HSR in larval activation, we determined the expression pattern of *Ac-hsb-1* in non-activated and activated L3s throughout the 24 h in vitro activation course.

Within 1 h of incubation, *Ac-hsb-1* expression was notably up-regulated in activated L3s compared with non-activated L3s (Fig. 5A). Interestingly, the increase in expression correlated with the stimulus and the degree of activation. Larvae incubated with a partial stimulus (GSM or serum filtrate alone) had intermediate expression levels between non-activated L3s and L3s exposed to the complete activation stimulus (Fig. 5A). Expression levels correlated to the feeding level of parallel incubations determined at 24 h, with L3s incubated with GSM alone feeding at 16% and L3s incubated with serum filtrate alone feeding at 26%, compared with 88% feeding in L3s exposed to both serum filtrate and GSM (Fig. 5A). Therefore, early expression of *Ac-hsb-1* is correlated with activation in hookworm L3s.

As in the heat shock experiments, elevated temperature alone had a slight effect on *Ac-hsb-1* levels. L3s incubated at 37°C in the nematode handling buffer BU (Hawdon and Schad, 1991b) showed slightly elevated levels of *Ac-hsb-1*, which returned to baseline by 24 h (Fig. 5B). However, non-activated L3s, which differ from the BU incubated L3s in that they were incubated in RPMI, show a much more robust up-regulation of *hsb-1* levels, rising to approximately eight-fold above untreated L3s at 12 h before returning to near baseline levels at 24 h. The highest expression was seen in activated L3s at 12 h, with a 15-fold up-regulation compared with the untreated controls. By 24 h, the expression levels had dropped to a six-fold up-regulation, but still remained higher than in non-activated L3s. These results show a significant up-regulation of *Ac-hsb-1* transcription in response to activation and indicate a clear impact of host-mimicking stimuli on its expression. At least a portion of this up-regulation is induced by complex incubation medium alone.

3.5. Ac-HSB-1 interacts differently with substrates under activating conditions

As activation increases transcription of *Ac-hsb-1*, we next asked whether HSB interactions with endogenous proteins differed during activation. We tested the ability of rAc-HSB-1 to interact with endogenous proteins during activation by immunoprecipitation and Far Western detection. Soluble lysates of 24 h non-activated and activated L3s were mixed with T7-tagged rAc-HSB-1, and complexes precipitated by anti-T7 antibody. Following electrophoresis, recombinant HSB-1 was used as a probe to detect interacting proteins, and anti-T7 antibody conjugated to HRP used to visualize the interactions by chemiluminescence. As shown in Fig. 6, the bound fraction of activated L3s contains significantly greater amounts of proteins with relative molecular weights of approximately 40 kDa and 70 kDa (compare lanes 1 and 4). These proteins are absent from the controls (lanes 5–11). This indicates that at least two HSB-1 substrates have an increased ability to interact with HSB-1 in activated worms.

4. Discussion

Elevated temperature elicits the expression of molecular chaperones and stress-associated proteins that help to mitigate heat-induced damage (Mager and De Kruijff, 1995). This universal HSR is regulated by the key transcription factor HSF-1, which induces expression of HSPs in response to stress (Pirkkala et al., 2001). The HSR of parasitic nematodes is poorly understood, especially given the role of temperature as a development cue during infection of homeothermic hosts. For example, microfilaria of the filarid nematode *B. malayi* express several HSPs at 37°C that are rapidly repressed upon transfer to the temperature equivalent to that of the mosquito host (Devaney et al., 1992; Jecock and Devaney, 1992). In the L3s, however, HSP gene expression coincides with transition to the homeothermic mammalian host, with elevated temperature being the primary trigger (Jecock and Devaney, 1992; Hunter et al., 2001; Devaney, 2006). Increased temperature is a required component of the hookworm activation signal (Hawdon and Schad, 1990; Hawdon and Hotez, 1996), suggesting a potential role for the HSR and HSP gene expression in hookworm development.

An important negative regulator of HSF-1, and consequently the HSR, is the HSF binding protein HSB-1. During attenuation HSB-1, together with HSP70, binds to the DNA-bound trimeric form of HSF-1 and causes its dissociation from the DNA, thereby repressing the HSR (Morimoto, 1998; Satyal et al., 1998). HSB-1 is required for proper regulation of stress responses in *C. elegans*, as over-expression of HSB-1 causes decreased survival following exposure to elevated temperature or chemical stress (Satyal et al., 1998), and HSB-1 is required for seed development in *Arabidopsis* (Hsu et al., 2010). Given the role of increased temperature in the life cycle of hookworms, and the importance of HSB-1 in regulation of

the stress response and development, we sought to identify and characterize an HSB-1 ortholog from the dog hookworm *A. caninum*.

Using an EST sequence, primers were synthesized and used to amplify the *Ac-hsb-1* cDNA in three separate reactions. The deduced amino acid sequence showed high levels of identity to *Ce*-HSB-1 and *Hs*-HSB-1 sequences, including the characteristic coiled-coil motif containing two interacting alpha helices and the heptad repeats of hydrophobic amino acids involved in HSF-1 binding (Tai et al., 2002; Fu et al., 2006). Recently, the crystal structure of the *Hs*-HSB-1 hexamer was solved (Liu et al., 2009). Individual HSB-1 molecules form parallel, symmetrical coiled-coil trimers that further associate as dimers in a head-to-head arrangement in solution, although HSB-1 probably binds to HSF-1 as a trimer (Liu et al., 2009). Comparison of *Ac*-HSB-1 primary sequence with that of *Hs*-HSB-1 indicates strict conservation of the critical Ser31 (Ser41 in *Ac*-HSB-1) and other residues involved in trimer formation.

As expected, *Ac-hsb-1* is expressed throughout the life cycle of *A. caninum*. However, we investigated whether expression levels changed during periods of elevated temperature, perhaps as a mechanism for regulating the HSR. Incubation at 42°C for 60 min induced a decrease in *hsb-1* expression levels, as would be expected if active, DNA-bound HSF-1 was required to induce an HSR. This decrease continued for the first 15 min of attenuation at 22°C, but increased during the later portion of attenuation, when HSB-1 would be required to repress HSF-1 activity. A similar increase in *Ath-sbp-1* was seen during attenuation of the HSR in *Arabidopsis thaliana* subjected to heat shock (Hsu et al., 2010). Interestingly, when we tested the expression levels at the host temperature of 37°C, we found that *Ac-hsb-1* transcript levels rose slightly by 60 min, but remained elevated throughout the 60 min attenuation. Assuming that higher levels of HSB-1 would repress the HSR by binding to HSF-1, this data suggests that host-like temperature does not induce a classic, short-term HSR. This is supported by the ability of r*Ac*-HSB-1 to interact with *Ce*-HSF-1 at 42°C, when HSF-1 would be a trimer bound to DNA, and not at 37°C when it exists as a monomer (Satyal et al., 1998) (Fig. 4).

In addition to regulating the HSR, HSF-1 is important for regulating development in several species (Jedlicka et al., 1997; Xiao et al., 1999). Knockdown of HSF-1 by RNAi causes a rapid aging and decreased lifespan phenotype in *C. elegans* adults (Garigan et al., 2002), and HSF-1 is required for longevity associated with insulin/insulin growth factor (IGF) signaling mutants (Hsu et al., 2003). Furthermore, *C. elegans* eggs grown on HSF-1 RNAi plates arrested development at the L2/L3 stage, and HSF-1 is required for entry into the dauer stage (Walker et al., 2003; Devaney, 2006). This suggests that repression of HSF-1 mediated gene expression would be required to exit developmental arrest. As a negative regulator of HSF-1, we asked whether HSB-1 functioned in hookworm larval activation.

When *A. caninum* L3s are incubated in tissue culture medium RPMI containing a serum filtrate and S-methyl-glutathione, greater than 90% resume feeding by 24 h at 37°C (Hawdon and Hotez, 1996; Hawdon and Datu, 2003). This “activation” models the early events of infection and the transition to parasitism, characterized by secretion of molecules associated with infection and changes in gene expression (Hawdon et al., 1995b, 1996, 1999; Datu et al., 2008). Incubation in RPMI medium alone at 37°C does not induce feeding or secretion. We asked what effect activation had on levels of *Ac-hsb-1*, as HSF-1 is required for proper development in *C. elegans* (Walker et al., 2003). We found that activation increased expression of *Ac-hsb-1*, and that expression levels were dependent on the percentage of the population that resumed feeding. This suggests that the increase in *Ac-hsb-1* expression is associated with activation and not only extended incubation at 37°C. However, incubation at 37°C in RPMI also increased *Ac-hsb-1* expression approximately

two-fold at 24 h, indicating that the complex nature of the RPMI medium itself influences expression of *Ac-hsb-1* under host-like temperatures and therefore host physiological conditions contribute to the activation stimulus.

The overall increase in *Ac-hsb-1* expression by 24 h is relatively small, reaching approximately three-fold higher levels than buffer alone. However, a time course of expression indicates that they reach their maximum, approximately eight-fold higher than buffer alone, at 12 h incubation. This coincides with the time when L3s have reached their maximal feeding levels (Hawdon and Schad, 1990). Feeding typically plateaus from 12-24 h, but *Ac-hsb-1* expression decreased over this period, reaching a final expression level approximately three-fold higher than the buffer control at 24 h. Down-regulation of *Ac-hsb-1* during the last half of activation would permit HSF-1 trimer formation and transcriptional activity, suggesting that extended incubations under activating, host-like conditions might require HSF-1 to induce a heat shock response, or up-regulate expression of arrest-promoting genes required for maintenance of the dauer arrested L3 state. Activated L3s do not resume development in vitro, and therefore extended incubations under host-like conditions might impart thermal stress in the absence of development. A similar situation would occur in a paratenic host, such as a mouse in the case of *A. caninum*. The L3s remain viable and infective, and begin feeding but fail to resume development. The ability to resume feeding but also protect against thermal stress and remain developmentally arrested would allow L3s to exploit a future predation event to complete transmission if it should end up in the wrong host unfortunatously. Interestingly, a co-IP using recombinant HSB-1 precipitated higher amounts of a 70 kDa unidentified protein from 24 h activated L3s than from non-activated L3s. While we have not identified this protein, it has a molecular weight similar to that of HSF-1, suggesting that HSF-1 might be present as a transcriptionally active trimer at 24 h incubation, when L3s are activated but not developing. Further investigations are required to confirm this speculation.

The transcription factor HSF-1, together with DAF-16, is central to regulating developmental arrest of *C. elegans*, and our data suggests similar functions for these transcription factors in hookworm development. We propose the following model for the role of HSB-1 and HSF-1 in the hookworm infective process (Fig. 7). In our model, recovery from arrest and the transition to parasitism in hookworm L3s is regulated by DAF-16, HSF-1 and an unknown transcription factor, TFX, which could be DAF-12. DAF-16 and HSF-1 target genes would be involved in maintaining developmental arrest, by positively regulating arrest-associated genes and repressing developmental genes. Several of these target genes may be co-regulated by both transcription factors, as is the case in *C. elegans* (Hsu et al., 2003). Hence in arrested L3s, DAF-16 and HSF-1 are occupying the promoters and regulating these arrest-associated genes. When *A. caninum* L3s enter a permissive host orally, they encounter the developmental signal in the intestine that stimulates ILS, resulting in the negative regulation of DAF-16. The concurrent shift to physiological temperature will induce HSB-1 expression and negatively regulate HSF-1, as our data indicate. The cumulative result of these events is repression of DAF-16 and HSF-1 arrest-associated target genes. A second, parallel pathway activates TFX, which induces expression of genes required for development. Therefore, development would occur only when HSF-1 and DAF-16 are inactive, and TFX is active and bound to DNA (Fig. 7).

Under activating conditions (i.e. host-like temperature and a parenteral stimulus), ILS and temperature negatively regulate DAF-16 and HSF-1 as above, but in the absence of the developmental signal, TFX is negatively regulated. This causes the de-repression of genes required for activation and feeding controlled by DAF-16 and HSF-1 but not development. The activated state allows the L3 to survive at the elevated temperature while it awaits the developmental signal. For example, an L3 that penetrates through the skin must undergo a

somatic migration to reach the intestine where it encounters the developmental signal. However, if the L3 is in an improper host, the developmental signal will not be present, so it would be advantageous to remain arrested. In this case, the elevated host temperature will induce the down-regulation of HSB-1 and the subsequent trimerization and binding of HSF-1 to the promoters of arrest-associated genes. This is supported by the decrease in HSB-1 levels after 12 h activation. In this case, when DAF-16 and TFX are not bound to DNA, but HSF-1 is, activation-associated genes regulated by DAF-16 are expressed but arrest-associated genes regulated by HSF-1 are induced. Presumably, some of these genes would protect the L3 from stress associated with the elevated temperature, whereas others allow it to remain arrested but feeding to survive extended periods of developmental arrest in a non-permissive host. Further investigations of HSF-1, HSB-1 and the HSR will be necessary to test this model.

In conclusion, we report the molecular cloning and characterization of an *A. caninum* HSB-1 protein. The *Ac*-HSB-1 shares extensive homology with other HSF binding proteins including conserved, functionally important residues. Recombinant *Ac*-HSB-1 immunoprecipitates *Ce*-HSF-1 expressed in heat shocked mammalian cells but not in cells incubated at 37°C, suggesting that *Ac*-HSB-1 binds to transcriptionally active trimeric HSF-1. Expression of *Ac*-*hsb-1* is increased approximately two-fold after L3s are incubated at 37°C for 1 h but decreases after heat shock at 42°C. When L3s are stimulated to feed in vitro, *Ac*-*hsb-1* expression reaches a maximum at 12 h, then decreases to slightly elevated levels at 24 h. We propose that an increase in *Ac*-*hsb-1* expression down-regulates HSF early in the activation process to allow feeding, but after extended times at host-like conditions in the absence of development, *Ac*-*hsb-1* levels decrease to allow for HSF-1 activity and an HSR to protect against thermal stress and to maintain developmental arrest.

Research Highlights

Ancylostoma caninum heat shock factor binding protein, *Ac*-HSB-1, is orthologous to *Caenorhabditis elegans* (*Ce*)-HSB-1

Ac-HSB-1 interacts with heat shock factor of *C. elegans* only during heat shock

Heat shock, but not host-like temperatures, down-regulates *Ac*-*hsb-1* expression

Larval activation increases *Ac*-*hsb-1* expression

Acknowledgments

The authors thank Devora Champa and Camille Dowling for parasite maintenance, and Xin Gao for technical assistance. Some nematode strains used in this work were provided by the *Caenorhabditis* Genetics Center, which is funded by the National Institutes of Health, National Center for Research Resources (NCRR), USA. The project was supported by Award Number R01AI069293 and 1R21AI062857 from the National Institute of Allergy and Infectious Diseases, USA. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of Allergy And Infectious Diseases or the National Institutes of Health. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

References

- Albonico M, Wright V, Bickle Q. Molecular analysis of the β -tubulin gene of human hookworms as a basis for possible benzimidazole resistance on Pemba Island. *Mol Biochem Parasitol.* 2004; 134:1–4.
- Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic Local Alignment Search Tool. *J Mol Biol.* 1990; 215:403–410. [PubMed: 2231712]

- Altschul SF, Madden TL, Schaeffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Res.* 1997; 25:3389–3402. [PubMed: 9254694]
- Bektesh S, Van Doren K, Hirsh D. Presence of the *Caenorhabditis elegans* spliced leader on different mRNAs and in different genera of nematodes. *Genes Devel.* 1988; 2:1277–1283. [PubMed: 3203906]
- Bethony J, Chen J, Lin S, Xiao S, Zhan B, Li S, Xue H, Xing F, Humphries D, Yan W, Chen G, Foster V, Hawdon JM, Hotez PJ. Emerging patterns of hookworm infection: influence of aging on the intensity of *Necator* infection in Hainan Province, People's Republic of China. *Clin Infect Dis.* 2002; 35:1336–1344. [PubMed: 12439796]
- Brand A, Hawdon JM. Phosphoinositide-3-OH-kinase inhibitor LY294002 prevents activation of *Ancylostoma caninum* and *Ancylostoma ceylanicum* third-stage infective larvae. *Int J Parasitol.* 2004; 34:909–914. [PubMed: 15217729]
- Brand AM, Varghese G, Majewski W, Hawdon JM. Identification of a DAF-7 ortholog from the hookworm *Ancylostoma caninum*. *Int J Parasitol.* 2005; 35:1489–1498. [PubMed: 16135366]
- Brooker S, Bethony J, Hotez PJ. Human Hookworm Infection in the 21st Century. *Adv Parasitol.* 2004; 58:197–288. [PubMed: 15603764]
- Bundy DAP, Chan MS, Savioli L. Hookworm infection in pregnancy. *Trans Royal Soc Trop Med Hyg.* 1995; 89:521–522.
- Cassada RC, Russell RL. The dauer larva, a post-embryonic developmental variant of the nematode *Caenorhabditis elegans*. *Dev Biol.* 1975; 46:326–342. [PubMed: 1183723]
- Datu BJ, Gasser RB, Nagaraj SH, Ong EK, O'Donoghue P, McInnes R, Ranganathan S, Loukas A. Transcriptional changes in the hookworm, *Ancylostoma caninum*, during the transition from a free-living to a parasitic larva. *PLoS Negl Trop Dis.* 2008; 2:e130. [PubMed: 18235850]
- De Clercq D, Sacko M, Behnke J, Gilbert F, Dorny P, Vercruysse J. Failure of mebendazole in treatment of human hookworm infections in the southern region of Mali. *Amer J Trop Med Hyg.* 1997; 57:25–30. [PubMed: 9242313]
- de Silva NR, Brooker S, Hotez PJ, Montresor A, Engels D, Savioli L. Soil-transmitted helminth infections: updating the global picture. *Trends Parasitol.* 2003; 12:547–551. [PubMed: 14642761]
- Delaney A, Williamson A, Brand A, Ashcom J, Varghese G, Goud GN, Hawdon JM. Cloning and characterisation of an aspartyl protease inhibitor (API-1) from *Ancylostoma* hookworms. *Int J Parasitol.* 2005; 35:303–313. [PubMed: 15722082]
- Devaney E. Thermoregulation in the life cycle of nematodes. *Int J Parasitol.* 2006; 36:641–649. [PubMed: 16620827]
- Devaney E, Egan A, Lewis E, Victoria Warbrick E, Jecock RM. The expression of small heat shock proteins in the microfilaria of *Brugia pahangi* and their possible role in development. *Mol Biochem Parasitol.* 1992; 56:209–217. [PubMed: 1484546]
- Devaney E, Lewis E. Temperature-induced refractoriness of *Aedes aegypti* mosquitoes to infection with the filaria *Brugia pahangi*. *Med Vet Entomol.* 1993; 7:297–298. [PubMed: 8369568]
- Dreyfuss ML, Stoltzfus RJ, Shrestha JB, Pradhan EK, LeClerq SC, Khatry SK, Shrestha SR, Katz J, Albonico M, West KP Jr. Hookworms, malaria and vitamin A deficiency contribute to anemia and iron deficiency among pregnant women in the plains of Nepal. *J Nutr.* 2000; 130:2527–2536. [PubMed: 11015485]
- Feder ME, Hofmann GE. Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. *Annu Rev Physiol.* 1999; 61:243–282. [PubMed: 10099689]
- Fu S, Rogowsky P, Nover L, Scanlon MJ. The maize heat shock factor-binding protein paralogs EMP2 and HSBP2 interact non-redundantly with specific heat shock factors. *Planta.* 2006; 224:42–52. [PubMed: 16331466]
- Gao X, Frank D, Hawdon JM. Molecular cloning and DNA binding characterization of DAF-16 orthologs from *Ancylostoma* hookworms. *Int J Parasitol.* 2009; 39:407–415. [PubMed: 18930062]
- Garigan D, Hsu AL, Fraser AG, Kamath RS, Ahringer J, Kenyon C. Genetic analysis of tissue aging in *Caenorhabditis elegans*: a role for heat-shock factor and bacterial proliferation. *Genetics.* 2002; 161:1101–1112. [PubMed: 12136014]

- Geary TG, Woo K, McCarthy JS, Mackenzie CD, Horton J, Prichard RK, de Silva NR, Olliaro PL, Lazdins-Helds JK, Engels DA, Bundy DA. Unresolved issues in anthelmintic pharmacology for helminthiasis of humans. *Int J Parasitol.* 2010; 40:1–13. [PubMed: 19932111]
- Hahn JS, Hu Z, Thiele DJ, Iyer VR. Genome-Wide Analysis of the Biology of Stress Responses through Heat Shock Transcription Factor. *Mol Cell Biol.* 2004; 24:5249–5256. [PubMed: 15169889]
- Hajarnavis A, Korf I, Durbin R. A probabilistic model of 3' end formation in *Caenorhabditis elegans*. *Nucleic Acids Res.* 2004; 32:3392–3399. [PubMed: 15247332]
- Hall TA. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucl Acids Symp Ser.* 1999; 41:95–98.
- Hawdon JM. Hookworm infectious process: host signals and parasite responses, Parasitology. University of Pennsylvania; Philadelphia: 1991.
- Hawdon JM, Datu B. The second messenger cyclic GMP mediates activation in *Ancylostoma caninum* infective larvae. *Int J Parasitol.* 2003; 33:787–793. [PubMed: 12865078]
- Hawdon JM, Hotez PJ. Hookworm: developmental biology of the infectious process. *Curr Opin Gen Dev.* 1996; 6:618–623.
- Hawdon JM, Jones BF, Hoffman DR, Hotez PJ. Cloning and characterization of *Ancylostoma*-secreted protein. A novel protein associated with the transition to parasitism by infective hookworm larvae. *J Biol Chem.* 1996; 271:6672–6678. [PubMed: 8636085]
- Hawdon JM, Jones BF, Hotez PJ. Cloning and characterization of a cDNA encoding the catalytic subunit of a cAMP-dependent protein kinase from *Ancylostoma caninum* third-stage infective larvae. *Mol Biochem Parasitol.* 1995a; 69:127–130. [PubMed: 7723782]
- Hawdon JM, Jones BF, Perregaux MA, Hotez PJ. *Ancylostoma caninum*: metalloprotease release coincides with activation of infective larvae *in vitro*. *Exp Parasitol.* 1995b; 80:205–211. [PubMed: 7895832]
- Hawdon JM, Narasimhan S, Hotez PJ. *Ancylostoma* secreted protein 2 : cloning and characterization of a second member of a family of nematode secreted proteins from *Ancylostoma caninum*. *Mol Biochem Parasitol.* 1999; 99:149–165. [PubMed: 10340481]
- Hawdon JM, Schad GA. Serum stimulated feeding *in vitro* by third stage infective larvae of the canine hookworm *Ancylostoma caninum*. *J Parasitol.* 1990; 76:394–398. [PubMed: 2112598]
- Hawdon JM.; Schad, GA. Developmental Adaptations in Nematodes. In: Toft, CA.; Aeschlimann, A.; Bolis, L., editors. *Parasitism: Coexistence or Conflict?*. Oxford University Press; Oxford: 1991a. p. 274–298.
- Hawdon JM, Schad GA. Long-term storage of hookworm infective larvae in buffered saline solution maintains larval responsiveness to host signals. *J Helm Soc Wash.* 1991b; 58:140–142.
- Hotez PJ, Bethony JM, Diemert DJ, Pearson M, Loukas A. Developing vaccines to combat hookworm infection and intestinal schistosomiasis. *Nat Rev Micro.* 2010; 8:814–826.
- Hsu AL, Murphy CT, Kenyon C. Regulation of aging and age-related disease by DAF-16 and heat-shock factor. *Science.* 2003; 300:1142–1145. [PubMed: 12750521]
- Hsu SF, Lai HC, Jinn TL. Cytosol-localized heat shock factor-binding protein, AtHSBP, functions as a negative regulator of heat shock response by translocation to the nucleus and is required for seed development in Arabidopsis. *Plant Physiol.* 2010; 153:773–784. [PubMed: 20388662]
- Hunter SJ, Thompson FJ, Tetley L, Devaney E. Temperature is a cue for gene expression in the postinfective L3 of the parasitic nematode *Brugia pahangi*. *Mol Biochem Parasitol.* 2001; 112:1–9. [PubMed: 11166381]
- Jecock RM, Devaney E. Expression of small heat shock proteins by the third-stage larva of *Brugia pahangi*. *Mol Biochem Parasitol.* 1992; 56:219–226. [PubMed: 1484547]
- Jedlicka P, Mortin MA, Wu C. Multiple functions of *Drosophila* heat shock transcription factor *in vivo*. *EMBO J.* 1997; 16:2452–2462. [PubMed: 9171358]
- Kelley LA, Sternberg MJ. Protein structure prediction on the Web: a case study using the Phyre server. *Nat Protoc.* 2009; 4:363–371. [PubMed: 19247286]
- Kimura KD, Tissenbaum HA, Liu Y, Ruvkun G. *daf-2*, an insulin receptor-like gene that regulates longevity and diapause in *Caenorhabditis elegans*. *Science.* 1997; 277:942–946. [PubMed: 9252323]

- Kiss J, Gao X, Krepp J, Hawdon J. Interaction of hookworm 14-3-3 with the forkhead transcription factor DAF-16 requires intact Akt phosphorylation sites. *Parasit Vectors*. 2009; 2:21. [PubMed: 19393088]
- Kopp SR, Kotze AC, McCarthy JS, Coleman GT. High-level pyrantel resistance in the hookworm *Ancylostoma caninum*. *Vet Parasitol*. 2007; 143:299–304. [PubMed: 17011128]
- Liu X, Xu L, Liu Y, Tong X, Zhu G, Zhang XC, Li X, Rao Z. Crystal structure of the hexamer of human heat shock factor binding protein 1. *Proteins*. 2009; 75:1–11. [PubMed: 18767159]
- Livak, KJ. ABI Prism 7700 Sequence Detection System, User Bulletin 2 PE Applied Biosystems. 2001.
- Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2⁻($\Delta\Delta C_T$) Method. *Methods*. 2001; 25:402–408. [PubMed: 11846609]
- Lozoff B. Iron and learning potential in childhood. *Bull NY Acad Med*. 1989; 65:1050–1066.
- Lozoff B, Jimenez E, Wolf AW. Long-term developmental outcome of infants with iron deficiency. *New Engl J Med*. 1991; 235:687–694. [PubMed: 1870641]
- Mager WH, De Kruijff AJ. Stress-induced transcriptional activation. *Microbiol Rev*. 1995; 59:506–531. [PubMed: 7565416]
- Morimoto RI. Cells in Stress: Transcriptional Activation of Heat Shock Genes. *Science*. 1993; 259:1409–1410. [PubMed: 8451637]
- Morimoto RI. Regulation of the heat shock transcriptional response: cross talk between a family of heat shock factors, molecular chaperones, and negative regulators. *Genes Dev*. 1998; 12:3788–3796. [PubMed: 9869631]
- Morley JF, Morimoto RI. Regulation of longevity in *Caenorhabditis elegans* by heat shock factor and molecular chaperones. *Mol Biol Cell*. 2004; 15:657–664. [PubMed: 14668486]
- Nollen EA, Morimoto RI. Chaperoning signaling pathways: molecular chaperones as stress-sensing 'heat shock' proteins. *J Cell Sci*. 2002; 115:2809–2816. [PubMed: 12082142]
- Paradis S, Ruvkun G. *Caenorhabditis elegans* Akt/PKB transduces insulin receptor-like signals from AGE-1 PI3 kinase to the DAF-16 transcription factor. *Genes Dev*. 1998; 12:2488–2498. [PubMed: 9716402]
- Petronijevic T, Rogers WP. Gene activity and the development of early parasitic stages of nematodes. *Int J Parasitol*. 1983; 13:197–199. [PubMed: 6853019]
- Pirkkala L, Nykanen P, Sistonen L. Roles of the heat shock transcription factors in regulation of the heat shock response and beyond. *FASEB J*. 2001; 15:1118–1131. [PubMed: 11344080]
- Reynoldson JA, Behnke JM, Pallant LJ, Macnish MG, Gilbert F, Giles S, Spargo RJ, Thompson RCA. Failure of pyrantel in treatment of human hookworm infections (*Ancylostoma duodenale*) in the Kimberley Region of North West Australia. *Acta Trop*. 1997; 68:301–312. [PubMed: 9492915]
- Rogers, WP.; Petronijevic, T. The infective stage and the development of nematodes. In: Symons, LEA.; Donald, AD.; Dineen, JK., editors. *Biology and Control of Endoparasites*. Academic Press; Australia: 1982. p. 3-28.
- Rogers WP, Sommerville RI. The infective stage of nematode parasites and its significance in parasitism. *Adv Parasit*. 1963; 1:109–177.
- Santoro MG. Heat shock factors and the control of the stress response. *Biochem Pharmacol*. 2000; 59:55–63. [PubMed: 10605935]
- Satyal SH, Chen D, Fox SG, Kramer JM, Morimoto RI. Negative regulation of the heat shock transcriptional response by HSBP1. *Genes Dev*. 1998; 12:1962–1974. [PubMed: 9649501]
- Schad GA, Page MR. *Ancylostoma caninum* : adult worm removal, corticosteroid treatment, and resumed development of arrested larvae in dogs. *Exp Parasitol*. 1982; 54:303–309. [PubMed: 7151941]
- Schaffer AA, Aravind L, Madden TL, Shavirin S, Spouge JL, Wolf YI, Koonin EV, Altschul SF. Improving the accuracy of PSI-BLAST protein database searches with composition-based statistics and other refinements. *Nucleic Acids Res*. 2001; 29:2994–3005. [PubMed: 11452024]
- Shi Y, Mosser DD, Morimoto RI. Molecular chaperones as HSF1-specific transcriptional repressors. *Genes Dev*. 1998; 12:654–666. [PubMed: 9499401]

- Stepek G, Buttle DJ, Duce IR, Behnke JM. Human gastrointestinal nematode infections: are new control methods required? *Int J Exp Pathol*. 2006; 87:325–341. [PubMed: 16965561]
- Tai LJ, McFall SM, Huang K, Demeler B, Fox SG, Brubaker K, Radhakrishnan I, Morimoto RI. Structure-function analysis of the heat shock factor-binding protein reveals a protein composed solely of a highly conserved and dynamic coiled-coil trimerization domain. *J Biol Chem*. 2002; 277:735–745. [PubMed: 11679589]
- Thompson FJ, Cockcroft AC, Wheatley I, Britton C, Devaney E. Heat shock and developmental expression of hsp83 in the filarial nematode *Brugia pahangi*. *Eur J Biochem*. 2001; 268:5808–5815. [PubMed: 11722567]
- Tissenbaum HA, Hawdon J, Perregaux M, Hotez P, Guarente L, Ruvkun G. A common muscarinic pathway for diapause recovery in the distantly related nematode species *Caenorhabditis elegans* and *Ancylostoma caninum*. *Proc Natl Acad Sci USA*. 2000; 97:460–465. [PubMed: 10618440]
- Trivedi S, Arasu P. Evaluation of endogenous reference genes for real-time PCR quantification of gene expression in *Ancylostoma caninum*. *Mol Biochem Parasitol*. 2005; 143:241–244. [PubMed: 15993958]
- Voellmy R, Kivie M. Transcriptional Regulation of the Metazoan Stress Protein Response. *Prog Nucleic Acid Res Mol Biol*. 2004; 78:143–185. [PubMed: 15210330]
- Walker GA, Lithgow GJ. Lifespan extension in *C. elegans* by a molecular chaperone dependent upon insulin-like signals. *Aging Cell*. 2003; 2:131–139. [PubMed: 12882326]
- Walker GA, Thompson FJ, Brawley A, Scanlon T, Devaney E. Heat shock factor functions at the convergence of the stress response and developmental pathways in *Caenorhabditis elegans*. *FASEB J*. 2003; 1096/fj.1003–0164fje
- WHO. Burden of disease in DALYs by cause, sex and mortality stratum in WHO regions, estimates for 2001. World Health Organization; Geneva: 2002. Reducing risks, promoting healthy life. The World Health Report 2002. Annex Table 3.
- Wolstenholme AJ, Fairweather I, Prichard R, von Samson-Himmelstjerna G, Sangster NC. Drug resistance in veterinary helminths. *Trends Parasitol*. 2004; 20:469–476. [PubMed: 15363440]
- Wu C. Heat Shock Transcription Factors: Structure and Regulation. *Ann Rev Cell Dev Biol*. 1995; 11:441–469. [PubMed: 8689565]
- Xiao X, Zuo X, Davis AA, McMillan DR, Curry BB, Richardson JA, Benjamin IJ. HSF1 is required for extra-embryonic development, postnatal growth and protection during inflammatory responses in mice. *EMBO J*. 1999; 18:5943–5952. [PubMed: 10545106]

Fig. 1.

Alignment and secondary structure prediction of *Ancylostoma caninum* heat shock factor binding protein 1 (Ac-HSB-1). Amino acid sequence alignment of Ac-HSB-1 with *Caenorhabditis elegans* (Ce-HSB-1, accession number **CAB01233**) and *Homo sapiens* (Hs-HSB-1, **NP_001528**) HSB-1 proteins. Amino acids that are identical to Ac-HSB-1 are shaded in black, and conserved residues are shaded in gray. Residue numbering and secondary structure prediction for Ac-HSB-1 are shown; h=helix, c=coil (Phyre webserver, <http://www.sbg.bio.ic.ac.uk/~phyre/index.cgi>)(Kelley and Sternberg, 2009). The N- and C-terminal heptad repeat regions (HR-N and HR-C) are denoted by overlines, and the a and d positions of the heptad repeats (abcdefg) are indicated for Ac-HSB-1.

Fig. 2. Expression profile of *Ancylostoma caninum* heat shock factor binding protein 1 (*Ac-hsb-1*) throughout the *A. caninum* life cycle. First strand cDNA from *A. caninum* life cycle stages was amplified by PCR using *Ac-hsb-1* or a control gene (*Ac-pka-1*, a portion of the 3' untranslated region of the protein kinase A catalytic subunit) specific primers and separated on a 1.6% agarose gel. A) *Ac-hsb-1* primers, B) *Ac-pka-1* primers. Lane 1, egg cDNA; lane 2, L1 cDNA; lane 3, non-activated L3 cDNA; lane 4, activated L3 cDNA; lane 5, adult cDNA, lane 6, *A. caninum* genomic DNA; lane M, 1 kb Plus ladder (Invitrogen). Sizes of representative markers are indicated in bp.

Fig. 3.

The expression of the gene for *Ancylostoma caninum* heat shock factor binding protein (*Ac-hsb-1*) is down-regulated in heat shock, but not at host-like temperature. Normalized fold expression of *Ac-hsb-1* was analyzed after exposing developmentally arrested *A. caninum* L3s to 37°C or 42°C for 15, 30 or 60 min. Also, L3s exposed for 60 min to higher temperatures were attenuated to room temperature (22°C) for 15, 30 or 60 min. Error bars represent the S.D.s of three independent experiments.

Fig. 4.

Ancylostoma caninum heat shock factor binding protein (*Ac-hsb-1*) interacts with *Caenorhabditis elegans* heat shock factor (*Ce-HSF-1*) during heat shock. HEK 293T cells were transfected with a pCMV-Tag4a-FLAG containing the full-length *Ce-hsf-1* cDNA or with an empty pCMV-Tag4a vector (mock). After 48 h at 37°C, *Ce-hsf-1* transfected cells were collected or subjected to a 1 h heat shock at 42°C and lysates prepared. T7 tagged recombinant (r)*Ac-HSB-1* was added and *Ce-HSF-1* immunoprecipitated by addition of anti-FLAG M2 resin (Lanes 1–3). Precipitated complexes were separated by electrophoresis and the separated proteins transferred to polyvinylidene difluoride membranes for Western blotting (WB) with FLAG antibody or T7 antibody. Lane 1, *Ce-hsf-1* transfected cells incubated at 37°C; lane 2, *Ce-hsf-1* transfected cells incubated at 42°C; lane 3, mock transfected cells grown at 37°C; lane 4, mock cells only; lane 5, *Ce-hsf-1* transfected cells only; lane 6, *Ce-HSF-1* lysate or r*Ac-HSB-1*.

Fig. 5.

Ancylostoma caninum heat shock factor binding protein (*Ac-hsb-1*) expression is up-regulated in in vitro activated L3s. Quantitative reverse transcriptase-PCR (qRT-PCR) was performed with cDNA from *A. caninum* L3s which were incubated at 37°C in RPMI-c alone (NON) or with 10% dog serum filtrate and 15 mM S-methyl-glutathione (GSM) (ACT). A) In vitro activation of L3s for 1 h in RPMI-c alone (NON), with GSM only (ACT w/o FIL), with FIL only (ACT w/o GSM), or with both (ACT) for 1 h. The percentage feeding (= activated) of parallel incubations at 24 h is shown for each condition. B) L3s were activated in vitro for 24 h and collected at the indicated time points. In addition, L3s were incubated in the nematode storage buffer 1x BU at 37°C for 3, 6, 12 and 24 h (BU). Error bars represent the S.D.s of three independent experiments

Fig. 6.

Ancylostoma caninum heat shock factor binding protein (*Ac-hsb-1*) interacts with two distinct proteins from *A. caninum* lysate during larval activation. T7 tagged recombinant (r) *Ac*-HSB-1 was incubated with hookworm larval lysates, and interacting proteins co-precipitated with anti-T7 antibody (α -T7). Following electrophoretic separation and blotting, the membrane was incubated with 50 μ g of T7-tagged r*Ac*-HSB-1, followed by detection using the anti-T7 antibody. Bound (B) refers to immunoprecipitated material, and unbound (U) refers to supernatants of the immunoprecipitation (IP) reactions. Lanes 1-4, IP with lysates, rHSB-1, and T7 antibody. Lanes 5 and 6, IP with T7 antibody only; Lanes 7-10, IP with lysate and T7 antibody only. Lane 1, non-activated lysate, bound; lane 2, activated lysate, unbound; lane 3, activated (ACT) lysate, bound; lane 4, non-activated (NA) lysate, unbound; lane 5, T7 antibody, bound; lane 6, T7 antibody, unbound; lane 7, non-activated lysate, bound; lane 8, non-activated lysate, unbound; lane 9, activated lysate, bound; lane 10, activated lysate, unbound; lane 11, r*Ac*-HSB-1 control.

	DAF16	HSF1	TFX	
Arrested	ON	ON	OFF	→ Expression of arrest associated genes
Developing	OFF	OFF	ON	→ Expression of developmental genes
Activated (pre-feeding)	OFF	OFF	OFF	→ Expression of feeding & activation genes
Activated (post-feeding)	OFF	ON	OFF	→ Expression of arrest & activation associated genes

Fig. 7.

Proposed model for the role of *Ancylostoma caninum* heat shock factor binding protein (*Ac-hsb-1*) and heat shock factor (HSF-1) in hookworm development. Pre-feeding refers to the first 12 h of activation, during which time feeding in the L3 population resumes and reaches its maximum. Post-feeding is the period of activation after 12 h, when feeding has reached its maximum level in the population. Off and on refer to the presence of the transcription factor on its corresponding target genes. TFX refers to an unknown transcription factor. See the Discussion for details.