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Molecular cloning and DNA binding characterization of DAF-16 orthologs from *Ancylostoma* hookworms^{*}

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Abstract

Infective hookworm L3s encounter a host-specific signal during infection that re-initiates a suspended developmental pathway, resulting in development to the adult stage. This resumption of development in the host is analogous to recovery of developmentally arrested *Caenorhabditis elegans* dauer larvae in response to favorable environmental signals. Dauer recovery in *C. elegans* dauers and hookworm L3s is mediated by insulin-like signaling (ILS). A key output of ILS in *C. elegans* is the forkhead transcription factor DAF-16, which controls the expression of genes required for maintenance of the dauer stage. The similarity between recovery pathways of L3s and dauers suggests that DAF-16 functions similarly in hookworm L3 activation. To test this, orthologs of *Ce*-DAF-16 were isolated from the hookworms *Ancylostoma caninum* and *Ancylostoma ceylanicum*. The protein sequences of hookworm DAF-16 DNA binding domains were identical, and shared 94% identity with the b and c isoforms of *Ce*-DAF-16. *Ac*-DAF-16 expressed in HEK293 kidney cells bound strongly to the conserved DAF family binding element (DBE), but not to a random DNA sequence. *Ac*-DAF-16 was able to drive transcription of a reporter gene located downstream of six copies of the DBE in NIH3T3 cells under starved conditions. Addition of serum caused a decrease in reporter gene expression, indicating that DAF-16 is negatively regulated by growth factor stimulation. These data confirm the presence of DAF-16 orthologs in hookworms, and demonstrate that *Ac*-DAF-16 binds to and drives transcription from a conserved DAF-16 family DNA binding element.

Keywords

Ancylostoma caninum; Hookworm; Insulin signaling; Dauer; Forkhead; Transition to parasitism

1. Introduction

Hookworms infect an estimated 740 million people, mostly in tropical and subtropical regions, making it among the most common infectious diseases worldwide (Bethony et al., 2006). Heavy infection with hookworm can create severe health issues for newborns, growing

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children, pregnant women and malnourished persons (Lozoff et al., 1991; Bundy et al., 1995; Dreyfuss et al., 2000; Bethony et al., 2002). The critical step for hookworm development and survival is to successfully establish a parasitic relationship. The infective form of hookworm is a free-living L3 that remains developmentally arrested in the environment until it enters a definitive host. In this host, it encounters host-specific signals that initiate development and maturation to adulthood (Rogers and Petronijevic, 1982; Hawdon and Schad, 1991a). An in vitro system using the canine hookworm *Ancylostoma caninum* has allowed the investigation of the early events of reactivation of hookworm L3s (Hawdon and Schad, 1990; Hawdon and Hotez, 1996). This system uses the resumption of feeding by infective L3s as a marker for activation (Hawdon and Hotez, 1996). Using this system, several molecules that are released during the infectious process have been identified (Hawdon et al., 1995b, 1996, 1999). More recently, the system was used to demonstrate that cGMP signaling is involved in larval activation (Hawdon and Datu, 2003). However, very little is known about the molecular mechanisms underlying the transition to parasitism, in large part due to the complexity of hookworm life cycles and the lack of genetic tools.

The free-living nematode *Caenorhabditis elegans* has served as a useful model for understanding the mechanisms of hookworm larval development. *Caenorhabditis elegans* adopts a non-feeding, developmentally arrested dauer stage under conditions of overcrowding and starvation (Cassada and Russell, 1975). The dauer stage is remarkably similar to infective L3s of hookworm in terms of behavior, morphology and function, except that development into the L3 is obligatory for hookworm (Rogers and Sommerville, 1963; Hawdon and Schad, 1991a). Exit from the dauer stage in *C. elegans* in response to improved environmental signals is hypothesized to be analogous to the reactivation of the arrested hookworm L3 in response to the host-specific signals during infection (Rogers and Sommerville, 1963; Hawdon and Schad, 1991a; Hotez et al., 1993). Studying the processes of signal transduction in *C. elegans* has yielded important information about the development of hookworm.

Insulin-like signaling (ILS) has been implied in regulation of *C. elegans* dauer arrest and recovery by epistasis analysis coupled with cloning of genes related to dauer formation (Gottlieb and Ruvkun, 1994; Morris et al., 1996; Kimura et al., 1997; Ogg et al., 1997; Ogg and Ruvkun, 1998; Paradis and Ruvkun, 1998; Paradis et al., 1999). A key target of the ILS pathway is the Forkhead box O (FOXO) transcription factor DAF-16, which is located in the nucleus under dauer-inducing conditions, where it binds to insulin response sequences in the promoters of target genes and causes entry into dauer (Lin et al., 1997; Ogg et al., 1997). Phosphorylation by the activated serine-threonine protein kinase Akt/protein kinase B (Akt/PKB) under permissive growth conditions results in nuclear exclusion and cytoplasmic retention of DAF-16, leading to reproductive growth (Brownawell et al., 2001; Cahill et al., 2001). An 8-bp consensus DAF-16 family member binding element (DBE) has been identified that binds both *C. elegans* and mammalian forkhead transcription factors (Furuyama et al., 2000).

Involvement of an ILS pathway in hookworm L3 activation was supported by experiments with specific inhibitors for PI3 kinase and Akt/PKB kinase (Brand and Hawdon, 2004). In the present study, *C. elegans* DAF-16 orthologs were cloned and sequenced from *A. caninum* and *A. ceylanicum*, two close relatives of human hookworms, and shown to bind to and drive transcription from a conserved binding element found in the promoters of FOXO/DAF-16 target genes in *C. elegans* and mammals. These data represent the first identification of a molecule involved in ILS from a hookworm, further support a role for insulin signaling in the hookworm activation, and suggest that DAF-16 plays a critical role in gene expression associated with the transition to parasitism.

2. Materials and methods

2.1. Nomenclature

Molecules are named in accordance to the guidelines established by the Society of Nematology (Bird and Riddle, 1994). The *C. elegans* name for the forkhead transcription factor DAF-16 was retained to indicate orthology. The gene names and DNA sequences are written in lowercase italics, with the species of origin as a prefix (*Ac-daf-16*). When referring to the protein, the name is written in uppercase letters (*Ac-DAF-16*).

2.2. Parasites

The Baltimore strain of *A. caninum* (US National Parasite Collection accession number 100655.00) was maintained as previously described in beagles (Schad and Page, 1982) and *A. ceylanicum* was maintained in Syrian hamsters (Garside and Behnke, 1989). Animals were housed and treated in accordance with institutional care and use committee guidelines. Infective L3s are recovered from coproculture by a modified Baermann technique and frozen until used. Adults were collected from intestines of infected animals following euthanasia, and frozen at -80°C until nucleic acids were isolated.

2.3. Cloning and characterization of hookworm DAF-16

An *A. ceylanicum* (*Ae*) expressed sequence tag (pk84h05) (Mitreva et al., 2005) with similarity to the *C. elegans* forkhead transcription factor DAF-16 was used to design specific forward and reverse oligonucleotide primers for PCR. The *Ae-daf-16* cDNA ends were amplified in two separate hemi-nested PCR reactions. The 3' end was amplified from an *A. ceylanicum* L3 directional cDNA library constructed in Lambda ZAP II (Hawdon et al., 1995a), with the nested forward primers DAF16-F1 (5'-ACAATCTCTGAGTGCCGT GCACC-3') and DAF16-F2 (5'-CATTGACATACACACAT CCATCCAC-3') together with a primer complementary to the T7 promoter sequence flanking the 3' end of the cDNA insert site in the pBluescript vector. In the first round PCR, DAF16-F1, T7, and the cDNA library (1 μl) were incubated with *Taq* polymerase (USB, Cleveland, OH) for 35 cycles of 1 min at 94°C , 1 min at 57°C , 2 min at 72°C , followed by a final extension for 5 min at 72°C . One μl of the first round reaction was used as template for the second round PCR, containing the reverse nested primer DAF16-F2 and T7, and incubated with JumpStart *Taq* polymerase (Sigma, St. Louis) for 30 cycles as above except the annealing temperature was lowered to 55°C .

The 5' end of *daf-16* was amplified from first strand cDNA synthesized from infective L3s of *A. ceylanicum* by standard methods (Hawdon et al., 2003). The nested reverse primers DAF16-R1 (5'-CTGCGACTGATTACGGACACTGC-3') and DAF16-R2 (5'-GACAGGCGATTCCGTCTTGA TTGG-3') and the forward conserved nematode spliced leader primer SL1 (5'-GGTTTA ATTACCAAG TTTGAG-3') (Bektesh et al., 1988; Hawdon et al., 1995a) were used in successive reactions. In the first PCR, DAF16-R1, SL1, and cDNA were incubated with JumpStart *Taq* for 2 min at 95°C , followed by 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 . One μl of the first round reaction was used as template for the second round PCR, containing the reverse nested primer DAF16-R2 and SL, and incubated as above. Amplicons of approximately 850 bp and 400 bp from the 5' and 3' reactions, respectively, were gel purified and cloned into a pGEM-T Easy vector (Promega, Madison, WI), and the DNA sequences determined (see below).

To complete the sequences, the section of the *daf-16* cDNA connecting the 5' and 3' ends was amplified. Primers DAF16-F3 (5'-GAAACCAATCAAGA CGGAATCGC-3') and DAF16-R3 (5'-TGCACGGCACTCAGAGATTGTCG-3') were incubated with L3 cDNA library (1 μl) and *Taq* polymerase for 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. An amplicon of approximately 350 bp was cloned in pGEM-T Easy vector and sequenced as described above.

The oligonucleotide primers designed from the *Ae-daf-16* sequence were used to amplify the full-length coding sequence of the *A. caninum daf-16* cDNA. Primer DAF16pic-FX (5'-ATCCTCGAGCATAATGTCTATGTCTGAATCACCTCTCGCAAACG-3') and DAF16pic-RX (5'-TCATTCTAGAAGATTGTCTGAAATTCAGGCG-3') were incubated together with *A. caninum* L3 first-strand cDNA and *Taq* polymerase for 35 cycles of 1 min at 94° C, 1 min at 55° C, 4 min at 72° C, followed by a final extension for 4 min at 72° C. An amplicon of approximately 1.8 kb was gel purified, cloned in pGEM-T Easy vector and sequenced.

2.3. Stage specificity

Ancylostoma caninum eggs were isolated from infected dog feces, and *A. ceylanicum* eggs from infected hamster feces, by sucrose floatation (Nolan et al., 1994), axenized by treatment with NaOCl, and plated on nematode growth medium (NGM) agar plates (Sulston and Hodgkin, 1988). Following incubation at 28 C for 16–18 h, the hatchlings (L1s) were washed from the plates with nematode handling buffer BU (50 mM Na₂HPO₄/22 mM KH₂PO₄/70 mM NaCl, pH 6.8) (Hawdon and Schad, 1991b) and snap-frozen in a dry ice/ethanol bath.

Reverse transcriptase (RT)-PCR was performed to determine the stage specificity of *Ac-daf-16* expression. Total RNA was isolated from *A. caninum* eggs, L1/L2, L3 and adult stages by homogenization in TRIzol Reagent according to the manufacturer's instructions (GibcoBRL). First-strand cDNA was synthesized from approximately 1 µg of total RNA using the Superscript First-Strand Synthesis System (GibcoBRL). Primers spanning a 360 bp intron were chosen so that products amplified from contaminating genomic DNA could be distinguished from those amplified from the stage-specific cDNA. PCR reactions contained gene-specific primers DAF16-F3 and DAF16-R3 (located at the 3' end of the open reading frame) and 1 µl of first strand cDNA from each *A. caninum* developmental stage. These primers are predicted to generate a product of ~ 384 bp from cDNA. Primers specific for the 3' untranslated region of the catalytic subunit of protein kinase A (Hawdon et al., 1995a), which is expressed in all stages, were used in positive control reactions.

2.4. Bacterial expression and antiserum production

A 492 bp fragment corresponding to amino acids 365 to 528 was amplified from a cDNA clone of *Ac-daf-16* for expression in bacteria. Primers DAF16E-FB (5'-TTAGGATCCGA GGAAGCCATG CAAGCATCATTCG-3') and DAF16E-RX (5'-TATACTCGAGAGTAAT CAGTGGTGTATGTGTCGGCCACGG-3'), containing the 5' restriction sites *Bam*HI and *Xho*I, respectively (underlined), were incubated in a PCR containing plasmid DNA and *Pfu* Ultra polymerase (Stratagene, La Jolla, CA) for 2 min at 95 C, followed by 30 cycles of 30 s at 95 C, 30 s at 55 C, 1 min at 72 and a final extension for 4 min at 72 C. The 492 bp amplicon was digested with *Bam*HI and *Xho*I (New England Biolabs Beverly, Massachusetts), ligated into the bacterial expression vector pET28a (Novagen, San Diego, CA) cut with the same restriction enzymes, and transformed into *E. coli* DH5α cells by standard methods. Clones containing *Ac-daf-16*_{365–528} were confirmed by DNA sequencing. For expression, plasmid DNA was transformed into *E. coli* BL-21 (DE3) competent cells, and protein expression induced by the addition of 1 mM isopropyl-beta-D-thio-galacto-pyranoside (IPTG) to log-phase bacterial cultures. An aliquot of cells was removed from the culture prior to induction to serve as a pre-induction control, and from an un-induced culture grown under the same conditions. The induced cultures were grown for 3 h at 37° C with continuous shaking at 220 revolutions per min. Aliquots (1 ml) were withdrawn at 0, 1, 2 and 3 h of induction, and soluble and insoluble fractions of the lysates analyzed on 12% SDS-PAGE as described (Hawdon et al., 1999). Recombinant DAF-16_{365–528} (rDAF-16_{365–528}), which was expressed in an

insoluble form, was solubilized by the addition of 1% SDS and 0.5% 2-mercaptoethanol, and purified as described elsewhere (Hawdon et al., 1996).

Polyclonal antiserum was raised in New Zealand white rabbits by immunization with purified rDAF-16₃₆₅₋₅₂₈. Approximately 150 µg of rDAF-16₃₆₅₋₅₂₈ were injected s.c. after emulsification with FCA. Additional boosts with recombinant protein (150 µg each) in incomplete Freund's adjuvant were administered at 3, 6 and 9 weeks.

2.5. Cell culture, expression of recombinant Ac-DAF-16 and antiserum characterization

HEK293T cells were maintained in 25-cm² flasks in RPMI1640 supplemented with 10% FBS (Biowhittaker, Walkersville, MD), 100 I.U. /ml penicillin (Mediatech, Herndon, VA), 100 µg/ml streptomycin (Mediatech) and 2mM L-glutamine (Mediatech), at 37°C in an atmosphere of 5% CO₂. The entire coding region of *Ac-DAF-16* was cloned into the mammalian expression vector pCMV-TAG4 (Stratagene) at the *Xho*I and *Bam*HI restriction sites to generate pCMV4-daf16 construct containing an in-frame C-terminal FLAG octapeptide tag (DYKDDDDK). The construct was confirmed by DNA sequencing. HEK293T cells grown in six-well dishes were transfected with 4 µg of pCMV4-daf-16 using Metafectene (Bionex, Munich) according to the manufacturer's instructions, and cultured for 48 h. Empty vector controls received 4 µg pCMV4 without an insert, and mock-treated cells received no DNA. The cells were disrupted in lysis buffer (20 mM Tris-HCl (pH7.5), 50 mM NaCl, 5 mM 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 1% Triton X-100, 0.075% SDS), and the protein concentration determined by micro-bicinchoninic acid (BCA) assay (Pierce, Rockford, IL). Samples containing equal amounts of protein were separated by electrophoresis through 4–20% Tris-glycine pre-cast Novex gradient gels (Invitrogen, Carlsbad, CA) and transferred to polyvinylidene fluoride (PVDF) membrane (Immobilon-P, Millipore, Billerica, MA). Membranes were blocked overnight with 5% nonfat milk in PBS containing 0.2% Tween 20 (PBST) and then incubated with anti-FLAG antibody M2 (1:2,000 dilution, Sigma) or DAF16 antiserum (1:20,000) for 2 h at 22 °C. Membranes were washed twice with PBST and probed with the corresponding secondary antibodies conjugated with horseradish peroxidase (anti-mouse IgG, Sigma; anti-rabbit IgG, Boehringer Mannheim) at room temperature for 1 h. Lastly, the membranes were washed several times with PBST and visualized using the ECL detection kit (Pierce).

2.6. Streptavidin bead pull-down assay

The following 5'-end biotin labeled forward oligonucleotides and their unlabeled complements were synthesized by Integrated DNA Technologies (Coralville, IA): forward DBE oligonucleotide (5'-Bio-TGCCAGTGCG GGTTGTTTACGCGTTTCAAGA-3'), reverse DBE oligonucleotide (5'-AAAATCTTGAAAACGCGTAAACAACCCGCACT-3'), forward random oligonucleotide (5'-Bio-CATACGGGCGCATGAATTACCGCAAG CACAGG-3'), reverse random oligonucleotide (5'-CCTGTGCTTGCGGTAATTCATGCG CCC GTATG-3'). Forward and reverse oligonucleotides were annealed to form double-stranded (ds) DNA and used in the pull-down assays. Concentrated complementary oligonucleotides (200 µM forward and reverse DBE oligonucleotides, 200 µM forward and reverse random oligonucleotides) were mixed at 1:1 molar ratio in microcentrifuge tube and annealed in a thermocycler. The oligonucleotides were incubated for 1 min at 95 °C, and then the incubation temperature was gradually reduced (−1 °C/min) for 70 cycles, followed by a final hold at 4 °C. Annealed oligonucleotides were stored at −20°C. Twenty µg of HEK293T cell lysate was incubated with 100 pmoles of biotin-labeled DBE dsDNA or 100 pmoles biotin-labeled random dsDNA in binding buffer (10 mM Tris pH7.5, 50 mM KCl, 1 mM DTT, 2.5% glycerol, 5 mM MgCl₂, 50 ng/µL poly (dI.dC), 0.05% Nonidet P-40) at 4°C for 2 h. After incubation, 25 µl of 30% Streptavidin-sepharose (GE Healthcare) slurry equilibrated with TNE₅₀ buffer (10 mM Tris, 50mM NaCl, 1mM EDTA, pH7.5) + 0.1% Nonidet P-40 were added to the mixture and

incubated for 2 h at 4°C. Beads were collected by centrifugation at $2,500 \times g$ and washed twice with TNE₁₀₀ buffer (10 mM Tris, 100 mM NaCl, 1 mM EDTA pH7.5) + 0.1% Nonidet P-40. The bound proteins were separated on a 4–20% SDS-PAGE and Western blotted with DAF16 antiserum.

2.7. Transfections of NIH3T3 cells and reporter assays

Reporter vector p6xDBE-*luc*, a derivative of the pGL3-basic firefly luciferase vector (Promega) containing six concatemerized copies of DAF-16 family DBE (Furuyama et al., 2000), was a gift from Professor B.M. Burgering (University Medical Centre Utrecht, Utrecht, The Netherlands). NIH3T3 cells grown in 96-well plates were transfected with 50 ng of reporter vector, 10 ng of pRL-TK-*Renella* luciferase vector (Promega) and 10 or 40 ng of pCMV4-DAF-16 clone 2J using Metafectene. Empty pcDNA3.1-V5 expression vector was included as a negative control, and to equalize the total amount of DNA transfected to 120 ng in co-transfections. For serum-response analysis, media was changed to 20% (v/v) bovine calf serum (ATCC, Manassas, VA) or serum-free media at 24 h after transfection. The relative luciferase activity (firefly luciferase activity divided by the *Renilla* luciferase activity) was determined 24 h later using the Dual Luciferase Assay System (Promega). To determine the effect of the PI3K inhibition on transcriptional activity, cells were pre-incubated for 2 h with 50 μ M of the specific PI3K inhibitor LY294002 (Calbiochem, San Diego, CA) in a final concentration of 0.05 % DMSO. Control cells were incubated with 0.05 % DMSO without inhibitor. After 2 h, the cells were either washed and incubated with 20% serum alone, or 20% serum and 50 μ M LY294002.

2.8. Sequence Analysis

Dideoxy DNA sequencing using the ABI BigDye Terminator Cycle Sequencing Ready Reaction Kit v3.1 was performed by the Nevada Genomics Center, and the reactions run on an ABI3730 DNA Analyzer. Sequence analysis was performed using BioEdit Sequence Alignment Editor version 5.0.9 (Hall, 1999), and multiple alignments performed using CLUSTAL W at Swiss EMBnet (<http://www.ch.embnet.org/software/ClustalW.html>). Neighbor-joining trees were constructed using MEGA version 3.1 (Kumar et al., 2004). Homology searches were done using BLASTP (Altschul et al., 1990; Altschul et al., 1997) at the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/BLAST/BLOSUM>) and WU-BLAST Parasite Genome Database Query at the European Bioinformatics Institute (<http://www.ebi.ac.uk/blast2/parasites.html>). Percentage identity and similarity was determined using the BLASTP algorithm with the BLOSUM62 matrix. Conserved motifs were identified by searching Scansite at the Massachusetts Institute of Technology (<http://scansite.mit.edu/>) using MotifScan software (Obenauer et al., 2003).

3. Results

3.1. Characterization of hookworm DAF-16 molecules

An *A. ceylanicum* expressed sequence tag (EST) sequence with homology to the *daf-16* cDNA from *C. elegans* was used to design oligonucleotide primers to isolate the orthologous cDNAs from the hookworms *A. ceylanicum* and *A. caninum*. The 3' end of *Ae-daf-16* was isolated from an *A. ceylanicum* L3 cDNA library using a hemi-nested PCR strategy. The second reaction, using a nested gene-specific primer and the T7 promoter sequence present in the vector flanking the cloning site, produced an amplicon of approximately 400 bp. DNA sequencing of this clone revealed that it contained the 3' end of *Ae-daf-16*, including the poly d(A) tail, and that it overlapped the EST sequence. A similar hemi-nested strategy was used to isolate the 5' end of *Ae-daf-16* from *A. ceylanicum* L3 first-strand cDNA. In this case, the 5' primer was the conserved nematode SL sequence, which has been used to identify several hookworm 5' ends, together with two nested gene-specific reverse primers. The second reaction produced an

amplicon of approximately 850 bp that contained the SL sequence and overlapped the 5' end of the *A. ceylanicum* EST sequence. New primers were designed to amplify the entire coding sequence of *Ae*- and *Ac*-*daf-16* cDNAs in a single piece, which were cloned into pGEM T Easy vector and confirmed by DNA sequencing.

The full-length *Ae*-*daf-16* cDNA contained the conserved 22 nucleotide nematode SL at the 5' end (Bektesh and Hirsh, 1988), which was followed by five untranslated nucleotides and an ATG codon encoding the starting methionine at nucleotide 29. The cDNA encodes an open reading frame of 584 amino acids, ending with a TGA termination codon at nucleotide 1781. This is followed by a 429 nucleotide 3' untranslated region containing a rare AATAAT polyadenylation signal 12 nucleotides upstream of the poly (dA) tail (nucleotides 2204 to 2209). This signal is found in only 1% *C. elegans* of cDNA 3' ends (Blumenthal and Steward, 1997; Hajarnavis et al., 2004).

Both *A. ceylanicum* and *A. caninum* *daf-16* cDNA sequences are predicted to encode proteins of 584 amino acids. The hookworm *daf-16* cDNA sequences of the open reading frames are 94% identical, and the deduced amino acid sequences are nearly the same (98% identical, 99% similar). Of the nine amino acid differences, only two were conserved. The *Ae*-DAF-16 deduced protein has a predicted mass of 62,664 Da and a calculated pI of 6.28, while the *Ac*-DAF-16 protein is similar, with a mass of 62,693 Da and a pI of 6.17. The N-terminus of the hookworm DAF-16 deduced amino acid sequences lack hydrophobic signal peptides (Bendtsen et al., 2004), consistent with its role as a transcription factor.

DAF-16 is a transcription factor characterized by a forkhead or "winged helix" DNA binding domain. This binding domain is unique, and is found in proteins involved in determining developmental fates (Hacker et al., 1992). The *A. ceylanicum* and *A. caninum* forkhead domain deduced amino acid sequences are identical. A search of GenBank (BLASTP algorithm) (Altschul et al., 1997) using the *Ac*-DAF-16 amino acid sequence confirmed the presence of a forkhead or "winged helix" transcription factor domain (architecture cd00059.3; Interpro IPR001766) from amino acids trp₂₁₆ to val₃₀₈. As predicted, the best matches from the GenBank search were isoforms of *Ce*-DAF-16. There are three major DAF-16 isoforms in the *C. elegans* genome, which have recently undergone changes in their letter designations between the published and Wormbase versions (<http://www.wormbase.org>, release WS178). Isoforms "b" (formerly "a2") and "c" (formerly "a1") differ by only two amino acids, whereas *Ce*-DAF-16a (formerly "b" isoform) is 530 amino acids (Ogg et al., 1997). The N-terminal 191 amino acids of *Ce*-DAF-16 b and c isoforms and 211 amino acids of *Ce*-DAF-16a are encoded by different exons and are only 32% identical, whereas the 319 C-terminal residues are from the same exons, and therefore nearly identical in all isoforms (Lee et al., 2001). Pairwise alignments indicate that the hookworm DAF-16 molecules are most closely related to *C. elegans* b (46% identity/59% similarity) and c (47/59) isoforms, and more distantly related to the *Strongyloides stercoralis* FKTF isoforms a (26/41) and b (27/43).

As expected based on database searching and alignments, phylogenetic analysis using aligned forkhead domain amino acid sequences grouped the hookworm DAF-16 proteins most closely with *Ce*-DAF-16b, *Ce*-DAF-16c and *Ss*-FKTF-1a (Fig. 1A). More distantly related were *Ce*-DAF-16a and *Ss*-FKTF-1b. The relationship is readily apparent from an alignment of the forkhead domains (Fig. 1B). The hookworm forkhead domain is 94% identical and 98% similar to *Ce*-DAF-16 b and c isoforms, and 87% identical and 91% similar to *Ss*-FKTF-1a, but only 66% identical and 89% similar to the *Ce*-DAF-16a isoform and 63% identical and 80% similar to *Ss*-FKTF-1b. This level of similarity suggests that the hookworm DAF-16 proteins represent orthologs of *Ce*-DAF-16.

At 584 amino acids, the hookworm DAF-16 proteins are larger than the *C. elegans* isoforms, which range from 508 to 530 amino acids, as well as *Ss*-FKTF-1b (566 amino acids), but not *Ss*-FKTF-1b (741 amino acids). Hookworm DAF-16 proteins have an N-terminal extension of approximately 56 amino acids, and an alanine-rich 17 amino acid insert following the forkhead domain that is absent from all of the other nematode DAF-16 isoforms. Ten of the 17 amino acids are alanines. Like *Ss*-FKTF-1a and *Ce*-DAF-16b and c, the N-terminal domain of the hookworm proteins is serine-rich, containing 38 serine residues out of 216 (17.6%) (Massey et al., 2003). There is a predicted nuclear export signal (NES) conforming to the canonical LXXLXL sequence (la Cour et al., 2004) at Leu₅₄₈ to Leu₅₅₃. NES sequences are common in proteins that are shuttled between the nucleus and cytoplasm, such as transcription factors (la Cour et al., 2004).

The serine/threonine kinase Akt/PKB phosphorylates *Ce*-DAF-16 in response to ILS signaling (Paradis and Ruvkun, 1998; Brunet et al., 1999). There are four potential Akt phosphorylation sites in *Ce*-DAF-16 that conform to the conserved RXRXXS/T motif (Alessi et al., 1996): Thr₅₄, Ser₂₄₀, Thr₂₄₂, and Ser₃₁₄ (isoform c numbering) (Cahill et al., 2001). At least three of those are phosphorylated by Akt (Cahill et al., 2001). MotifScan software at Scansite (<http://scansite.mit.edu/>) identified three potential Akt phosphorylation sites in *Ac*-DAF-16, at Ser₁₀₇, Thr₃₁₂, and Ser₃₈₁. There is also a serine (Ser₃₁₁) adjacent to Thr₃₁₂ that is not in the proper Akt context, and therefore may not be phosphorylated. In the *C. elegans* and *S. stercoralis* molecules, the analogous serine and threonine residues are separated by a single amino acid, and both are located in conserved Akt phosphorylation motifs.

In *C. elegans*, phosphorylation at the Thr₅₄ and Ser₃₁₄ sites was shown to be required for binding of a 14-3-3 protein that negatively regulates *Ce*-DAF-16 by promoting its exclusion from the nucleus (Cahill et al., 2001; Henderson and Johnson, 2001). The Ser₃₈₁ site in *Ac*-DAF-16 is probably homologous to Ser₃₁₄ of *Ce*-DAF-16, whereas Ser₁₀₇ is most likely equivalent to the Thr₅₄ of *Ce*-DAF-16. Interestingly, all of the *Ac*-DAF-16 Akt phosphorylation sites are located in consensus 14-3-3 binding sites (Muslin et al., 1996), suggesting that the hookworm DAF-16 molecules are regulated by a similar mechanism as *Ce*-DAF-16. It remains to be determined which of these sites are phosphorylated in *Ac*-DAF-16.

3.2 Stage specificity by RT-PCR

PCR was performed on first-strand cDNA from egg, L1/L2, L3 and adult *A. caninum* life cycle stages to determine when *Ac-daf-16* was expressed during the hookworm life cycle. The *daf-16* primers were designed to span an intron, thereby amplifying a larger band if genomic DNA contaminated the cDNA preparation (Fig. 2B). Primers that amplify a portion of the 3' untranslated region of the protein kinase A catalytic subunit (Hawdon et al., 1995a) were used as a positive control. As shown in Fig. 2A, both the control primers and the *daf-16* primers amplified products of the appropriate size (~380 bp) from all life cycle stages, indicating that *Ac-daf-16* mRNA is transcribed throughout hookworm development.

3.3. *Ac*-DAF-16 polyclonal antiserum

A 163 amino acid peptide from the C-terminal portion of *Ac*-DAF-16 was expressed as a polyhistidine fusion protein in *E. coli*, purified, and used to make a polyclonal antiserum in a rabbit. A Western blot using this antiserum is shown in Fig. 3A. The DAF-16 antiserum recognizes a protein of approximately 62 kD in *A. caninum* L3 soluble extracts. A second, smaller band of approximately 37 kD is recognized weakly, and may represent an as yet undefined cross-reacting isoform similar to *Ce*-DAF-16e (33 kD).

3.4. Ac-DAF-16 expression in HEK293T cells

Active, properly folded recombinant Ac-DAF-16 protein was required for functional studies. Attempts to express soluble rAc-DAF-16 in the yeast *Pichia pastoris* were unsuccessful (data not shown), so we used mammalian cell culture for in vitro expression. The mammalian expression construct pCMV4-*daf-16* was verified by sequencing the inserted DNA fragment. The full-length coding region of 1,782 bp pCMV4-*daf-16* encodes a 584-amino acid fusion protein of Ac-DAF-16 with a FLAG tag at the C-terminus. The calculated molecular mass of the fusion protein is 63933.4 Da. Two sequence-verified clones (Daf16.4Ba.pCMV.2J and Daf16.4Ba.pCMV.1D) were used for transient transfection of HEK293T cells. Transfected, empty vector transfected and non-transfected (mock-treated) cell lysates were separated by SDS-PAGE and blotted to PVDF membranes for Western blots. An immunoreactive band corresponding to the predicted molecular weight (64 kDa) of the expressed fusion protein was detected by both anti-FLAG antibody M2 (Fig. 3B) and Ac-DAF-16 antiserum (Fig. 3C) in the transfected cells, but not in the mock treated cells or the cells transfected with empty vector.

To determine the titer of anti-Ac-DAF-16 antiserum, Western blots of cell lysates expressing Ac-DAF-16 were probed with increasing dilutions of the polyclonal antiserum. The antiserum recognized Ac-DAF-16 as dilutions as low as 1:30,000 (not shown).

3.5. rAc-DAF-16 DBE binding and transcriptional activation

The consensus DBE is located in the promoter region of DAF-16/forkhead-regulated genes. It was identified by a binding/PCR amplification/selection strategy, and is conserved between mammals and *C. elegans* (Furuyama et al., 2000). We hypothesized that Ac-DAF-16 would function like *Ce*-DAF-16 and other forkhead transcription factors by binding to the DBE and driving transcription of the downstream gene. To determine whether Ac-DAF-16 binds to consensus DBE, oligomers containing the DBE (5'-TTGTTTAC-3') and a random sequence were synthesized with biotin label at the 5'-end. The biotin-labeled oligomers were incubated with soluble HEK293T cell lysates, followed by streptavidin beads to pull-down biotin-labeled oligonucleotide/protein complexes. Recombinant Ac-DAF-16 bound strongly to the biotinylated DBE oligo, as indicated by recognition of several bands of approximately 64 kDa by anti-DAF-16 antiserum in the pull-down assay containing DBE and pCMV4-DAF16 transfected cell lysate (Fig. 4, lane 2). The bands were absent in the pull-down assays containing the random oligomer and Ac-DAF16 transfected cell lysates or mock-treated cell lysate (Fig. 4, lanes 5 and 6), or DBE and mock-treated cell lysate (lane 3). These pull-down assay results suggest that Ac-DAF-16 recognizes and binds strongly to the conserved DBE.

To determine whether Ac-DAF-16 could drive transcription from the DBE promoter element in vivo, pCMV4-daf16 and the luciferase reporter assay p6xDBE-luc were co-transfected into NIH3T3 cells together with a *Renilla* luciferase reporter plasmid. In starved NIH3T3 cells, DAF-16 expression activated transcription of the DBE by five-fold (in cells transfected with 10 ng of pCMV4-daf16) or nine-fold (40 ng of pCMV4-daf16) (Fig. 5). Treatment of transfected cells with 20% serum significantly inhibited transcription from the DBE. This indicates that Ac-DAF-16 can bind to and drive expression from the *C. elegans* DBE when they are co-expressed in mammalian cells. Furthermore, endogenous cell signaling pathways are able to negatively regulate transcription activity by Ac-DAF-16 in response to serum in NIH3T3 cells. However, treatment with the specific PI3K inhibitor LY294002 (Vlahos et al., 1994) had no effect on the serum-stimulated negative regulation of transcription (not shown).

4. Discussion

Developmentally arrested hookworm infective L3s encounter a host-specific signal during invasion of their definitive host that re-activates suspended developmental programs. These

programs culminate in maturation to the parasitic adult stage. Timely and proper response to this signal is critical for the successful establishment of a parasitic relationship. Failure to initiate development in the correct host would represent a missed infection opportunity, and resumption of development in a non-permissive host would result in either direct death of the parasite or “reproductive death” due to isolation from potential mates. Therefore, proper execution of the events of this “transition to parasitism” is critical.

The molecular signaling events associated with the transition to parasitism in hookworms are only now beginning to be deciphered, primarily because of insights about nematode development gained from studies of *C. elegans*. Exit from the developmentally arrested dauer stage in *C. elegans* has been compared with the transition to parasitism that occurs when free-living infective stages enter their definitive host (Rogers and Sommerville, 1963; Hawdon and Schad, 1991a; Hotez et al., 1993; Hawdon and Hotez, 1996). In *C. elegans*, recovery from dauer requires ILS, but not TGF- β , signaling (Tissenbaum et al., 2000). Using pharmacological agents and an in vitro feeding assay, we have shown previously that hookworm L3 use an analogous ILS pathway during larval activation (Tissenbaum et al., 2000; Hawdon and Datu, 2003; Brand and Hawdon, 2004). Given this conserved signaling pathway, we hypothesized that a hookworm ortholog of the *C. elegans* transcription factor DAF-16 was the terminus of ILS signaling during activation. To investigate this hypothesis, we have cloned a DAF-16 ortholog from the hookworms *A. caninum* and *A. ceylanicum*, and expressed recombinant Ac-DAF-16 in mammalian cells to investigate its function in larval activation.

An *A. ceylanicum* expressed sequence tag (pk84h05) with similarity to *Ce-daf-16* was used to design gene-specific oligonucleotide primers for PCR. The 5' and 3' ends of *Ae-daf-16* cDNA were amplified in separate reactions, and a second set of primers design from those sequences to complete the entire sequence. Oligonucleotide primers designed from the complete *Ae-daf-16* sequence were used to amplify the full-length coding sequence of *daf-16* from *A. caninum* cDNA. The deduced amino acid sequence was used to search the non-redundant GenBank database, and found to be a member of the forkhead family of transcription factors, characterized by a forkhead or “winged helix” DNA binding domain. Originally identified in *Drosophila*, this binding domain is found in proteins involved in determining developmental fates (Hacker et al., 1992). In *C. elegans*, DAF-16 controls longevity and resistance to stress, as well as dauer development (Lin et al., 1997; Ogg et al., 1997; Henderson and Johnson, 2001). Phylogenetic analysis of the binding domains indicates that the hookworm DAF-16 molecules are most closely related to isoforms b and c of *C. elegans*, as well as FKTF-1a of the parasitic nematode *S. stercoralis* (Massey et al., 2003). Interestingly, *Ss*-FKTF-1b, but not *Ss*-FKTF-1a, partially rescued the dauer defective phenotype of *daf-2*; *daf-16* double mutants when expressed in *C. elegans* (Massey et al., 2006). The *C. elegans* DAF-16 isoforms appear functionally interchangeable (Lee et al., 2001), so differences in biological effects are likely due to differential tissue expression. However, Estevez et al. (2006) suggested that the *C. elegans* isoforms a and b, which differ in their DNA binding domains, bind to different parts of the tryptophan hydroxylase (*tph-1*) promoter and have opposite effects on *tph-1* expression. We are currently seeking additional isoforms in hookworms.

There are also several sequence differences between the isoforms outside the DNA binding domain that may alter as yet undetected regulatory interactions. For example, the hookworm DAF-16 molecules contain a serine-rich N-terminal domain, found also in *Ss*-FKTF-1a, *Ce*-DAF16b and c, but not in *Ce*-DAF-16a *Ss*-FKTF-1b or the mammalian orthologs (Massey et al., 2003). Also, the hookworm DAF-16s contain a unique alanine-rich sequence in the C-terminal domain missing from the other orthologs. The functional significance of these sequences is unknown.

In *C. elegans*, DAF-16 regulates aging and development. When environmental conditions are inhospitable for development, ILS is inactive and DAF-16 enters the nucleus and binds to DNA, resulting in expression of genes required for the dauer stage. When conditions improve, the serine/threonine kinase Akt/PKB phosphorylates *Ce*-DAF-16 in response to ILS through the insulin receptor DAF-2 (Paradis and Ruvkun, 1998; Brunet et al., 1999). Phosphorylation creates binding sites for 14-3-3 proteins, which escort DAF-16 from the nucleus and sequester it in the cytoplasm (Cahill et al., 2001; Li et al., 2007). The hookworm DAF-16 orthologs contain three serine or threonine residues in the proper context for AKT phosphorylation (Ser₁₀₇, Thr₃₁₂, and Ser₃₈₁), all of which are located in consensus 14-3-3 binding sites (Muslin et al., 1996). This also suggests a role for 14-3-3 proteins in the regulation of hookworm DAF-16 (Cahill et al., 2001; Li et al., 2007). *Ac*-DAF-16 is expressed in all developmental stages, similar to *C. elegans* DAF-16 and *S. stercoralis* forkhead transcription factor (FKTF) (Ogg et al., 1997; Lee et al., 2001; Massey et al., 2003), consistent with their regulation by phosphorylation and cellular relocation, rather than at the level of transcription.

The near identity of the hookworm DAF-16 DNA binding domain to that of *Ce*-DAF-16b suggested that *Ac*-DAF-16 might bind to the same DNA sequence. *Ce*-DAF-16 and mammalian orthologs bind, via the forkhead domain, to a specific consensus DBE with the sequence 5'-TTGTTTAC-3' (Furuyama et al., 2000). The core sequence of the DBE is TGTT, and a change of any base in TGTT to C eliminated the binding affinity of DAF-16 homologues. Using streptavidin bead pull-down assay, we demonstrated that recombinant *Ac*-DAF-16 expressed in mammalian cells strongly bound to the conserved DBE. The multiple bands seen on the Western blot may represent differentially phosphorylated forms of *Ac*-DAF-16. Given its ability to bind to the DBE, we next tested whether *Ac*-DAF-16 could drive transcription of a reporter gene from this binding element. A reporter plasmid (p6xDBE) containing six consecutive DBE sequences cloned upstream of the firefly luciferase gene (Furuyama et al., 2000) was co-transfected with pCMV4-daf16:FLAG into NIH3T3 cells. Under starved conditions, increasing amounts of *Ac*-DAF-16 plasmid increased transcription of the reporter gene, as determined by luciferase assay. Addition of serum inhibited transcription to control levels. While these results were expected, we were unable to block inhibition of transcription with the specific PI3K inhibitor LY294002 (Vlahos et al., 1994). This suggests that while endogenous serum-activated signaling pathways were able to negatively regulate *Ac*-DAF-16 transcriptional activity, it is unlikely to require ILS in this cell line. AKT kinase, which phosphorylates DAF-16, can be activated by pathways other than ILS in response to serum growth factors (Van Der Heide et al., 2004). For example, the serum and glucocorticoid regulated kinase (SGK) recognizes the same sequence motif as Akt (Alessi, 2001), and also phosphorylates mammalian FoxO factors (Van Der Heide et al., 2004). Investigations of *Ac*-DAF-16 function in other cell lines are ongoing.

In summary, we have identified an ortholog of *C. elegans* DAF-16 from the hookworms *A. caninum* and *A. ceylanicum*. Recombinant *Ac*-DAF-16 binds to a conserved DNA binding element, and can drive transcription from this promoter sequence in mammalian cells. Transcription is negatively regulated by serum in this system, indicating that *Ac*-DAF-16 functions similarly to *Ce*-DAF-16. Given the similarity with the *C. elegans* dauer, genes that are controlled by DAF-16 are probably important for the maintenance of the developmentally arrested "dauer" state in hookworms, and consequently represent an interesting area of investigation. Identification and subsequent manipulation of these genes may provide novel avenues for intervention in the hookworm life cycle. The interaction between *Ac*-DAF-16 and DBE will provide a valuable tool in the search for target genes regulated by DAF-16.

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A.

B.

<i>Ac</i> -DAF-16	1	NAWGNLSYADLITQAIMQSPEKRLTLSQVYEWVQNVVPYFRDKGDSNSSAGWKN SIRHNL
<i>Ce</i> -DAF-16b	1	NAWGNMSYAE LITTAIMASPEKRLTLAQVYEWVQNVVPYFRDKGDSNSSAGWKN SIRHNL
<i>Ss</i> -FKTF-1a	1	NAWGSHSYADLITQAIQSPEKRLTLSQVYEWVMTNVPYFRDKGDSNSSAGWKN SIRHNL
<i>Ce</i> -DAF-16a	1	NPWGEESYSDTI AKALESAPDGR LKLN EYQWFS DNIPYFGERSSPEEAAGWKN SIRHNL
<i>Ss</i> -FKTF-1b	1	NPWGEESYSDLI AKALECAPDKRMKLN EYQWFS ENIPYFNDRSSQEEAAGWKN SIRHNL
<i>Ac</i> -DAF-16	61	SLHSRFRMRIQNEGAGKSSWWVINPDAKPGR
<i>Ce</i> -DAF-16b	61	SLHSRFRMRIQNEGAGKSSWWVINPDAKPGR
<i>Ss</i> -FKTF-1a	61	SLHNRFRMRIQNEGAGKSSWWVINPDAKNGR
<i>Ce</i> -DAF-16a	61	SLHSRFRMRIQNEGAGKSSWWVINPDAKPGR
<i>Ss</i> -FKTF-1b	61	SLHNRFRMRIQNEGAGKSSWWVINPDAKNGR

Fig. 1.

The phylogenetic relationship between nematode forkhead transcription factor DAF-16 and selected forkhead protein family members. A) Neighbor joining tree of forkhead domains. Forkhead domains were aligned using CLUSTAL W software on the Swiss EMBnet server (<http://www.ch.embnet.org>). Amino acid distances were calculated using the Poisson correction model in the MEGA program version 3.1 (Kumar et al., 2004). Bootstrap values (1,000 replications) are shown at each node. *Ac*, *Ancylostoma caninum*; *Ae*, *Ancylostoma ceylanicum*; *Ce*, *Caenorhabditis elegans*; *Ss*, *Strongyloides stercoralis*; *Hs*, *Homo sapiens*. Accession numbers: *Ce*-DAF-16a, [AAK82917](#); *Ce*-DAF-16b, [AAK82918](#); *Ce*-DAF-16c, [AAK82919](#); *Ss*-FKTF-1a, [AAQ23177](#); *Ss*-FKTF-1b, [AAQ23178](#); *Hs*-AFX1, [P98177](#); *Hs*-

FOXO-1, **NP_002006**; *Ce*-FKH-9, **AAA81139**; *Ce*-FKH-2, **AAA82436**; *Hs*-HNF-3, **AAD51979**; *Ce*-UNC-130, **CAA88935**; *Hs*-FOXO3, **AAH68552**; *Ce*-let-381, **AAB37792**; *Ce*-PHA-4, **CAB07378**. B) Alignment of representative nematode forkhead domains. Shading indicates residues that are identical (black) or similar (gray) to *Ac*-DAF-16. Protein sequences were aligned using CLUSTAL W software and displayed using BOXSHADE software located on the Swiss EMBnet server. The *A. ceylanicum* and *C. elegans* isoform c were omitted from the alignment because the forkhead domains are identical to *A. caninum* and *C. elegans* b isoforms, respectively.

A.

B.

Fig. 2.

Expression of *Ac-daf-16* mRNA during the hookworm life cycle. A) First strand cDNA from *Ancylostoma caninum* life cycle stages was amplified with the gene-specific primers DAF16-F3 and DAF16-R3 (lanes 1–4) or control primers PKA5'-4 and PKA3'-1 (lanes 5–10) and separated on a 1.5% agarose gel. Lanes 1 and 5 = egg cDNA; lanes 2 and 6 = L1/L2 cDNA; lanes 3 and 7 = L3 cDNA; lanes 4 and 8 = adult cDNA; lane 9, genomic DNA; lane 10, no DNA control. B) Primers DAF16-F3 and DAF16-R3 amplify a larger amplicon from genomic DNA. Lane 1, *Ac-daf-16* plasmid DNA; lane 2, *A. caninum* L3 cDNA; lane 3, *A. caninum* genomic DNA.

Fig. 3.

Forkhead transcription factor DAF-16 expression in mammalian cells and antiserum characterization. The arrowheads indicate the position of *Ac*-DAF-16. A) Western blot of *Ancylostoma caninum* L3 extract and recombinant DAF-16 C-terminus (rDAF-16₃₆₅₋₅₂₈) expressed in *Escherichia coli*. Proteins were detected with *Ac*-DAF-16 polyclonal antiserum. B) Expression of *Ac*-DAF-16 in HEK293T human embryonic kidney cells. Lanes 2J and 1D contain lysates of HEK293T cells transfected with clones Daf16.4Ba.pCMV.2J and Daf16.4Ba.pCMV.1D. The mock lane contains lysates from non-transfected cells. The blot was probed with antibody (M-2) against the FLAG epitope tag expressed on the recombinant protein C) Western blot of HEK293T cells expressing recombinant *Ac*-DAF-16. Lane 2J contains lysate of HEK293T cells transfected with clone Daf16.4Ba.pCMV.2J, and Lane Con contains lysate from cells transfected with empty pCMV mammalian expression vector DNA. The blot was probed with *Ac*-DAF-16 polyclonal antiserum (1:20,000).

Fig. 4.

Streptavidin bead pull-down to detect DBE binding activity of recombinant *Ac*-DAF-16. Biotin labeled double-strand DNA oligonucleotides were incubated with cell lysates from pCMV4-Daf16 transfected HEK293T human embryonic kidney cells or untransfected (mock) cells, and DAF-16-oligo complexes pulled down with streptavidin conjugated Sepharose beads. Complexes were detected by Western blotting with DAF-16 polyclonal antiserum. DBE, DAF-16 binding element; random, random sequence oligonucleotide.

Fig. 5.

Recombinant *Ancylostoma caninum* forkhead transcription factor DAF-16 drives expression from the DAF-16 binding element (DBE). Plasmid pCMV4-DAF-16-2J encoding full-length FLAG epitope-tagged *Ac*-DAF-16 was cotransfected (10 or 40 ng) with 50 ng of reporter vector p6xDBE-luc and 10 ng of pRL-TK-*Renella* luciferase vector into NIH 3T3 cells. After 24 h, cells were incubated in either medium alone (black bars) or with medium plus 20% bovine calf serum (gray bars). The relative luciferase activity (firefly luciferase activity divided by the *Renilla* luciferase activity) was determined 24 h later.