daf-7-related TGF-β homologues from Trichostrongyloid nematodes show contrasting life-cycle expression patterns

H. J. McSORLEY[1]†, J. R. GRAINGER[1], Y. HARCUS[1], J. MURRAY[1], A. J. NISBET[2], D. P. KNOX[2] *and* R. M. MAIZELS[1]*

[1] *Centre for Immunity, Infection and Evolution, and Institute for Immunology and Infection Research, Ashworth Laboratories, University of Edinburgh, West Mains Road, Edinburgh EH9 3JT, Scotland*
[2] *Moredun Research Institute, Pentlands Science Park, Bush Loan, Penicuik, Midlothian, EH26 0PZ, Scotland*

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SUMMARY

The transforming growth factor-β (TGF-β) gene family regulates critical processes in animal development, and plays a crucial role in regulating the mammalian immune response. We aimed to identify TGF-β homologues from 2 laboratory model nematodes (*Heligmosomoides polygyrus* and *Nippostrongylus brasiliensis*) and 2 major parasites of ruminant livestock (*Haemonchus contortus* and *Teladorsagia circumcincta*). Parasite cDNA was used as a template for gene-specific PCR and RACE. Homologues of the TGH-2 subfamily were isolated, and found to differ in length (301, 152, 349 and 305 amino acids respectively), with variably truncated N-terminal pre-proteins. All contained conserved C-terminal active domains (>85% identical over 115 amino acids) containing 9 cysteine residues, as in *C. elegans* DAF-7, *Brugia malayi* TGH-2 and mammalian TGF-β. Surprisingly, only the *H. contortus* homologue retained a conventional signal sequence, absent from shorter proteins of other species. RT-PCR assays of transcription showed that in *H. contortus* and *N. brasiliensis* expression was maximal in the infective larval stage, and very low in adult worms. In contrast, in *H. polygyrus* and *T. circumcincta, tgh-2* transcription is higher in adults than infective larvae. The molecular evolution of this gene family in parasitic nematodes has diversified the pre-protein and life-cycle expression patterns of TGF-β homologues while conserving the structure of the active domain.

Key words: development, immune modulator, stage-specific expression.

INTRODUCTION

The TGF-β family is intimately involved in development and homeostasis of both vertebrate and invertebrate organisms (Newfeld *et al*. 1999; Massagué *et al*. 2000). In vertebrates, one branch of the family mediates immunological functions, in particular dampening potential immune pathology (Li *et al*. 2006). In *Drosophila*, the TGF-β family member *decapentaplegic* (DPP) acts in morphogenesis (Affolter and Basler, 2007) while in *Caenorhabditis elegans*, *daf*-7 regulates entry into the arrested Dauer larval stage (Ren *et al*. 1996; Inoue and Thomas, 2000) and *dbl-1* controls body size (Suzuki *et al*. 1999; Morita *et al*. 2002). There is considerable interest in TGF-β homologues of parasitic helminths, and in *Schistosoma mansoni* one member of this family (SmInAct) is required for embryonic development (Freitas *et al*. 2007). It has been suggested that other homologues may control parasite-arrested development, in particular the hiatus of the infective third-stage nematode larvae (L3) awaiting a mammalian host (Viney *et al*. 2005). Furthermore, parasitic species may have even adapted TGF-β genes to interact with, and suppress, the host immune system (Maizels *et al*. 2001).

To address these possibilities, nematode TGF-β homologues have been studied from *Brugia malayi* (Gomez-Escobar *et al*. 1998, 2000), *Ancylostoma caninum* (Brand *et al*. 2005; Freitas and Arasu, 2005), two *Strongyloides* species and *Parastrongyloides trichosuri* (Crook *et al*. 2005; Massey *et al*. 2005). Only the *B. malayi* TGF-β homologue-2 (*Bm*-TGH-2) shows binding to mammalian TGF-β receptors (Gomez-Escobar *et al*. 2000) with all other homologues appearing to fulfill a developmental function in the parasite, as is the case for *C. elegans daf*-7 and *dbl-1*. However, the contrasting gene expression patterns suggests a reversal in developmental roles between *C. elegans* and parasitic species (Viney *et al*. 2005). In the former, *daf*-7 is expressed in early stages, but down-regulated when entering the arrested Dauer larval form (Ren *et al*. 1996). In parasitic species expression is maximal in diapausal stages, including the arrested L3 larvae which may be developmentally analogous to Dauer (Hotez *et al*. 1993). As larval arrest is constitutive in parasitic life cycles, but facultative in *C. elegans*, parasite TGF-β homologues may be required not to control

* Corresponding author: Institute of Immunology and Infection Research, University of Edinburgh, Ashworth Laboratories, West Mains Road, Edinburgh EH9 3JT, Scotland. Fax: +44 131 650 5450. E-mail: r.maizels@ed.ac.uk
† Current address: Queensland Institute for Medical Research, Brisbane, Australia.

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entry into diapause, but rather to maintain arrest until the opportunity for infection arises (Viney *et al.* 2005). This evolutionary switch is made more plausible by the recent discovery that the common molecular pathway controlling Dauer formation in *C. elegans* and parasite infective larval development is not *daf-7*-related, but signals through dafachronic acid ligation of the nuclear hormone receptor *daf-12* (Ogawa *et al.* 2009).

The importance of TGF-β homologues in development and immune modulation may be dissected in model organisms such as the mouse intestinal helminth *Heligmosomoides polygyrus* and the rat parasite *Nippostrongylus brasiliensis*. Both are closely related to other Trichostrongyloid nematodes of great economic significance as veterinary pathogens, including *Haemonchus contortus* and *Teladorsagia circumcincta*. These species offer fascinating and contrasting developmental and immunological features. *H. polygyrus*, *H. contortus* and *T. circumcincta* all share transmission via the faecal/oral route, with L3 larvae entering orally, developing to L4 larvae in the duodenum of mice or the abomasum (4th stomach) of sheep, following which, they emerge into the lumen and deposit eggs which egress in faeces. *H. contortus*, like many cattle nematodes (Armour and Duncan, 1987), may enter environmentally-induced arrest and *T. circumcincta* can undergo immune-induced developmental delay in the mammalian host. In *N. brasiliensis* infections, larvae penetrate the skin, and migrate through the lungs, trachea and oesophagus to reach the gastrointestinal tract. In the lumen, parasites develop to sexually reproducing adults, depositing eggs that are voided in the faeces. *H. polygyrus* establishes chronic infections in mice, associated with immune suppression (Elliott *et al.* 2004) and regulatory T cell activity (Wilson *et al.* 2005; Finney *et al.* 2007; Rausch *et al.* 2008); however, *N. brasiliensis* is rapidly expelled from mice by a strong Th2-dependent immune response (Finkelman *et al.* 2004; Anthony *et al.* 2007). In both rodent and ovine hosts, a protective memory response can be generated following chemotherapy (Schallig, 2000; Finkelman *et al.* 2004; Anthony *et al.* 2006), which is also characterized by a dominant Th2 effector mechanism. With the definition of a *daf-7* homologue from the dog hookworm *A. caninum* (Brand *et al.* 2005; Freitas and Arasu, 2005) and with extensive genomic sequence data available for *H. contortus*, we initiated a project to identify new family members within this extremely important group of parasites, as we now report below.

MATERIALS AND METHODS

Parasites and life cycles

H. polygyrus bakeri was maintained in CBAxC57BL/6 F1 mice by infection with 500 larvae through a gavage tube; adults were recovered at day 14 post-infection. *N. brasiliensis* was maintained as described (Lawrence *et al.* 1996), by infecting SD rats with 4000 L3 subcutaneously. L1, L2 and L3 of *H. polygyrus* and L3 of *N. brasiliensis* were collected from faecal-charcoal cultures using the Baermann technique. *N. brasiliensis* L4 larvae were recovered from lungs 42 h post-infection, and adult parasites from the gut 6 days post-infection. Eggs were collected from adults cultured *in vitro* as described previously (Holland *et al.* 2000). *H. contortus* and *T. circumcincta* lifecycles were maintained as described (Knox and Jones, 1990). L3 larvae from faecal cultures were exsheathed in 8% sodium hypochlorite solution and washed 4 times in saline at 38 °C. Fourth-stage larvae and adult parasites were harvested 8 days and 21 days respectively after infecting worm-free sheep (7–12 months old) with 50 000 L3 of either *H. contortus* or *T. circumcincta*.

cDNA preparations

H. polygyrus and *N. brasiliensis* harvested as above, were washed 5 times in PBS, and then stored in TRIzol reagent (Invitrogen) at –80 °C, before use for RNA extraction following the manufacturer's methods. Briefly, around 100 μl packed volume of parasites (equivalent to around 50 adults or 1000 larvae) were first homogenized on ice, and after addition of 200 μl of chloroform, centrifuged at 12 000 ***g*** for 15 min at 4 °C. The aqueous layer was then precipitated with isopropanol and centrifuged at 12 000 ***g*** for 10 min at 4 °C. The RNA pellet was then washed in 500 μl of 70% ethanol, air-dried and re-suspended in 20 μl of DEPC water. The DNA-free kit (Ambion) was used to degrade DNA: 2 μl of DNAse with 2·4 μl DNAse buffer was added, and incubated at 37 °C for 30 min. Then 5 μl of DNAse inactivation reagent was added, incubated at room temperature for 2 min, spun down at 12 000 ***g*** for 2 min, and the supernatant removed. The concentration was determined by spectrophotometry at 260 nm.

For reverse transcription to cDNA, 500 ng of RNA was mixed with 2 μl of 10× reverse transcriptase buffer, 2 μl of 25 mM dNTP mix, 1 μl of 50 U/μl MMLV reverse transcriptase (Stratagene), 0·5 μl of 40 U/μl RNAsin (Promega), 1 μl of 0·4 μg/ml oligo-dT primer (Promega) and DEPC-treated water to 20 μl. The reaction was incubated on a PCR block at 20 °C for 10 min, 37 °C for 60 min and 99 °C for 5 min. cDNA from *H. contortus* and *T. circumcincta* was prepared as described previously (Redmond *et al.* 2006).

PCR amplifications and RACE

Polymerase chain reactions (PCRs) were run on an MJ Research DNA Engine with denaturing at 94 °C for 1 min, annealing at 55–65 °C for 1 min

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(depending on primers and product), and extended at 72 °C for 0·5–2 min (depending on length of product). Reaction mixes comprised 0·2 μl of 5 U/μl Taq (QIAGEN), 0·2 μl of 25 mM dNTP mix, 2 μl of 10 X PCR buffer (QIAGEN), 1 μl of forward and reverse primers (10 μm each), 1 μl of template (cDNA, colony pick or plasmid miniprep) and water to 20 μl. RACE-ready cDNA was made using the Invitrogen Generacer Core kit from adult parasites of all 4 species.

For 3′ RACE reactions, reverse transcription was carried out with a modified oligo-dT primer containing a sequence insert for subsequent amplification (5′-GCTGTCAACGATACGCTACGT-AACGGCATGACAGTG$(T)_{24}$-3′). The resulting cDNA was RNAse-treated and then used in PCR with the gene-specific forward primers set out in Table 1. For 5′ RACE, an oligonucleotide 5′-CGACUGGAGCACGAGGACACUGACAUGG-ACUGAAGGAGUAGAAA-3′ was ligated to the 5′ end of the RNA molecule prior to reverse transcription; 5 μg RNA was dephosphorylated with CIP (calf intestinal phosphatase), phenol : chloroform precipitated, and the 5′ cap structure then removed with TAP (tobacco acid pyrophosphatase). Free phosphate groups created at the 5′ end of mRNA were then ligated to the 5′ RACE primer. The resulting cDNA was RNAse-treated and used in PCR with the gene-specific reverse primers shown in Table 1.

Cloning

PCR products were excised under UV transillumination, purified using the QIAGEN gel extraction kit and ligated into pGEM-T (Promega) at a 3 : 1 molar ratio. Ligated plasmid preparations were then transformed into *E. coli* JM109 cells for overnight colony formation. Colonies positive by PCR screening were cultured overnight, and plasmid mini-prep DNA sequenced.

Real-time PCR

Real-time PCR was performed on 1 μl of a 1 in 5 dilution of cDNA, mixed with 5 μl of Platinum SYBR green qPCR supermix (Invitrogen), 0·2 μl of forward and reverse primers (10 μM), and water to 10 μl. Triplicate reactions were set up, with standards of a mixture of all cDNA, with doubling dilutions from neat cDNA mix. The reactions were run on a Chromo4 real-time PCR machine (Alpha Innotech). Reactions were denatured for 20 sec at 94 °C, annealed for 20 sec at 65 °C and extended for 20 sec at 72 °C (for SYBR green fluorescence) for 50 cycles. Primers used for the actin, tubulin and *tgh-2* genes of all 4 parasite species are given in Table 1. In all reactions a melting curve analysis was also carried out, in which the fluorescence profile was measured at 0·5 °C increments from the annealing temperature until 94 °C, to ensure a single peak, indicative of a single amplified product, was detected. Reaction products were also run on agarose gels to confirm that a single product had been amplified.

Bioinformatics

Homology searches of the partially sequenced *H. contortus* genome were performed using the Wellcome Trust Sanger Institute Blast server (www.sanger.ac.uk/Projects/H_contortus), using the datasets published on 27/01/2006, and more recently on 12/11/2007. Searches of nematode ESTs (including *T. circumcincta*) were carried out at http://www.ebi.ac.uk/Tools/blast2/nucleotide.html, selecting the 'nucleic acid' and 'EMBL EST Invertebrate' options. Signal peptide predictions were performed using the SignalP 3.0 server (http://www.cbs.dtu.dk/services/SignalP/). Alignments, translational predictions and sequence analysis were carried out using MacVector 9.5.2 (Symantec). The phylogenetic tree shown was constructed by best tree analysis, using a Poisson distribution.

Nomenclature

Naming of genes and proteins followed standard nomenclature for nematode species (Bird and Riddle, 1994), with a 2-letter species code followed by a 3-letter gene name and a numeral if required. Gene names for DNA and mRNA are referred to in lower-case italics, while proteins and amino acid sequences are Roman capitals. Names and numerals are conserved between orthologues from different species wherever possible. Hence, the orthologues of *B. malayi transforming growth factor homologue-2* (*Bm-tgh-2*) are each given the gene name *tgh-2*. In TGF-*β* superfamily members, proteolytic cleavage at an internal tetrabasic site is required to produce the receptor-binding C-terminal domain, variously termed mature, ligand or active domain; we use the latter term to denote this domain.

RESULTS

Identification of TGH-2 in Haemonchus contortus

Nematode homologues of TGF-*β* identified in recent years include *Ac-tgh-2* (also designated *Ac-daf-7*) from the dog hookworm *A. caninum* (Brand *et al.* 2005; Freitas and Arasu, 2005). Because *Ancylostoma* is within the same taxonomic suborder as *H. contortus* (Strongyloida), the *Ac*-TGH-2 protein sequence was selected to search for TGF-*β* homologues in *H. contortus*. A closely related sequence was found on a contig in the Wellcome Trust Sanger Institute dataset (http://www.sanger.ac.uk/Projects/H_contortus), covering 3 exons in the active domain,

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Table 1. Gene-specific primers used in 3′ and 5′ RACE (A) and in RT-PCR (B)

(RACE reactions used a single gene-specific primer with a second primer to a sequence ligated onto all cDNA molecules; products and sizes are shown in Fig. 2. RT-PCR used the indicated primer pairs and yielded products of the sizes indicated. Numerals in parentheses correspond to the nucleotide positions in the full-length cDNA sequences as deposited at GenBank.)

(A) RACE Primers

Transcript	3′ RACE (Forward primer)	5′ RACE (Reverse primer)
H. contortus tgh-2	5′-ATGTTCATGTCGGTTACGCTGG-3′ (652-673)	5′-TTCAGCGGGTGACAGCACATTC-3′ (954-932)
H. polygyrus tgh-2	5′-CCCGGATGTTGCCTCTACGAC-3′ (589-609)	5′-CCCCTGATTTCCAGTTGCGTC-3′ (789-769)
N. brasiliensis tgh-2	5′-GGTTGCTGTCTCTACGATCTC-3′ (148-168)	5′-CCCTTGGTTACCCGTTGCGTC-3′ (342-322)
T. circumcincta tgh-2	5′-GGGTGATTGTTCTGTGAATCAC-3′ (699-720)	5′-GTGATTCACAGAACAATCACCC-3′ (720-699)

(B) Real-time PCR Primers

Transcript	Forward primer	Reverse primer	Product size
Actin (all species)	5′-TGAGCACGGTATCGTCACCAAC-3′ (216-237)	5′-TTGAAGGTCTCGAACATGATCTG-3′ (386-364)	171 bp
T. circumcincta β-tubulin	5′-TTCCATTCCCTCGTCTTCAC-3′ (773-792)	5′-AGCCATTTTCAATCCACGAG-3′ (1092-1073)	320 bp
H. contortus tgh-2	5′-ATGTTCATGTCGGTTACGCTGG-3′ (652-673)	5′-TTCAGCGGGTGACAGCACATTC-3′ (954-932)	303 bp
H. polygyrus tgh-2	5′-CCCGGATGTTGCCTCTACGAC-3′ (589-609)	5′-CCCCTGATTTCCAGTTGCGTC-3′ (789-769)	201 bp
N. brasiliensis tgh-2	5′-GGTTGCTGTCTCTACGATCTC-3′ (148-168)	5′-CCCTTGGTTACCCGTTGCGTC-3′ (342-322)	195 bp
T. circumcincta tgh-2	5′-GGGTGATTGTTCTGTGAATCAC-3′ (699-720)	5′-TCAAGAGCATGTACATTTACGG-3′ (918-897)	220 bp

as shown in Fig. 1A. No ESTs from *H. contortus* (Parkinson *et al*. 2004), *N. brasiliensis* (Harcus *et al*. 2004), or *T. circumcincta* (Nisbet *et al*. 2008) were found with similarity to either *Ac-tgh-2* or the *H. contortus* contig. When, as described below, 5′ and 3′ RACE were used to extend the cDNA sequence to the N- and C-termini of the protein, the additional sequences matched two additional contigs containing five 5′ and one 3′ exons. The organization of *Hc-tgh-2* into 10 exons closely resembles that of *Ac-tgh-2*, with all but the first exon being of identical length. There is considerable variation, however, in the intronic tracts, as far as genomic information permits comparison (Freitas and Arasu, 2005).

Isolation of TGH-2 homologues from other species

To isolate cDNAs coding for TGH-2 homologues from other gastrointestinal nematode parasites, an alignment of known human and nematode TGF-β amino acid sequences was made, including the newly defined *H. contortus* homologue (Fig. 1B). Degenerate primers were designed for regions that were best conserved and offered the lowest degeneracy. Where sequences diverged between homologues, the *H. contortus*/*A. caninum* TGH-2 consensus was taken. These primer sequences are shown in Fig. 1C.

Combinations of these degenerate primers were used in PCRs with *H. polygyrus*, *N. brasiliensis* and *T. circumcincta* adult parasite cDNA, and products (Fig. 1D) excised from gels and sequenced. From both *H. polygyrus* and *N. brasiliensis*, bands of ~250 bp were amplified which, on sequencing, showed similarity to *Ac-tgh-2*. Using a different forward primer, a 200-bp product was amplified from *T. circumcincta* cDNA, with homologous sequence. Non-degenerate primers (Table 1) were then designed to these newly sequenced regions of each homologue, and were used in 5′ and 3′ rapid amplification of cDNA ends (RACE) to identify flanking coding sequences.

Full sequences of TGF-β homologue genes

RACE was used to isolate the 5′ and 3′ ends of each gene, in separate reactions with products as shown in Fig. 2. The *H. contortus* and *T. circumcincta* 5′ and 3′ RACE products were amplified by single PCR reactions, while the *H. polygyrus* and *N. brasiliensis* 5′ and 3′ RACE products required amplification by nested PCR, using a second set of primers. Products were sequenced, and sequences of the newly assigned *Hc*-TGH-2, *Hp*-TGH-2, *Nb*-TGH-2 and *Tc*-TGH-2 aligned with other TGF-β family members at the amino acid level (Fig. 3). The 4 trichostrongyloid parasite proteins were all identical at 100/117 positions (85·4%) of the C-terminal domain, but only 50 amino acids (42·7%) were identical to *Bm*-TGH-2 over the same tract.

Particular scrutiny was given to the 5′ termini of these genes, as in 3 cases they were truncated at the 5′ end compared to canonical TGF-βs (Fig. 3). To verify the full-length sequence of *Hp-tgh-2* and *Nb-tgh-2*, 5′ RACE was repeated several times and the 5′ ends of each gene were also amplified from cDNA libraries prepared as described elsewhere (Harcus *et al*. 2004); in no case did we detect a longer transcript. Furthermore, we were able to amplify *Hp-tgh-2* with an internal gene-specific reverse primer, and a forward primer for the nematode 22-nt spliced leader sequence, and this product contained the same start codon as present in all other PCR products (data not shown). Significantly, the SmInAct gene is also truncated at the 5′ end (Freitas *et al*. 2007), so this is not a feature confined to trichostrongyloid TGF-β homologues.

The truncated N-termini of *Hp*-TGH-2, *Nb*-TGH-2 and *Tc*-TGH-2 also result in the absence of a conventional signal peptide; interestingly, the N-terminus of *Nb*-TGH-2 fulfils the criteria for a predicted signal peptide, although this sequence corresponds to an internal hydrophobic region in the longer homologues (Fig. 3). Notably, a potential N-glycosylation site is present in the active domain of the trichostrongyloid proteins, which is not observed in other family members (Fig. 3). N-glycosylation is known to be important for secretion of mammalian TGF-β (Brunner *et al*. 1992) and it would be interesting to establish if this also pertains to the parasite products.

Phylogenetic analysis

Within the TGF-β superfamily, each of the homologues described here were found by phylogenetic analysis of the conserved C-terminus to group as a closely-spaced set on the same branch as *Bm-tgh-2* and *C. elegans daf-7* (Fig. 4). Morphological characteristics group *H. contortus* and *T. circumcincta* together in the Family Haemonchidae, while *H. polygyrus* and *N. brasiliensis* are placed in separate Families, Heligmosomatidae and Heligmonellidae respectively ((Hoberg and Lichtenfels, 1994) and http://beta.uniprot.org/taxonomy/). Our analysis lends support to this classification, but not at the highest level of confidence because of the limited number of variant positions available for comparison.

Real-time PCR analysis

To assess the expression profile of the novel TGF-β homologues in parasites at different developmental points, cDNA was prepared from eggs, larvae and adult worms of each organism. Larval-stage availability varied between species, but it was possible to analyse L1, L2 and L3 larvae of *H. polygyrus*, L3 and lung L4 larvae of *N. brasiliensis*, L3 and

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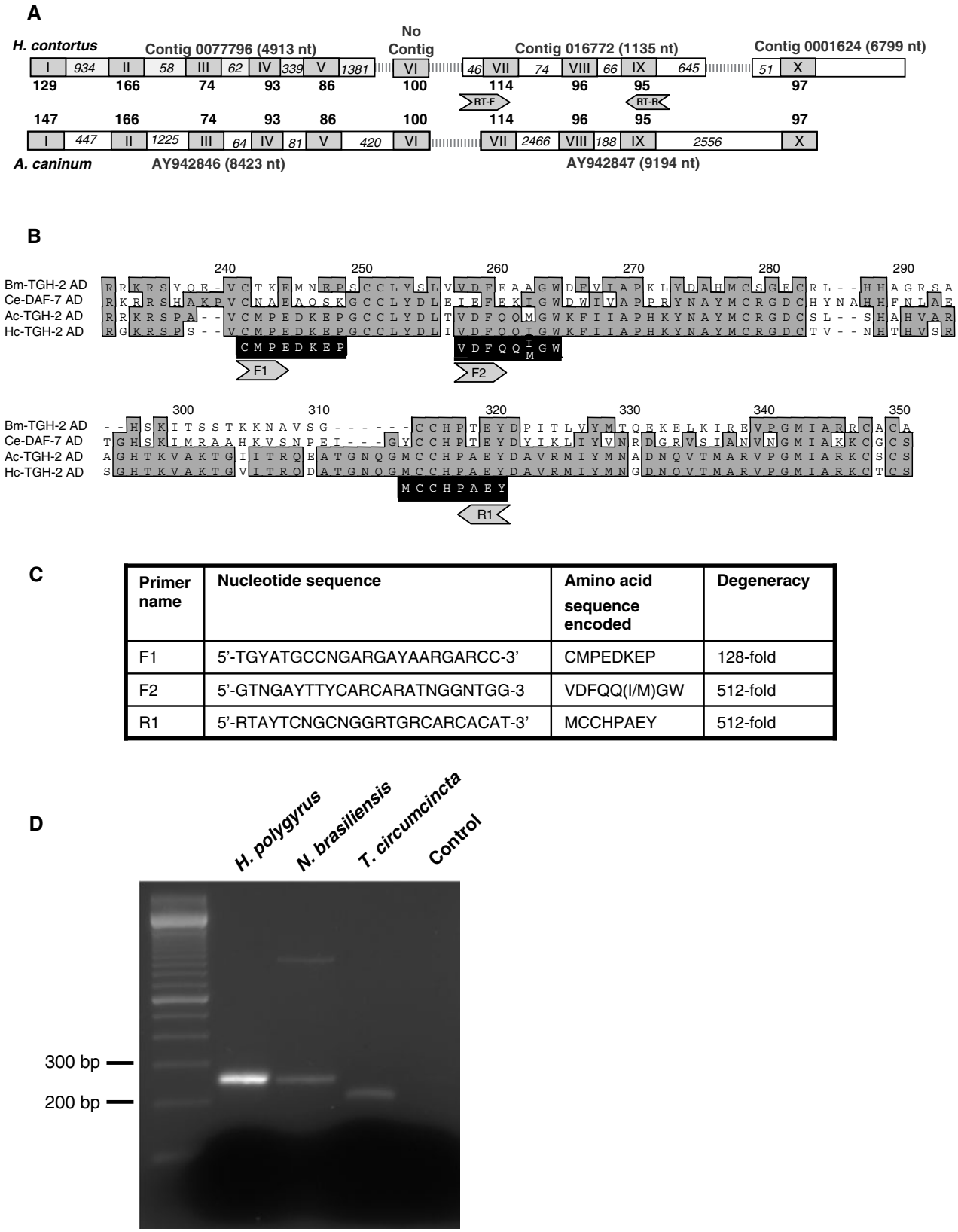

Primer name	Nucleotide sequence	Amino acid sequence encoded	Degeneracy
F1	5'-TGYATGCCNGARGAYAARGARCC-3'	CMPEDKEP	128-fold
F2	5'-GTNGAYTTYCARCARATNGGNTGG-3	VDFQQ(I/M)GW	512-fold
R1	5'-RTAYTCNGCNGGRTGRCARCACAT-3'	MCCHPAEY	512-fold

Fig. 1. Primer design and amplification of *tgh-2* sequences. (A) Schematic of *H. contortus tgh-2* genomic organization, in comparison to the corresponding organization of the *A. caninum* homologue, *Ac-tgh-2*, according to Freitas and Arasu (2005). Based on cDNA sequence of *H. contortus tgh-2* (Accession no. FJ391183), exons were identified on contigs 0077796, 016772 and 001624. Nucleotide lengths of exons (below or above box, bold) and introns (within box, italic) are given. Intron boundaries invariably conformed with the GT....AG consensus. Longer intronic tracts that contain gaps in existing sequence are indicated with broken lines. Note that no genomic sequence yet corresponds with exon VI of *Hc-tgh-2*. The active domain is encoded within Exons VII-X. The positions of forward and reverse primers

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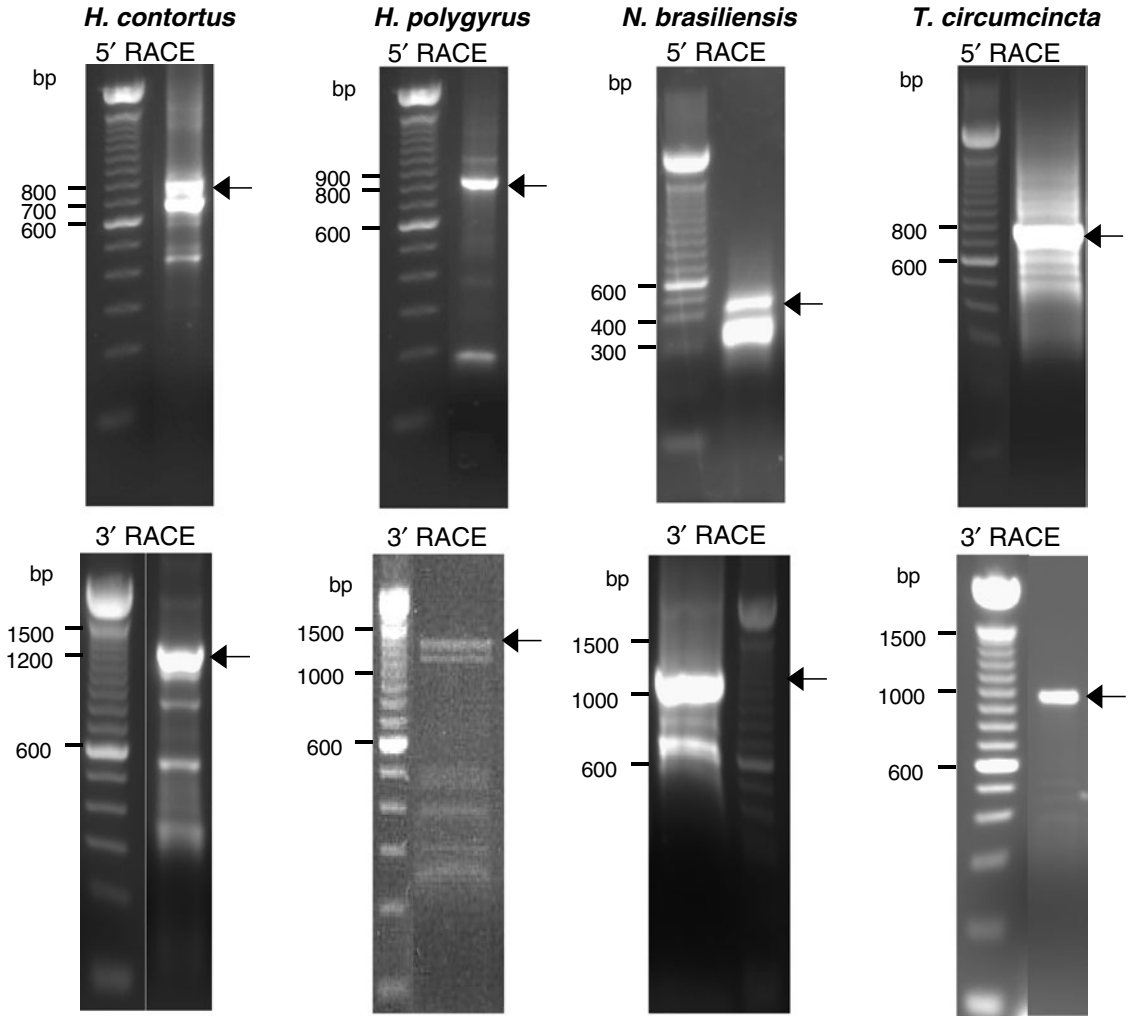

Fig. 2. 5′ and 3′ RACE products of TGF-β homologues. Upper panel: 5′ RACE of *H. contortus*, *H. polygyrus*, *N. brasiliensis* and *T. circumcincta* adult cDNA, amplified and visualized by ethidium bromide staining of agarose gels. Following sequencing, the indicated bands were found to contain *tgh-2*-like sequences. Lower panel: 3′ RACE from the same cDNA samples as above.

exsheathed L3 (xL3) and abomasal L4 of *H. contortus*, and xL3 and abomasal L4 of *T. circumcincta*. Using real-time PCR, *tgh-2* gene transcription was quantified, and normalized by comparison to parasite β-actin gene expression. In *H. polygyrus*, transcription of *Hp-tgh-2* is lowest in the early larval stages, and increases through later stages to reach highest levels in the adults and eggs (Fig. 5A). In contrast, *N. brasiliensis tgh-2* expression was maximal in L3 larvae and eggs, with lower expression in L4 larvae and minimal levels in adults (Fig. 5B). RT-PCR analysis of cDNA from *H. contortus* and *T. circumcincta* also revealed a different developmental pattern of gene expression, the former species showing a larval maximum (Fig. 5C), as has also been shown for *S. ratti* (Crook *et al.* 2005), while in *T. circumcincta* adult stages contained high levels of *tgh-2* transcript (Fig. 5D).

used for RT-PCR analysis (see Fig. 5 below) are indicated beneath the *H. contortus* diagram. (B) Alignment of the active domains (AD) of TGF-β family members from *C. elegans* (*Ce*-DAF-7, AAC47389), *B. malayi* (*Bm*-TGH-2, AF104016), *A. caninum* (*Ac*-TGH-2, AAY79430 and AAX36084, identical depositions), and the newly-identified *H. contortus* (*Hc*-TGH-2, Accession number FJ391183). Numbering corresponds to the *Ce*-DAF-2 sequence. Identical amino acids are shaded, and the amino acid sequences coded by the degenerate primers shown in solid blocks. (C) Degenerate primer sequences used in this study (Y = C/T, R = A/G and N = A/C/G/T). (D) Ethidium bromide gels of degenerate PCR products amplified from *H. polygyrus*, *N. brasiliensis* and *T. circumcincta*. Sizes of marker polynucleotides are given in bp. *H. polygyrus* and *N. brasiliensis* products were amplified using F1 and R1 degenerate primers, while the *T. circumcincta* product was amplified using F2 and R1 degenerate primers as shown in (B).

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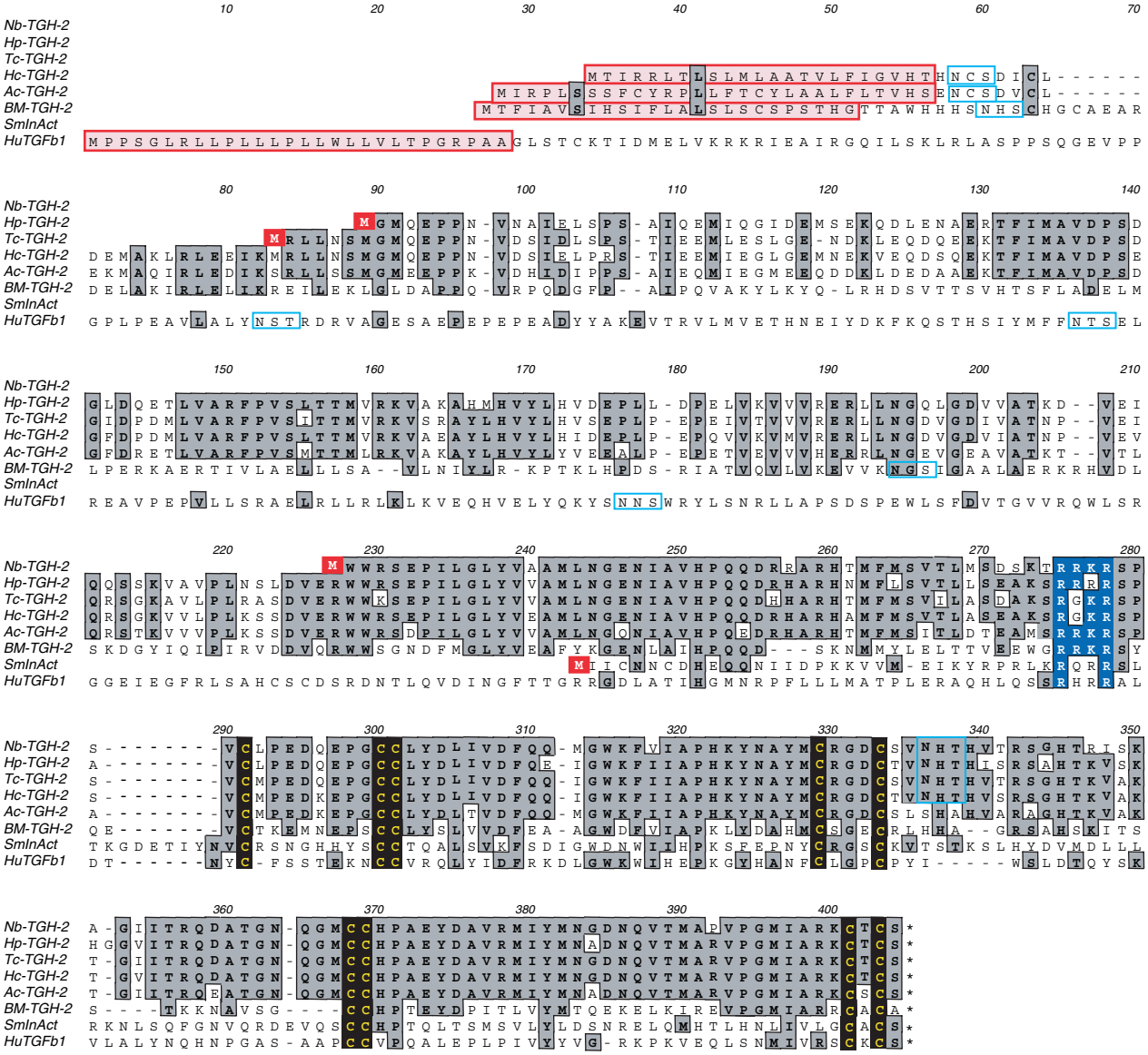

Fig. 3. Full amino acid sequences and alignment of TGF-β homologues. Protein alignments of known TGF-β homologues from *Homo sapiens*, *B. malayi*, *A. caninum*, and novel TGF-β homologues from *H. contortus*, *H. polygyrus*, *N. brasiliensis* and *T. circumcincta*. Numbering corresponds to the human protein sequence. Predicted signal sequences are shown in red boxes, or where no signal sequence is predicted, start methionines are indicated in solid red boxes. Solid blue box indicates tetrabasic protease cleavage site, black boxes indicate conserved cysteines. Open turquoise boxes denote potential N-glycosylation motifs (N-X-S/T). Accession numbers for novel sequences are: *Hp-tgh-2* FJ410912, *Nb-tgh-2* FJ410913 and *Tc-tgh-2* FJ410914.

DISCUSSION

Over recent years, it has become established that members of the TGF-β gene superfamily are required in helminth organisms to direct a suite of developmental processes, most clearly observed in the free-living nematode *C. elegans* (Patterson and Padgett, 2000). Strikingly, the complex signalling pathway, with 2 receptor subunits and intracellular kinases, is conserved from mammals to nematodes (Estevez *et al.* 1993; Gomez-Escobar *et al.* 1997; Newfeld *et al.* 1999), trematodes (Beall and Pearce, 2001; Osman *et al.* 2006; Freitas *et al.* 2007) and cestodes (Zavala-Gongora *et al.* 2006), indicating the potential for molecular cross-talk between evolutionarily distant organisms (Luckhart *et al.* 2003). An important, but unproven, hypothesis is that parasites may have adapted genes first involved in endogenous body organization to interact with the immune system of their host (Gomez-Escobar *et al.* 2000). TGF-β proteins may exemplify this process, if it is the case that helminth homologues can ligate host TGF-β receptors and thereby diminish immune responsiveness.

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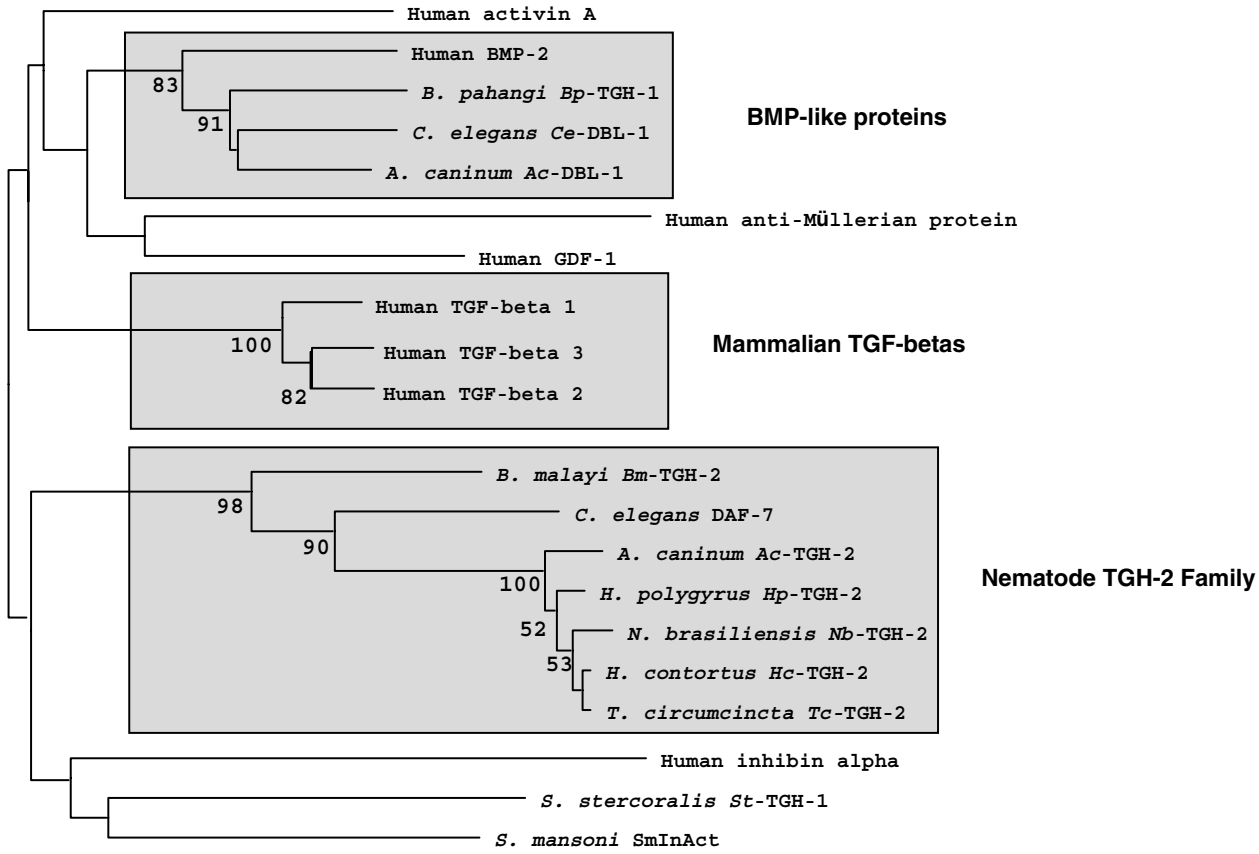

Fig. 4. Phylogenetic tree of TGF-β superfamily members. Neighbour-joining phylogenetic tree of TGF-β superfamily members, generated using Poisson correction on MacVector; numbers indicate bootstrap values (percentage of calculated trees branching at each point, based on 1000 repetitions. Where no numbers are shown, bootstrap values are below 50%.

A number of TGF-β homologues have been characterized from parasitic nematodes, including the human filarial nematode *B. malayi* (Gomez-Escobar *et al*. 1998, 2000), *S. stercoralis* and *S. ratti*, and *P. trichosuri* (Crook *et al*. 2005; Massey *et al*. 2005), as well as the dog hookworm *A. caninum* (Brand *et al*. 2005; Freitas and Arasu, 2005). Some important groups of parasites, however, have not been examined in this regard. For example, *H. polygyrus* and *N. brasiliensis* are species widely used for experimental infections with rodents and known to drive strong regulatory T cell (Wilson *et al*. 2005; Finney *et al*. 2007; Rausch *et al*. 2008) and Th2 differentiation (Lawrence *et al*. 1996; Voehringer *et al*. 2004) respectively in laboratory mice. It is possible that parasite-derived TGF-β family members play a role in this very pronounced skewing of immune responsiveness. Two related nematodes, found worldwide in sheep and other ruminants and responsible for very considerable economic damage, are *H. contortus* and *T. circumcincta*. Identification of TGF-β homologues in these species could pave the way for new strategies to control infections which are proving recalcitrant to drug clearance (Wolstenholme *et al*. 2004). These 4 species are closely related taxonomically within the Trichostrongyloidea superfamily of nematodes, and are each found in specialized habitats within the host gastrointestinal tract.

Our identification of new homologues was made possible, firstly, by the release of substantial genomic sequence from *H. contortus*, and secondly by the availability of sequences from closely related species, most particularly *A. caninum* (Brand *et al*. 2005; Freitas and Arasu, 2005). While the 4 new homologues were found to be highly similar to each other in the conserved active domain, it was surprising to find that 3 of the 4 contained truncated pro-protein N-terminal domains, without conventional signal sequences. Although in *N. brasiliensis* it is possible that the truncated product employs an alternative hydrophobic tract as a signal sequence, no alternative is apparent for either *H. polygyrus* or *T. circumcincta*. However, it has recently been reported that the *S. mansoni* homologue SmInAct, which is a functional TGF-β like protein, is also truncated and lacks a conventional signal sequence (Freitas *et al*. 2007). In the context of signal peptide-independent secretion, it should be noted that in a recent proteomic study of *B. malayi* secreted proteins, some 33% lacked signal peptides, including one of the most abundant secreted molecules (Hewitson *et al*. 2008).

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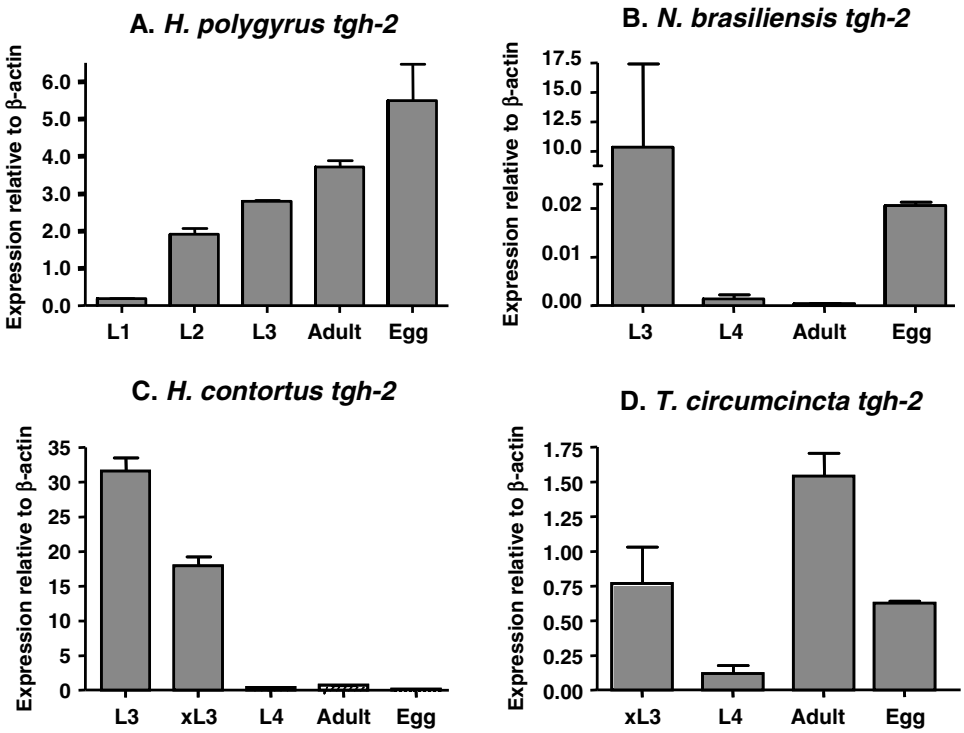

Fig. 5. Expression levels of *tgh-2* transcript in different life-cycle stages. Levels of *tgh-2* and actin transcription were measured by real-time PCR in different developmental stages of *H. polygyrus* (A), *N. brasiliensis* (B), *H. contortus* (C) and *T. circumcincta* (D). The genomic position of the RT-PCR primers used for *H. contortus* are shown in Fig. 1 (RT-F and RT-R). The relative transcription levels were calculated by dividing the relative levels of *tgh-2* transcription by relative levels of actin transcription. Standard errors shown are calculated from replicate determinations of *tgh-2* levels divided by the mean of replicate determinations of actin levels. Relative levels of *T. circumcincta tgh-2* were very similar when β-tubulin was used as the reference gene (data not shown). Data are representative of at least 2 independent experiments in each case.

Studies are now under way to ascertain whether the trichostrongyloid parasites secrete TGH-2 proteins in a signal peptide-independent manner.

A notable level of diversity between the trichostrongyloid species was seen in the patterns of *tgh-2* expression throughout their developmental cycles. Real-time PCR showed that in *N. brasiliensis* and *H. contortus* expression is highest in the arrested L3 larvae, which is similar to the profile reported for *A. caninum*, *P. trichosuri*, *S. ratti* and *S. stercoralis* (Brand *et al.* 2005; Crook *et al.* 2005; Freitas and Arasu, 2005; Massey *et al.* 2005). In contrast, the TGF-β homologue *daf-7* is down-regulated in *C. elegans* Dauer larvae which have entered developmental arrest (Ren *et al.* 1996). Thus, in parasitic species in which larval arrest is constitutive, TGF-β homologues may regulate later developmental steps that are exquisitely dependent on the environmental or immunological cues accompanying infection (Freitas and Arasu, 2005; Viney *et al.* 2005). Interestingly, among these cues may be host TGF-β (Arasu, 2001). In distinction to *N. brasiliensis* and *H. contortus*, 2 other species display continued *tgh-2* expression beyond the infective larval stage. Adult worms of *H. polygyrus* and *T. circumcincta* show high levels of gene transcription, as do the eggs. Because *H. contortus* eggs do not express *tgh-2*, it is difficult to attribute its function in either adult worms or eggs to any fundamental, conserved developmental pathway.

An alternative scenario is that these gene products exert an immunological effect in the host. Thus, expression by infective larvae allows them to release high levels of TGF-β homologue on infection of the host, thereby impairing the host immune response from the very outset. Where, as with *H. polygyrus*, the expression increases through the duration of mammalian infection, this could explain the dominance of regulatory T-cell activity in mice harbouring adult worms of this parasite (Wilson *et al.* 2005; Finney *et al.* 2007; Rausch *et al.* 2008). It is interesting to speculate whether the failure of *N. brasiliensis* adult worms to establish beyond 6–8 days in the murine gut is related to the absence of TGF-β homologue expression by this stage of the parasite.

These alternative hypotheses now require experimental investigation. While conventional genetic knockout techniques cannot be used with helminth parasites, RNAi offers a promising route to study individual gene function, and most recently

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knock-down of the *S. mansoni* TGF-β family member SmInAct confirmed its role in parasite development (Freitas *et al*. 2007). Although progress with RNAi on trichostrongyloid nematodes has been limited (Hussein *et al*. 2002; Geldhof *et al*. 2006; Zawadzki *et al*. 2006; Lendner *et al*. 2008), in some instances this technique has been successful (Kotze and Bagnall, 2006) indicating that it may be possible to knock down TGF-β expression in a species such as *H. contortus*. Further, heterologous transfection of helminth genes into other organisms such as *Leishmania* has permitted the immunological function of individual genes to be elucidated (Gomez-Escobar *et al*. 2005; Maizels *et al*. 2008). We are pursuing these and other means of establishing the biological role of TGF-β family members in these important helminth parasite species.

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