# The isolation of differentially expressed cDNA clones from the filarial nematode *Brugia pahangi*†

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SUMMARY

A cDNA library constructed from 3 day post-infective L3 of the filarial nematode *Brugia pahangi* was screened by differential hybridization with cDNA probes prepared from different life-cycle stages. Five cDNA clones hybridizing selectively to the mosquito-derived L3 probe were isolated and characterized. Northern blot analysis of 4 of the clones confirmed that each was most highly expressed in the mosquito-derived L3. The expression of each mRNA during parasite development in the mosquito vector was investigated using RT–PCR, and all were shown to be abundant in the immature L3. Four of the 5 cDNAs cloned coded for structural proteins: 2 cuticular collagens, and the muscle proteins tropomyosin and troponin. Further studies on troponin using an antiserum raised to the recombinant protein demonstrated that the protein, unlike the mRNA, was present in all life-cycle stages examined, while immunogold labelling demonstrated that it was localized to the muscle blocks.

Key words: *Brugia pahangi*, L3, differentially expressed genes, troponin, tropomyosin, cuticular collagens.

## INTRODUCTION

Filarial nematodes are blood and tissue-dwelling parasites, which are transmitted by arthropod vectors. The human lymphatic filarial nematodes *Wuchereria bancrofti*, *Brugia malayi* and *Brugia timori*, infect an estimated 120 million persons world-wide (Michael, Bundy & Grenfell, 1996) and cause a significant degree of morbidity in infected individuals. Studies in animal models have shown that immunization with the L3, the infective form for the mammalian host, induces significant levels of protective immunity (Oothuman *et al.* 1979; Bancroft & Devaney, 1993). Furthermore, data from field studies support the concept that the L3 is the target of protective immune responses (Day, Gregory & Maizels, 1991), while epidemiological data suggest the development of an age-dependent immune response in endemic areas (Vanamail *et al.* 1989). However, the antigens responsible for eliciting protective immune responses have not been identified.

Many of the *Brugia* antigens characterized to date are shared between different life-cycle stages, while the antigens involved in protective immunity are thought to be L3-specific (Maizels & Lawrence, 1991). For this reason, we have attempted to isolate cDNAs which are highly expressed in the L3 stage of the life-cycle, with the long-term aim of determining whether any of these genes encode proteins with protective potential. Several previous studies have sought to identify L3-specific antigens (Lal & Ottesen, 1988). This is not an easy task because of the small numbers of L3 usually available, and most efforts have focused on studying surface components of the parasites (Carlow *et al.* 1987; Devaney & Jecock, 1991). In addition, until recently few cDNA libraries from the L3 were available. In this paper we describe the isolation of 5 genes from a cDNA library prepared from 3 day post-infective (p.i.) L3 of *Brugia pahangi* (Martin *et al.* 1996). The library was screened by differential hybridization with cDNA probes prepared from a variety of life-cycle stages. In this study the clones which hybridized selectively to the mosquito-derived L3 probe were picked and further characterized. Four of the differentially expressed cDNAs were characterized and their expression patterns confirmed in representative life-cycle stages.

## MATERIALS AND METHODS

### *Parasites*

Adults, microfilariae and a range of different post-infective life-cycle stages were isolated from the peritoneal cavity of infected jirds. The parasites were washed in HBSS (Gibco/BRL) at 37 °C and stored in liquid nitrogen. Vector-derived L3 were harvested

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† Nucleotide sequence data reported in this paper are available in the EMBL and GenBank™ data bases under the accession numbers: SJ1 AJ224966; SJ5 AJ224967; clone A AJ130821; clone C AJ224968; clone F AJ224969; clone EE4 X91066.

 

at room temperature from infected mosquitoes in Graces Insect TC Medium (Gibco/BRL) by the mass harvesting technique (Ash, 1974).

*RNA isolation*

Total RNA was extracted from mf, adult and post-infective larval stages using a modified hot TRIzol™ method (Gibco/BRL) as described previously (Martin, Thompson & Devaney, 1995). RNA was extracted from infected mosquito thoraces (24 h, day 3 and day 8 p.i.) by a similar method, except in this case the RNA lysis buffer contained 2 % $\beta$-mercaptoethanol.

*cDNA library construction*

The construction of the 3 day p.i. L3 library has been described by Martin *et al*. (1996). In brief, RT–PCR using the conserved nematode spliced leader and oligo dT primers was used to generate the input cDNA. The library was screened by differential hybridization with cDNA probes generated from mosquito-derived L3, 3 day p.i. L3 and adult parasites. Triplicate filter lifts were screened exactly as described by Martin *et al*. (1996). The positive *Bluescript™* plasmids were recovered by *in vivo* excision (Stratagene) and the insert sizes determined by restriction digestion.

*Sequencing reactions*

Double-stranded sequencing was performed using the SequiTherm EXCEL™ II Long-Read™ DNA Sequencing Kit-LC (Epicentre Technologies, Cambio) and fluorescently labelled (IRD 800) M13 reverse and T7 primers (MWG-Biotech) on an automated sequencer (Li-cor).

*Sequence analysis*

Partial nucleotide sequences were searched for homology against the EMBL, Genbank and FilGenNet data bases using the FASTA search programme of the GCG package. Translated amino acid sequences were searched for in the SWISS PROT data bank using the same programme.

*PCR amplification*

First strand cDNA was generated from 2 $\mu$g of total RNA using Superscript II™ and conditions recommended by Gibco/BRL. Reverse transcription was primed with an oligo dT primer ($^{5'}$**GCCG**CTCGAG$T_{17}^{3'}$) (500 $\mu$g/ml). PCR reactions were performed on 2 $\mu$l of a 1:20 dilution of each first strand cDNA sample. The reactions contained 200 $\mu$M of each dNTP, 5 $\mu$l 10 × *Amplitaq*™ DNA polymerase buffer, 1·25 units *Amplitaq*™ DNA polymerase (all Perkin Elmer) in a final volume of 50 $\mu$l. Gene-specific primers (final concentration 20 ng/$\mu$l) were designed for tropomyosin TR1F 5′ CTAAAGCAAAAATGGATGCGATCAAG 3′ and TR3R 5′ TCCAAATTAGTATTTGCAACAGC 3′, and for troponin ER1F 5′ GATTGCATAGAGAGAATGGC 3′ and ER3R 5′ GCTTCAGTTTGTTCAGCCGG 3′. For Clone C the cuticular collagen precursor PC1F 5′ CGAAACAGCTTCTCATTGAGGC 3′ and PC3R 5′ CCAATTGCGCGGGTCCTGC 3′, for Clone F the cuticular collagen CC1F 5′ GGAGTTGGATACATCCATGG 3′ and CC3R 5′ GCATGTATCATCATCATCCGG 3′.

A constitutive ribosomal protein gene was amplified using primers (R-forward: 5′ GCATTGTTCTCAAATAGAGC 3′, R-reverse: 5′ CCCATATCATCATCTGATTCC 3′). Preliminary reactions showed that 23 rounds of PCR gave conditions where reagents were not exhausted. Cycling was performed as follows: 94 °C, 1 min, 55 °C, 1 min, 72 °C, 3 min, with a final extension at 72 °C for 10 min. PCR reactions were separated on 2 % gels and blotted on to nylon membrane (Genetic Research Instrumentation Ltd). Blots were pre-hybridized at 65 °C for 4 h in 20 ml of pre-hybridization solution (5 × SSC, 5 × Denhardt's solution, 0·5 % SDS containing 20 mg/ml of denatured salmon sperm DNA). The inserts from the positive clones were excised by restriction digest and gel purified by standard procedures. These fragments were used to generate probes using High Prime™ (BCL). The probes were denatured by boiling for 10 min, added to the bottles containing pre-hybridization solution ($1 \times 10^7$ cpm/ml) and hybridized at 65 °C overnight. Blots were washed at 65 °C to 0·1 × SSC, 0·5 % SDS and the resulting autoradiographs were used as templates to excise the corresponding filter spots. The counts from each filter were measured and the relative abundance of the test gene was expressed as a ratio relative to the expression level of the control gene in each different life-cycle stage.

*Northern blot analysis*

Two $\mu$g of total RNA was separated on a 1·2 % denaturing formaldehyde gel and transferred to nylon membrane in 20 × SSC. Following UV cross-linking the blots were pre-hybridized and probed at 65 °C as described above. The blots were washed to 0·2 × SSC, 0·5 % SDS at 65 °C and exposed to X-ray film.

*Antibody production*

A 420 bp fragment of the SJ5 troponin cDNA was amplified by PCR using primers sj5pf 5′ CCATGGATCCATGGCAGACGAAGAAGAAGAG 3′ and sj5pr 5′ CCATGGTACCTTATCGCAACTCTTCTTCGAATCTG 3′. This fragment was then

digested using *Bam*HI and *Kpn*I (Gibco/BRL) and subcloned into the PQE30 expression vector (Qiagen). The recombinant protein was purified through a Ni-NTA resin column (Qiagen) and then used to raise an antibody in rabbits, by standard immunization protocols.

### *Western blot analysis*

Equivalent amounts of L3, L4 and adult SDS sample cocktail extracts were run out on 12·5 % SDS–PAGE gels. The separated proteins were then transferred onto nitrocellulose paper (Gelman) by immunoblotting. The blot was stained with Ponceau S and cut into strips before non-specific antibody binding sites were blocked with TBS/Tween 20/5 % BSA. Individual strips were probed with control or test rabbit serum at 1 : 200. Goat anti-rabbit alkaline phosphatase conjugate (ICN) was used at a dilution of 1 : 6000 and then the blot was developed using BCIP/NBT substrate (Sigma). Washing with TBS/Tween 20 was carried out between each incubation, (3 × 15 min, RT).

### *Immunoelectron microscopy*

Larvae and adult worms were fixed in freshly prepared 4 % (w/v) paraformaldehyde, 0·1 % glutaraldehyde in phosphate-buffered saline (PBS), pH 7·4 at 4 °C for 30 min. Larvae were concentrated to a loose pellet, embedded in low temperature gelling agarose at 37 °C, and brought to 4 °C. Parasites were cold processed (modified from Bannister & Kent, 1992) to 100 % ethanol at −20 °C, then infiltrated with increasing concentrations of LR resin and ethanol at −20 °C. This was followed by 100 % resin for 8 h at 20 °C and infiltration overnight with agitation prior to polymerization at 20 °C under 365 nm indirect UV light for 2 days. Ultrathin sections were blocked using 0·2 M glycine and 1 % acetylated bovine serum albumin (Aurion) in PBS (PBS/aBSA), then incubated for 30 min in a 1 in 100 dilution of primary antibody/PBS + aBSA followed by a 1 in 10 dilution of goat-anti-rabbit 10 nm gold conjugate (Aurion)/PBS + aBSA buffer (Griffiths, 1993). Sections were briefly counterstained in 0·5 % uranyl acetate and images examined and photographed using a Zeiss 902 EFTEM.

## RESULTS

### *Isolation of differentially expressed cDNAs*

Approximately 20000 plaques from the 3 day p.i. L3 library (Martin *et al*. 1996) were screened in triplicate using cDNA probes prepared from mosquito-derived L3, 3 day p.i. L3 and adult parasites. Plaques which selectively hybridized to the mosquito-derived L3 cDNA, but not to the adult cDNA, and which were very faint with the 3 day p.i. L3 probe were picked for further analysis. After taking positive plaques through to a tertiary screen, 14 clones were isolated, all of which contained inserts. Two clones contained inserts of ∼ 100 bp and were not characterized further. Cross-hybridization, restriction digest and sequence analysis demonstrated that 5 different genes were represented by the remaining 12 clones. Three of the clones (SJ1) contained inserts which showed homology to nematode muscle tropomyosin mRNA (Frenkel *et al*. 1989), while 6 (SJ5) shared homology with the muscle protein troponin from a variety of species.

Of the 3 remaining clones, clone A contained a 700 bp insert, which at the time that this study was carried out, showed no homology to any other sequences in the database and consequently was not further characterized. More recent analysis on clone A has shown a high degree of homology to sequences from other filarial nematodes. These include a *B. malayi* cDNA (AF072679), an *O. volvulus* cDNA (U77675) and a cDNA (U29533) corresponding to a protein expressed in the dog heartworm *Dirofilaria immitis*. The *D. immitis* protein is a secreted molecule found in L3/L4 ES products. Clone C was homologous to a cuticular collagen precursor gene (*col-12*) from *C. elegans* (Park & Kramer, 1990) and clone F shared homology with *col-1*, another cuticular collagen gene from *C. elegans* (Kramer, Cox & Hirsch, 1982).

### *Characterization of the L3 cDNAs*

The longest of the *Brugia* tropomyosin inserts was 303 bp (SJ1). This region showed 98 % identity with a *B. malayi* EST and shared a high degree of homology with full length sequences for tropomyosin from *A. viteae* (86 % identical), and *T. colubriformis* (80 % identical). As with all the other cDNAs isolated during the course of this work, clone SJ1 contained the nematode spliced leader sequence (SL1) at the 5′ end. However, none of the differentially expressed cDNAs isolated were full-length; in all cases a run of A's, but no polyadenylation signal, was present towards the 3′ end of each gene. This presumably relates to the ability of the oligo dT primer, used in the library construction, to anneal to A-rich sequences in the nematode genome (Joshua & Hsieh, 1995; Martin *et al*. 1996). Northern blot analysis of SJ1 revealed a transcript size of 2·3 kb, while the pattern of hybridization of SJ1 on Southern blots suggested that it belongs to a small multi-gene family (data not shown). Attempts were made to obtain a longer length tropomyosin clone by PCR on different cDNA libraries, but this approach yielded no further sequence information.

The longest troponin sequence obtained was 415 bp (SJ5), while the transcript size from Northern blots was 1·8 kb. The clone contains many GAA

Table 1. The characteristics of the cDNA clones isolated

| Gene | Accession no.* | Insert (bp)/ Transcript† (kb) | Homology | Identity at nucleotide level (%)‡ | | |
|---|---|---|---|---|---|---|
| SJ1 | AJ224966 | 303/2·3 | Tropomyosin | *B. malayi* (EST $L_3$) | AA585557 | 98 % |
| | | | | *A. viteae* (cds) | AF000607 | 86 % |
| | | | | *T. colubriformis* (cds) | JO4669 | 80 % |
| SJ5 | AJ224967 | 404/1·84 | Troponin | *B. malayi* (cds) | AI234402 | 93 % |
| | | | | *O. volvulus* (EST $L_3$) | AI205465 | 81 % |
| | | | | *C. elegans* (cds) | U44759 | 60 % |
| A | AJ130821 | 700/N.D. | 24 kDa Secreted protein P22U P22U mRNA | *B. malayi* (cds $L_3$) | AF072679 | 95 % |
| | | | | *D. immitis* (cds) | U29533 | 72 % |
| | | | | *O. volvulus* (cds) | U77675 | 70 % |
| C | AJ224968 | 294/1·7 | Collagen 12/13 | *B. malayi* (EST $L_3$) | U80975 | 99 % |
| | | | | *C. elegans* (12) (cds) | X51622 | 63 % |
| | | | | *C. elegans* (13) (cds) | X51623 | 63 % |
| | | | | *O. circumcincta* (cds) | X96732 | 63 % |
| F | AJ224969 | 436/2·0 | Collagen 1 | *B. malayi* (EST $L_3$) | AA280451 | 96 % |
| | | | | *C. elegans* (cds) | J01047 | 76 % |
| EE4 | X91066 | 560/0·56 | 60S Ribosomal Protein P1 | *D. melanogaster* (cds) | Y00504 | 60 % |

* Accession numbers in Genbank.
† Transcript size determined from Northern blot.
N.D., Not determined.
‡ EST, expressed sequence tags; cds, coding sequence.

repeats, coding for glutamic acid (E), and in fact 33 % of the predicted partial protein sequence comprised glutamic acid. The clone shares homology with other genes which are rich in glutamic acid residues, such as the GARP gene from *Plasmodium falciparum* (Triglia *et al*. 1988) and a number of troponins and neurofilament proteins from a variety of different species (Smillie, Golosinska & Reinach, 1988). As with the tropomyosin gene, the highest homology is with other nematode genes which most likely encode troponins (see Table 1). The *C. elegans* homologue codes for the troponin T isoform, which is 60 % identical to the *Brugia* troponin. Southern blots suggest that SJ5 is a single copy gene (data not shown).

Clones C and F both code for cuticular collagens. Clone C shows 63 % identity to cDNAs encoding cuticle collagens 12 and 13 from *C. elegans*, since these two proteins are identical when mature (Park & Kramer, 1990). The *Brugia* clone does not contain the characteristic Gly-X-Y- repeats; presumably this region would have been found had a longer clone been obtained. Only 294 bp of clone C were obtained while the transcript size from Northern blots is 1·7 kb. As expected from Southern blots, both clones C and F hybridized to a number of bands in each digest, suggestive of a multi-gene family (data not shown). Clone F is 436 bp long and shares a 76 % identity with a cDNA thought to encode cuticle collagen 1 from *C. elegans*. This clone contained the characteristic Gly-X-Y repeats. Northern blots indicated a transcript size of 2·0 kb for clone F. The characteristics of the cDNAs cloned are summarized in Table 1.

### *Identification of a constitutively expressed cDNA*

Since it was important to be able to verify the expression patterns of the differentially expressed genes by Northern blotting, a constitutively expressed cDNA was required for comparison. In order to identify such a clone, the initial screening of the cDNA library was repeated as described previously, except that this time plaques which appeared to hybridize equally to all 3 cDNA probes were selected. After secondary screening, 6 clones were picked, 4 of which contained inserts of sufficient size to warrant sequencing. Of these only 1 (EE4) contained an insert which showed reasonable homology to other sequences in the data bases. This clone coded for a ribosomal protein with significant homology to a *Drosophila* 60S ribosomal protein P1 gene (Wigboldus, 1987). In contrast to each of the differentially expressed cDNAs isolated in this study, EE4 was full length. The insert size was 560 bp, as was the transcript size observed by Northern blot.

### *Confirmation of the expression pattern of the L3 cDNAs*

The expression pattern of each of the cDNA clones isolated was analysed by Northern blotting of total

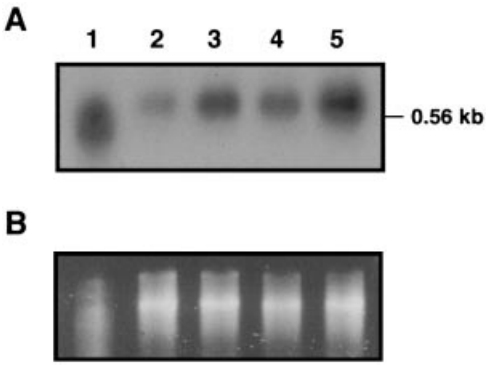


Fig. 1. Northern blot analysis of the constitutive ribosomal protein gene. RNA was isolated from different life-cycles stages of *Brugia pahangi* as described in the Materials and Methods section. Lane 1, mf RNA; Lane 2, mosquito-derived L3 RNA; Lane 3, 3 day p.i. L3 RNA; Lane 4, 15 day p.i. L4 RNA; Lane 5, Adult RNA. (A) Hybridization pattern obtained with the labelled ribosomal protein gene cDNA probe. The size of the transcript is indicated in kb on the right-hand side. (B) Ethidium bromide-stained gel before blotting.

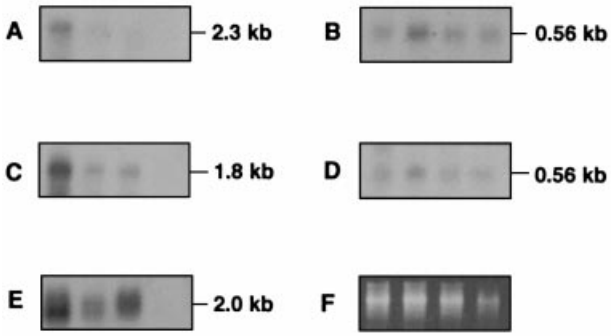


Fig. 2. Northern blot analysis of the differentially expressed clones. *Brugia pahangi* RNA was isolated from different life-cycle stages as described previously. Lane 1, mosquito-derived L3 RNA; Lane 2, 3 day p.i. L3 RNA; Lane 3, 15 day p.i. L4 RNA; Lane 4, Adult RNA. Northern blots were probed with tropomyosin (A), troponin (C) or clone F (E). (A and C) Stripped and re-probed with the labelled ribosomal protein gene, shown in B and D respectively. (F) Ethidium bromide-stained gel corresponding to E. The size of the transcript is indicated in kb on the right-hand side of A–E.

RNA isolated from different life-cycle stages of the parasite. In most cases RNA was obtained from mosquito-derived L3, 3 day p.i. L3, 15 day p.i. L4 and adult parasites, as described in the Materials and Methods section. To confirm the expression profile of the constitutive gene a Northern blot was carried out with the ribosomal protein probe; in this experiment RNA isolated from the microfilarial stage of the life-cycle was also included. A representative blot, shown in Fig. 1, demonstrates that the ribosomal protein mRNA is expressed in all life-cycle stages examined. The corresponding ethidium bromide stained gel is shown in the lower panel (Fig. 1). The probe hybridizes to a single transcript of $\sim 0{\cdot}56$ kb; the apparent slight difference in transcript size in the microfilariae lane probably reflects partial degradation of this RNA sample (panel B, lane 1).

Fig. 2 shows a composite of Northern blots for 3 of the differentially expressed cDNAs. In the case of the tropomyosin (panel A) and troponin genes (panel C), the strongest signal was obtained from the mosquito-derived L3 with minimal signals from the other life-cycle stages. RNA was present in all the lanes as demonstrated by the signal obtained when both blots were re-probed with the ribosomal protein gene (panels B and D respectively). The pattern of expression of the two cuticular collagen mRNAs was different from the other genes isolated. Both collagens (clones C and F) were highly expressed in mosquito-derived L3 and in 15 day p.i. L4 parasites, with a reduced level of expression in 3 day p.i. L3 and no signal apparent in the adult RNA. Clone F is shown in panel E whilst panel F is the corresponding ethidium stained gel for this blot. These results confirmed that the differential screen had been successful in isolating cDNAs which were up-regulated in the vector-derived L3.

The cluster analysis (Blaxter *et al*. 1999) shown in Table 2, (kindly provided by Dr M. Blaxter), shows the percentage of each *B. malayi* EST data set which corresponds to the *B. pahangi* gene studied. These results, by and large, confirm the Northern blot analysis of the different *B. pahangi* life-cycle stages. The *B. malayi* homologues of 4 of the 5 differentially expressed *B. pahangi* genes are most abundant in the L3 EST data set, while tropomyosin is most abundant in the *B. malayi* L4 EST data set. The constitutive ribosomal protein gene is expressed in all data sets.

### *Expression of the L3 genes during parasite development in the mosquito*

As all the genes isolated in this study were highly expressed in the mosquito-derived L3, it was of interest to examine their expression pattern during development to the L3 in the mosquito vector. For these experiments, total RNA was isolated from the thoraces of infected mosquitoes at different time-points p.i., as described in the Materials and Methods section. RT–PCR was carried out using gene-specific primers and the abundance of each of the differentially expressed mRNAs was expressed relative to that of the ribosomal protein mRNA for each life-cycle stage tested. As well as developmental stages in the mosquito, vector-derived L3, 3 day p.i. L3, 15 day p.i. L4 and adult parasites were analysed. This analysis was repeated on at least 2 separate occasions with similar results. Fig. 3 shows an example of the relative abundance of tropomyosin (A) and troponin (B) mRNAs compared to the

Table 2. Percentage of each *Brugia malayi* EST set which corresponds to the *B. pahangi* genes

| Bp acc. no.* | Protein | Bm gene | Bm cluster | All ESTs (%) | MF (%) | L2 (%) | L3 (%) | L4† (%) | AM (%) | AF (%) |
|---|---|---|---|---|---|---|---|---|---|---|
| AJ224966 | Tropomyosin | *tmy-1* | BMC04766 | 0·01 | 0 | 0 | 0 | 1·0 | 0 | 0 |
| AJ224967 | Troponin | *tin-1* | BMC00135 | 0·2 | 0·1 | 0 | 0·7 | 0·1 | 0 | 0 |
| AJ130821 | Alt | *alt* | BMC03432 | 0·2 | 0 | 0 | 0·7 | 0 | 0·1 | 0 |
| AJ224968 | Collagen | *col-12* | BMC00164 | 0·1 | 0 | 0 | 0·5 | 0·1 | 0 | 0 |
| AJ224969 | Collagen | *col-2* | BMC00160 | 0·04 | 0 | 0·2 | 0·1 | 0 | 0 | 0 |
| X91066 | Ribosomal | *rpp-1* | BMC00166 | 0·6 | 0·4 | 1·0 | 0·6 | 1·2 | 0·4 | 0·3 |

* Bp, *Brugia pahangi*; Bm, *Brugia malayi*; AM, adult male; AF, adult female; MF, microfilaria.
† L4 SL1 dataset only.

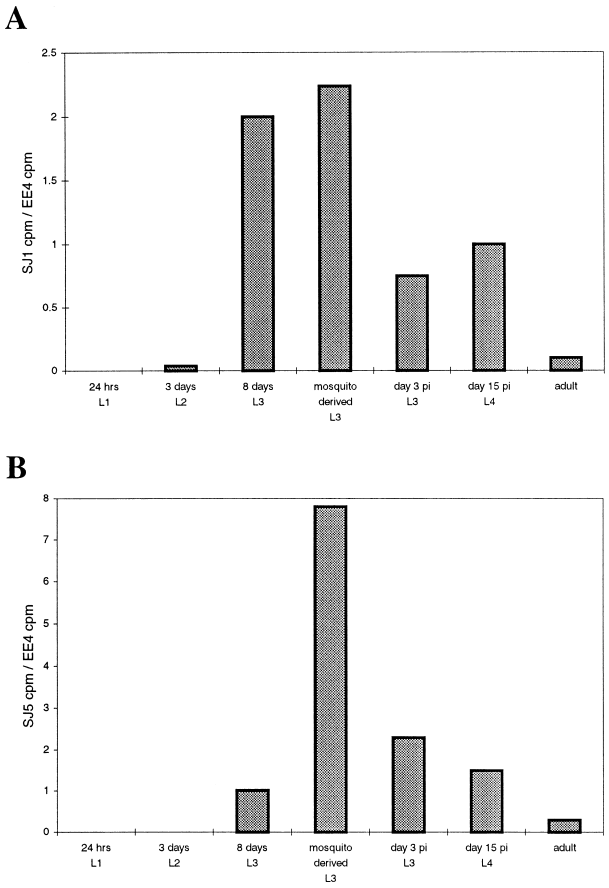


Fig. 3. Expression patterns of the *Brugia pahangi* tropomyosin and troponin genes by RT–PCR. The graphs show the ratio of the expression of (A) tropomyosin and (B) troponin compared to that of the constitutively expressed ribosomal protein gene at various points throughout the nematode life-cycle, determined as described in the Materials and Methods section.

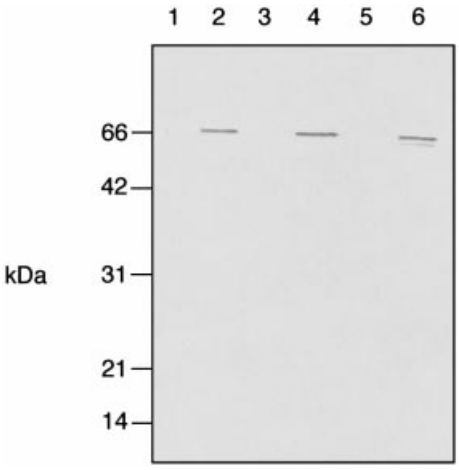


Fig. 4. Western blot analysis of troponin expression. *Brugia pahangi* L3 (Lanes 1 and 2), L4 (Lanes 3 and 4) and adult (Lanes 5 and 6) worms were used to make SDS extracts. The protein extracts were separated on a 12·5 % SDS polyacrylamide gel, blotted onto nitrocellulose paper and then incubated in either pre-bleed (Lanes 1, 3 and 5) or anti-troponin serum (Lanes 2, 4 and 6) at a 1/200 dilution. The sizes of the molecular weight standards are indicated in kDa on the left-hand side of the blot.

constitutively expressed ribosomal protein mRNA. Significant levels of expression were only detected for both tropomyosin and troponin at day 8 p.i., by which time the parasites are immature L3. In general the results obtained with the mammalian stages of the life-cycle were similar to those obtained by Northern blot, in that very low levels of expression of all the mRNAs were detected in adult worms. Transcripts for one of the cuticular collagens (clone C) were detectable at 24 h p.i. of the mosquito but, as with the tropomyosin and troponin genes, significant levels of expression were only detected at day 8 p.i. (data not shown).

### *Further analysis of the* Brugia *troponin*

A 420 bp fragment containing the partial *Brugia* troponin cDNA was amplified and expressed in a pQE30 (Qiagen) vector, with the aim of raising a polyclonal antiserum to the recombinant protein. As similar E-rich proteins had been identified during the course of the *B. malayi* genome sequencing project, and appeared to be particularly well represented in cDNA libraries prepared from the L3 stage of the life-cycle (Blaxter *et al*. 1996), it was of interest to further characterize one member of the family. While the results of the Northern blot demonstrated that the troponin mRNA was differentially

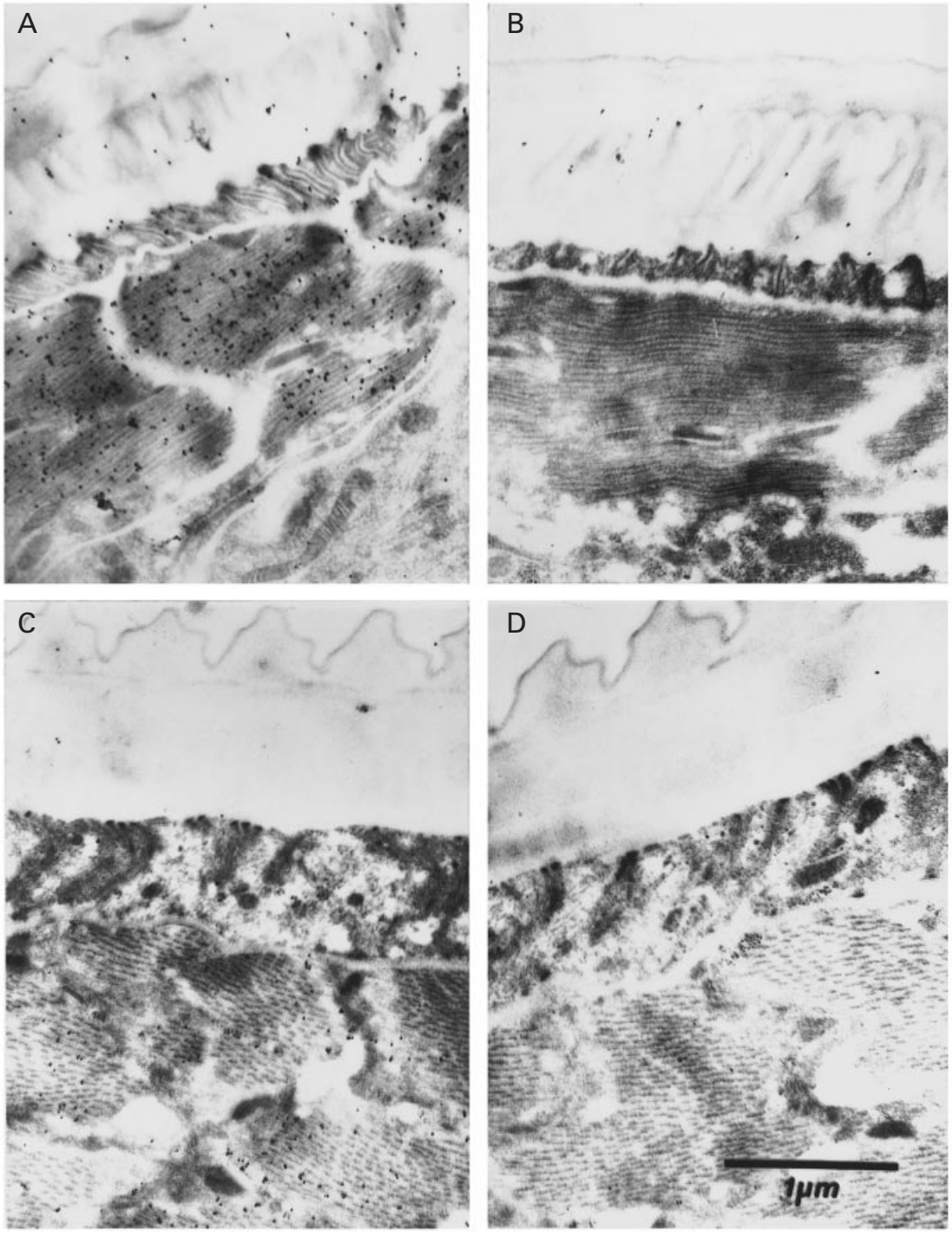


Fig. 5. Immunoelectron micrographs showing the distribution of troponin in *Brugia pahangi*. Day 6 p.i. L3 (A and B) or adult worms (C and D) were prepared using the low-temperature embedding protocol, as described in the Materials and Methods section. Sections were labelled with rabbit anti-troponin antibody (A and C) or the corresponding pre-bleeds (B and D) at a 1/100 dilution. The distribution of bound antibody was visualized using 10 nm goat anti-rabbit gold conjugate.

expressed, analysis of the pattern of protein expression, by Western blot, showed that the protein was present in each life-cycle stage tested. The polyclonal antiserum bound to a single band at 66 kDa (Fig. 4), the correct predicted size for the mRNA transcript of 1·8 kb. Immunogold labelling on sections of day 6 p.i. L3 (Fig. 5, panels A and B) and adult parasites (panels C and D) further confirmed the identity of the E-rich protein cDNA as a muscle protein. As can be seen in the figure, the gold particles specifically labelled the muscle fibres in the body wall muscle blocks of both the larval and adult parasites (panels A and C, respectively). Sections incubated with the pre-bleed from the same rabbit, showed only a background level of staining (panels B and D).

## DISCUSSION

Each of the 4 clones characterized in this study was highly expressed in the mosquito-derived L3 of *B. pahangi*. Although the cDNA library from which these clones were isolated was prepared by RT–PCR on 3 day p.i. L3, the differential screening approach proved successful in isolating cDNAs which were up-regulated in the vector-derived L3. Presumably this reflects the fact that the library contains genes which are expressed throughout the L3 stage of the life-cycle, as well as genes which are up-regulated in the p.i. L3. Although none of the genes were full length it was possible to assign identities to them all. Interestingly, 4 of the 5 differentially expressed cDNAs encoded structural proteins of the worm, a finding which may reflect the fact that the mosquito-derived L3 are developmentally blocked until transmission to the mammalian host. mRNAs coding for structural proteins may be relatively more abundant in the L3 compared to the p.i. L3, in which expression of many new mRNAs will be required for survival/adaptation to the mammalian host. In contrast to these results, when the same library was screened using a similar protocol to identify cDNAs up-regulated in the 3 day p.i. L3, only 1 of 4 genes isolated showed homology to known sequences (Martin *et al*. 1996). An additional screen was carried out with the aim of identifying a cDNA clone which was expressed in all life-cycle stages and which could be used as a constitutive control for Northern blot analysis and semi-quantitative RT–PCR. Clone EE4 was isolated for this purpose and was shown to be expressed in all life-cycle stages tested. The same gene was previously identified in this laboratory by screening an adult *Brugia* cDNA library with an antiserum raised to the L3 (S. J. Hunter, unpublished observations). For each of the genes isolated, a corresponding *B. malayi* EST exists. In all cases the most homologous EST derives from a gene expressed in the L3 stage of the life-cycle.

By Northern blot analysis it was possible to confirm the expression pattern of the genes isolated. As predicted from the screening procedure, each was more highly expressed in the mosquito-derived L3. The mRNAs for the collagen genes were also abundant in day 15 p.i. L4, as well as in the mosquito-derived L3, but transcripts could not be detected in the adult parasite. A similar collagen gene (*Bm-col-2*) has been cloned from a *B. malayi* SL-1 L3 cDNA library and was also shown to be transcribed at higher levels in the larval stages than in the adult worm (Scott *et al*. 1995). Studies on the free-living nematode *C. elegans* have demonstrated that expression of different collagen genes peaks at different times of the inter-moult period and that the collagen genes expressed at each moult may vary (Johnstone & Barry, 1996). The L3 of *B. pahangi* moult to the L4 around day 6, while male worms commence the L4 to adult moult around day 19 (Schacher, 1962). Thus the *Brugia* collagen genes cloned in this study appear to be expressed well in advance of the moult. The absence of detectable collagen transcripts in the adult parasite confirms the results of Selkirk *et al*. (1989) who demonstrated that synthesis of cuticular collagens takes place at a very low level in the adult worm.

Two of the differentially expressed genes coded for the muscle proteins, tropomyosin and troponin. Tropomyosin has been relatively well characterized from other nematodes because of its potential as a protective antigen. O'Donnell *et al*. (1989) reported that a detergent extract of *T. colubriformis* L3, enriched in tropomyosin, stimulated host immunity in a guinea pig model. Studies in a mouse model of *Onchocerca lienalis* infection, demonstrated that immunization with an *O. volvulus* tropomyosin–MBP fusion protein resulted in a significant reduction in microfilariae levels in the skin (Taylor, Jenkins & Bianco, 1996). More recent data suggest that antibody responses to tropomyosin are correlated with reduced mf densities in the skin of *O. volvulus* patients (Jenkins *et al*. 1998).

Tropomyosin interacts during muscle contraction with troponin, which was also isolated in the current screen. Both tropomyosin and troponin would be expected to be expressed in muscle cells from all life-cycle stages of *Brugia*. Additional studies in which a fragment of the troponin cDNA was expressed as a recombinant protein and used to make a polyclonal antiserum, revealed that the protein was indeed present in all life-cycle stages examined (L3, L4 and adult worms). Thus the pattern of expression of the mRNA differs from that of the protein, as the Northern blot and RT–PCR analyses revealed only a minimal troponin signal in adult worms. Presumably the troponin mRNA is not continuously transcribed throughout the life-cycle, and periods of synthesis may preceed periods of growth, as with the collagens. Immunogold localization using the polyclonal antiserum, demonstrated that troponin was localized to the muscle blocks in both life-cycle stages examined.

The present study also demonstrated that it was possible to detect the *Brugia* genes by RT–PCR during parasite development in the mosquito. Although previous workers have developed PCR methods for detecting filarial-infected mosquitoes for use in epidemiological studies these have relied on the amplification of a highly repeated DNA sequence, which is estimated to account for approximately 12% of the *Brugia* genome (McReynolds, DeSimone & Williams, 1986). In this study, RT–PCR was carried out on total RNA isolated from the mosquito thorax, in which mosquito mRNA molecules must greatly outnumber filarial-derived mRNAs. This method does not give actual expression levels of the mRNA but rather gives a measure of the relative abundance of a particular

mRNA species compared to a constitutively expressed gene (Johnstone & Barry, 1996). Although a signal could be obtained with the early developmental stages of the parasite in the mosquito, the strongest signal with each of the genes was obtained with immature L3 at day 8 p.i. This confirms the data of others (Bianco *et al*. 1990; Ibrahim, Richie & Scott, 1992) who demonstrated that many L3-specific proteins in filarial nematodes are synthesized during parasite development in the mosquito, in readiness for the transfer to the mammalian host.

Although 4 of the 5 genes cloned in this study coded for structural proteins, it has proved possible to identify *Brugia* transcripts which are highly expressed in the mosquito-derived L3 using the differential screening approach. Vector-derived L3 cDNA libraries are now available through the filarial genome sequencing consortium (Blaxter *et al*. 1996), and are yielding a large number of potentially interesting cDNA clones for further study.

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