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A novel cDNA clone encoding a specific excretory/secretory antigen of larval *Trichinella pseudospiralis*

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Abstract A cDNA library for *Trichinella pseudospiralis* was constructed to study the expression of specific antigens. Four positive clones were identified using antibodies against the excretory/secretory (ES) products of the nematode as probe. Sequence analysis showed that they contained identical cDNA inserts of 606 bp, including a 5′ non-translated region of 96 bp, a core translated segment of 408 bp and a poly(A)$^+$ 3′ terminus. It encoded a novel 136-amino-acid polypeptide. Southern blot analysis indicated that the cDNA did not cross-hybridize to the genomic digests of *T. spiralis*, mouse, or rat. A single copy only of its complementary sequence was found in the genome of *T. pseudospiralis*. Using the lambda ZAP expression system, the cDNA was induced to express a 23-kDa β-galactosidase-fusion protein which did not cross-react with polyclonal and monoclonal antibodies against *T. spiralis*, heat shock proteins, or four heterologous species of nematodes. The antiserum against the fusion protein recognized a 15-kDa band from the ES products of *T. pseudospiralis* in immunoblotting. Immunocytolocalization demonstrated that the anti-fusion protein serum only recognized an epitope in the stichosome of *T. pseudospiralis* and not in *T. spiralis*. The protein can therefore serve as a specific antigen for the differential diagnosis of trichinellosis.

Introduction

Trichinella pseudospiralis is a relatively new species which was only officially recognized as distinct from *T. spiralis* at the 7th International Congress of Parasitology held in Paris in 1990. The parasite was originally recovered from a raccoon (*Procyon lotor*) by Garkavi (1972). The two trichinellids are closely related, with a similar morphology and habitat. Both worms can invade the small intestine and striated muscles of mammals, including humans. Although trichinellosis caused by *T. spiralis* is commonly documented, the first case of human infection by *T. pseudospiralis* was only reported in New Zealand in 1991 (Andrews et al. 1994). Since then, outbreaks have been reported in Thailand and Russia (information from the International Commission on Trichinellosis). At present, it is not possible to distinguish the two types of trichinellosis by serological methods.

Specific antigens have been isolated from the excretory/secretory (ES) products of *T. spiralis* by various authors (Gamble 1985; Ko 1997; Leung and Ko 1997). Genes encoding 43-, 46-, 49- and 53-kDa immunodominant antigens of *T. spiralis* have been characterized (Su et al. 1991; Sugane and Matsuura 1990; Zarlenga and Gamble 1990; Vassilatis et al. 1992). Antibodies raised against the 43 fusion proteins reacted strongly with the stichocytes of the infective-stage larvae. Besides being useful in serodiagnosis, the ES molecules of *T. spiralis* have also been suggested to play a role in reprogramming the genomic expression of host muscles (Lee et al. 1991; Ko et al. 1994), induction and maintenance of nurse cells (Lee and Shivers 1987; Despommier 1990), development of the encysting worm, and degradation of muscle-specific proteins (Jasmer 1990; Ko et al. 1994).

Although the ES proteins of *T. pseudospiralis* have been suggested to be involved in modifying the tubular systems of host muscles (Garbryel et al. 1978), and in immunosuppression (Alkarmi and Faubert 1981; Stewart et al. 1985, 1988), their usefulness in serodiagnosis is not known. The antigenic composition of the ES products has not been well defined. The present study was undertaken to identify and clone a specific antigen which can provide a differential diagnosis of *T. pseudospiralis* infection.

Materials and methods

Source of worms

The *T. pseudospiralis* used in the experiment was originally established in the Department of Biology, University of Leeds, UK. The

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strain of *T. spiralis* was isolated by R.C. Ko from a pig in Ontario, Canada, in 1967. The worms were maintained in the laboratory in ICR mice and Wistar rats.

Infective-stage larvae were recovered from muscles of experimentally infected mice (about 30 days post-infection) by the standard pepsin digestion method. After recovery, the worms were washed three times with saline (0.85% NaCl) before they were given orally to ICR mice (6 week old) using a Pasteur pipette.

Isolation of mRNA

Infective-stage larvae recovered from mice were frozen quickly in liquid nitrogen, and then stored at −70 °C. Approximately 20,000 frozen worms, which were suspended in denaturing solution (4 *M* guanidinium thiocyanate, 25 m*M* sodium citrate, pH 7.0, 0.1 *M* 2-mercaptoethanol, 0.5% w/v *N*-lauroylsarcosine), were broken into fine pieces using a high-speed homogenizer (Polytron T25). The homogenate was thoroughly mixed with 1 ml sodium acetate (2 *M*, pH 4.0) and 1 ml of water-saturated phenol was added. After incubation for 15 min at 4 °C, the mixture was centrifuged for 20 min at 4 °C and 12,000 rpm. The RNA was precipitated by adding an equal volume of ice-cold isopropanol. It was then recovered by centrifugation for 15 min at 4 °C and 12,000 rpm. It was washed in 75% ethanol, air-dried and resuspended in DEPC-treated water and stored at −70 °C. Poly(A)$^+$ RNA was purified from total RNA using the PolyATract according to the protocol provided by Promega (USA). Streptavidin-paramagnetic particles were used to capture oligo(dT)-mRNA hybrids.

The absorbance of the extracted RNA was measured at 260 and 280 nm using a spectrophotometer (Hitachi 2000). RNA preparations, with an OD_{260}/OD_{280} of 1.8 or above, were used for experimentation. The quality of the RNA was also monitored by formaldehyde gel electrophoresis (Lehrach et al. 1977).

Construction of cDNA libraries

cDNA libraries were prepared using a ZAP-cDNA synthesis kit (Stratagene), following the protocol provided. The sizes of the single- and double-stranded cDNAs were determined using alkaline gel electrophoresis under denaturing conditions. Since the newly synthesized cDNA did not possess *Eco*RI and *Xho*I termini, they had to be linker-modified before being ligated to Uni-ZAP XR vector. Blunt or ''non-sticky'' cDNA termini were obtained using the end-filling approach. The vector-attached cDNAs were packaged in Gigapack II Gold packaging extract (Stratagene), and amplified in *Escherichia coli* XL1-Blue MRF′ strain. The transformants were grown in LB medium containing $MgSO_4$ (10 m*M*) and ampicillin (100 μg/ml).

The cDNA libraries were titered, plated, and screened, with some modifications of the protocols supplied with the kit. The library was plated on XL1-Blue *E. coli*, and approximately 10^5 plaques were screened in duplicate, using antiserum against the ES products of *T. pseudospiralis* (TpES) as probe and alkaline-phosphatase as conjugate. All positive clones were rescreened until a single plaque could be isolated. Positive clones were counter-screened with antiserum against the ES products of *T. spiralis* (TsES). Clones that were confirmed immunoreactive to anti-TpES (but not anti-TsES) sera were selected, resuspended in 1 ml of sterile SM buffer (50 m*M* Tris-HCl, pH 7.5, 100 m*M* NaCl, 10 m*M* $MgSO_4$), and stored at 4 °C with 50 μl of chloroform.

Plasmids were excised from the Uni-ZAP XR phage vector (Stratagene) under in vivo conditions. Excision was carried out by mixing 10^5 recombinant phages with ExAssist helper phage (10^6 pfu/ml) and freshly prepared XL1-Blue MRF cells (OD_{600} = 1.0). The excised phagemids were transformed to the *E. coli* SOLA strain, and were maintained on LB plates containing ampicillin (50 μg/ml).

Plasmid DNA containing the cDNA insert was isolated and purified using the Qiagen plasmid isolation kit. The nucleotide sequence of the cDNA clones was determined using the dideoxy chain-termination method (Sanger et al. 1977) with a Sequenase DNA Sequencing Kit and a Bio-Rad sequencing apparatus.

Genomic blot

Approximately 20,000 frozen infective-stage larvae were homogenized in 2 ml of lysis buffer (50 m*M* Tris-HCl, pH 8.0; 100 m*M* EDTA; 100 m*M* NaCl; 1% w/v SDS). Proteinase K was added to a final concentration of 100 μg/ml. The homogenate was digested for 20 h at 55 °C, before the DNA was extracted once with phenol, followed twice by phenol/chloroform (pH 8.0, 1:1 v/v). The ethanol-precipitated DNA, which was removed using a Pasteur pipette, was washed in 70% ethanol and resuspended in TE (10 m*M* Tris-HCl, pH 7.5, 1 m*M* EDTA) containing 20 μg/ml RNase. It was then incubated for 2 h at 37 °C. The DNA was further extracted twice with phenol, precipitated with ethanol, and resuspended in TE.

Genomic DNA (10 μg) isolated from *T. pseudospiralis*, *T. spiralis*, mouse, and rat was restricted with various restriction endonucleases (*Xho*I *Eco*RI, *Bam*HI, *Hind*III, *Bgl*II, *Sal*I, *Eco*RV, *Not*I and *Bss*HII) overnight at 37 °C. The restricted products were separated on a 0.8% agarose gel before being transferred onto nylon membranes (Boehringer Mannheim), using 0.4 *M* NaOH as transfer buffer. The blots were prehybridized for 1 h at 68 °C, and then hybridized overnight with DIG-labelled cDNA at the same temperature. After hybridization, the membranes were washed twice (5 min each) with 2× saline sodium citrate (SSC) and 0.1% (w/v) SDS. After two washes (15 min each) with 0.1× SSC and 0.1% (w/v) SDS at 68 °C, the membranes were developed using alkaline phosphatase.

Expression and analysis of fusion protein

Transformants were grown in 100 ml of LB medium with ampicillin at 37 °C until an A_{600} of 0.4 was obtained. Isopropyl-1-thio-β-*D*-galactopyranoside was then added to a final concentration of 0.5 m*M*. The cells were incubated for 3 h at 37 °C. The bacterial lysate was separated by SDS-PAGE under reducing conditions. The proteins were stained with Coomassie blue (Sigma).

The antigenicity of the fusion product was studied by electro-immunotransfer blotting (EITB) (described in more detail below). The following were used as developing antibodies: polyclonal and monoclonal antibodies (mAbs) against somatic (C) and ES antigens of *T. pseudospiralis* (Tp) and *T. spiralis* (Ts); heterologous antisera against *Trichuris suis*, *Metastrongylus apri*, *Stephanurus dentatus*, and *Gnathostoma hispidum*. The polyclonal antibodies were produced in rabbits according to Ko and Yeung (1989). Four of the five mAbs used (1H7, 3B2E6, 1D11, 5D11) were produced in our laboratory (Lee et al. 1990; hispidum 1991). $7C_2C_5$, originally produced by Gamble and Graham (1984), was purchased from the American Type Cell Collection. The latter mAb and 1H7 were IgM antibodies whereas all the others were IgG_1. All mAbs recognized the 45-, 49- and 53-kDa specific antigens in the ES products of the infective-stage larvae of *T. spiralis*. Anti-β-galactosidase mAb (Gibco) served as the control. Bacterial lysate without IPTG induction served as the negative control.

Since heat shock proteins (HSPs) have been reported in the ES products of *T. spiralis* and *T. pseudospiralis* (Ko and Fan 1996), the fusion product was also tested using mAbs against HSP 25, 60, 70 and 90 families (Sigma).

Production of polyclonal antibodies against the fusion protein

Sixteen-week-old, male New Zealand White rabbits were injected intramuscularly with a mixture of an equal volume of 1 mg of the bacterial lysate containing the fusion product and complete Freund's adjuvant. Two additional booster injections of 1 mg of protein in incomplete Freund's adjuvant were given to the animals at 30-day intervals. Pre-immune serum was collected prior to the immunizations. Sera were kept at −20 °C until use.

The specificity of the antiserum against the fusion protein was studied by EITB in a mini-transblot cell (Bio-Rad). Four micrograms of somatic extract or ES product of *T. spiralis* and *T. pseudospiralis* was first separated by SDS-PAGE under reducing conditions. After electrophoresis, the gel was equilibrated for 30 min with a 4 °C transfer buffer [25 m*M* Tris, 192 m*M* glycine, 20% (v/v) methanol, pH 8.3]. Nitrocellulose membranes (0.45 μm pore size, Scheicher and Schnell), filter papers (Whatman no.1), and fiber pads were saturated for 30 min in the same buffer. Transfer was carried out at 110-V constant voltage (250/2.5 power supply, Bio-Rad) for 1 h. The paper strips were exposed for 2 h to 10 ml of antiserum (diluted 1/200) or mAb (neat) in PBS. They were then incubated for 2 h in horseradish peroxidase (HRPO) conjugated to goat anti-rabbit/mouse IgG (Sigma, diluted 1/4,000) before development, using 0.3% (w/v) 4-chloro-1-naphthol (Sigma) as substrate.

Immunocytolocalization

The immunoperoxidase method was used. Paraffin sections (6 μm in thickness) of mouse diaphragm infected with *T. pseudospiralis* and *T. spiralis* were dewaxed in xylene. The tissue sections were given a final rinse in distilled water before adding 3% normal goat serum to block non-specific binding. Anti-fusion protein serum (1:64 and 1:128 dilutions) was applied to the sections for overnight incubation at 4 °C. HRPO-conjugated anti-rabbit IgG (Sigma) (1:360 dilution) was allowed to react for 1 h at room temperature before adding the substrate (diaminobenzidine tetrahydrochloride, Sigma). The following served as negative controls: *T. spiralis* sections, normal rabbit serum, and antiserum against bacterial lysate.

Results

Screening of library and sequence analysis

The cDNA library was screened using anti-TpES sera as probe. After tertiary screening, four clones were confirmed as immunoreactive. The four positive clones, designated Tp3-1, were found to contain the same nucleotide sequence. The encoded amino acids are shown in Fig. 1. A start codon, ATG, was identified at nucleotides 97–99. It was translated into methionine, the leader amino acid of the mature polypeptide. A terminator, or 'nonsense' codon (TGA) was further located at nucleotides 505–507.

The nucleotide sequence of the 43-kDa (Vassilatis et al. 1992; GenBank M95499), 49-kDa (Su et al. 1991; GenBank M37729) and 53-kDa (Zarlenga and Gamble 1990; GenBank M33386) proteins of *T. spiralis* were compared with that of the clone obtained in this study by 'BestFit homology search' using the GCG programme. The clone shared no sequence homology with the main bodies of these proteins. BLAST searches revealed a homology over a stretch of around 20 nucleotides between the cDNA and a number of nucleotide sequences in GenBank: *Borrelia burgdorfei* (GenBank AE001123, AE001158), *Onchocerca volvulus* (GenBank U00693) and *Caenorhabditis elegans* (GenBank U58754, AF016415, U60059).

Genomic analysis (Southern blot)

The genomic DNA of *T. pseudospiralis* after digestion with various endonucleases is shown in Fig. 2. More distinctive banding patterns were observed in the samples digested by *Eco*RI, *Hind*III, *Bgl*II and *Eco*RV. The 606-bp cDNA insert was used as probe in hybridization experiments to determine the occurrence of complementary sequences in various genomic DNAs. The DIG-labelled cDNA probe hybridized extensively to the genomic fragments of *T. pseudospiralis* (Fig. 3). These fragments included the 7-, 15-, 16- and 10-kb bands from the *Eco*RI, *Bgl*II, *Sal*I and *Eco*RV digests, respectively. Two hybridizing bands of 2.8 and 10 kbp were also detected in the *Hind*III digests. The probe failed to hybridize to the genomic DNAs of *T. spiralis*, rat, and mouse.

Immunoblotting and characterization of fusion protein

A comparison of the antigenic profiles of both ES products and crude somatic extracts of *T. pseudospiralis*

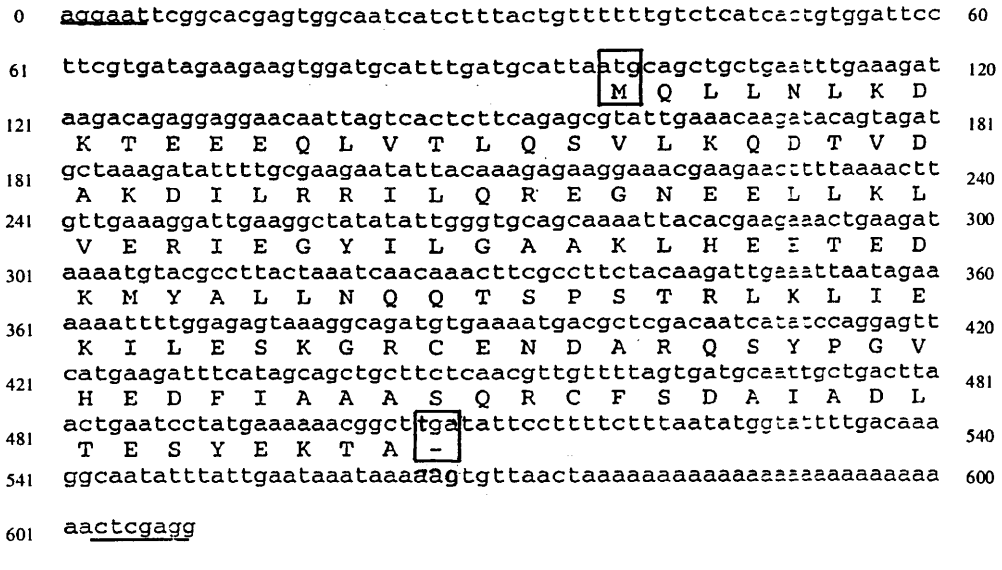

Fig. 1 Nucleotide sequence of the positive clone (Tp3-1) isolated from a *Trichinella pseudospiralis* cDNA library and its predicted amino acid sequence. The cloning sites are *underlined*. The start and stop codons are indicated by the *boxes*

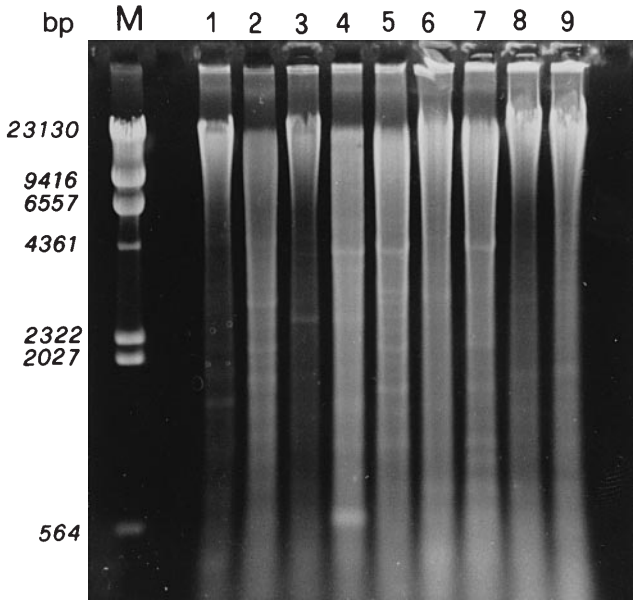

Fig. 2 Agarose gel showing the genomic DNA of *T. pseudospiralis* after digestion by various restriction endonucleases: *lane 1 Xho*I; *2 Eco*RI; *3 Bam*HI; *4 Hind*III; *5 Bgl*II; *6 Sal*I; *7 Eco*RV; *8 Not*I; *9 BssH*II. DNA was stained with ethidium bromide

and *T. spiralis* by immunoblotting (using both polyclonal and monoclonal antibodies) showed that the two species share numerous conserved epitopes (Figs. 4, 5). However, the ES products *of T. pseudospiralis* contains at least one major band (M_r < 20 kDa) which is absent in the *T. spiralis* samples.

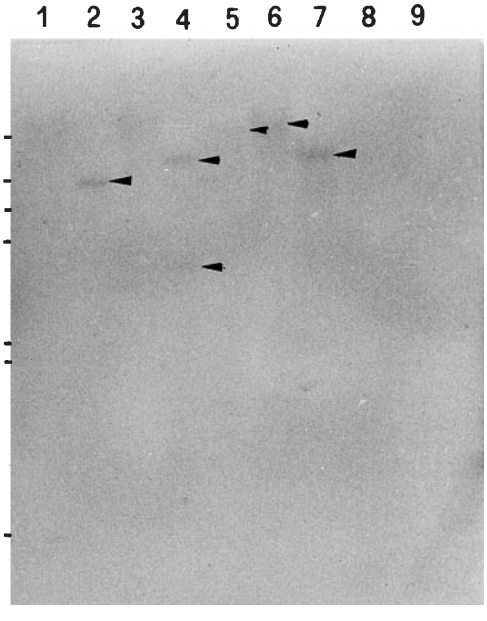

Fig. 3 Southern blot hybridization of the genomic DNA, using cDNA as probe. Bands are present in *lane 2* (*Eco*RI, 7 kb), *4* (*Hind*III, 2.8 and 10 kb), *5* (*Bgl*II, 15 kb), *6* (*Sal*I, 16 kb), and *7* (*Eco*RV, 10 kb) (*arrowheads*). *Dashes on left* indicate positions of molecular markers, from top to bottom: 23,130, 9,416, 6,557, 4,361, 2,322, 2,207 and 564 bp

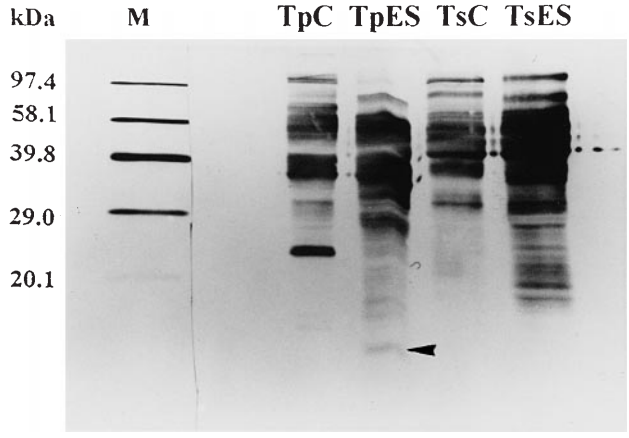

Fig. 4 Immunoblots of crude somatic worm extracts and excretory/secretory products (*ES*) of *T. pseudospiralis* (i.e., *TpC*, *TpES*) and *T. spiralis* (i.e., *TsC*, *TsES*). Antiserum against ES products of *T. pseudospiralis* was used as developing antibody. Note the presence of a discrete band in TpES with M_r < 20 kDa (*arrowhead*)

When the fusion product was analyzed using anti-*T. pseudospiralis* ES serum as the developing antibody, a distinct band of 23 kDa was observed in the bacterial lysate with the recombinant plasmid at 1 and 2 h after chemical induction. However, negative reactions were observed in the samples with bacterial cells only, and in transformed cells with non-recombinant plasmids (Fig. 6).

The 23-kDa protein (in lysate) was further tested using various mAbs and polyclonal antisera as developing antibodies. Of the four polyclonal antibodies (anti-TpES, TpC, TsES, and TsC) used, only the first was able to recognize the 23-kDa protein after chemical induction. Anti-TsES failed to detect any epitope from the lysate. Both anti-TpC and TsC recognized a clear band of 50 kDa before the induction of host cells. The

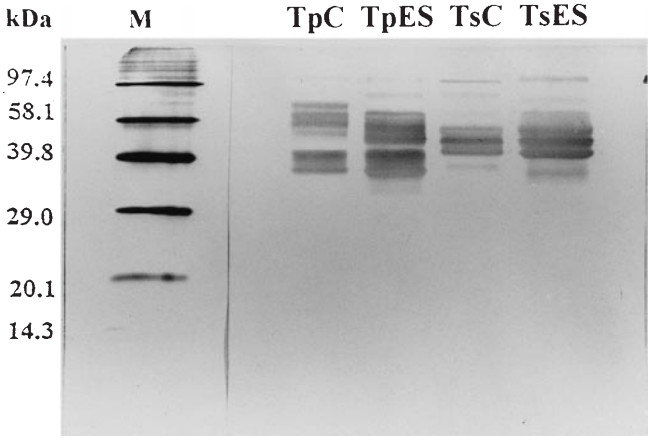

Fig. 5 Immunoblots of crude somatic worm extracts and excretory/secretory products (*ES*) of *T. pseudsopiralis* (i.e., *TpC*, *TpES*) and *T. spiralis* (i.e., *TsC*, *TsES*) using monclonal antibody $7C_2C_5$ as developing antibodies. Note the presence of two to five cross-reactive epitopes (M_r > 37 kDa) in the *T. pseudospiralis* and *T. spiralis* samples

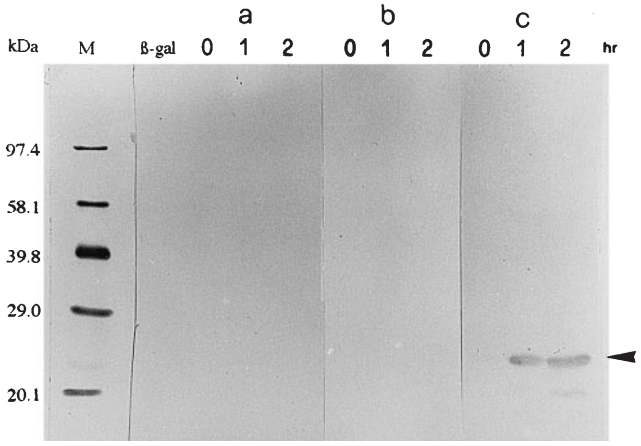

Fig. 6 Immunoblots of fusion protein, using antiserum against excretory/secretory products of *T. pseudospiralis* as developing antibody, before (0 h) and after (1 h, 2 h) IPTG induction: lysate from non-transformed bacterial cells (*a*); lysate from transformed bacterial cells with non-recombinant plasmid (*b*); lysates from bacterial cells with recombinant plasmid (*c*) (β-gal β-galactosidase protein; *M* markers)

band, however, was absent in the induced samples. Monoclonal antibodies against *T. spiralis* (i.e., 7C2C5, 3B2E6, 1D11, 1H7, and 5D11) failed to recognize the 23-kDa fusion product in the lysate. mAbs against HSPs of 25, 60, 70, and 90 kDa also yielded negative reactions. Heterologous antisera against *T. suis*, *M. apri*, *S. dentatus*, and *G. hispidum* sera did not cross-react with the 23-kDa protein (data not shown).

When the anti-fusion protein serum was used to develop the following trichinellid antigens, i.e., TpES, TpC, TsEs and TsC, a band of 15 kDa was observed only in the TpES sample. The remaining antigens yielded negative reactions (Fig. 7).

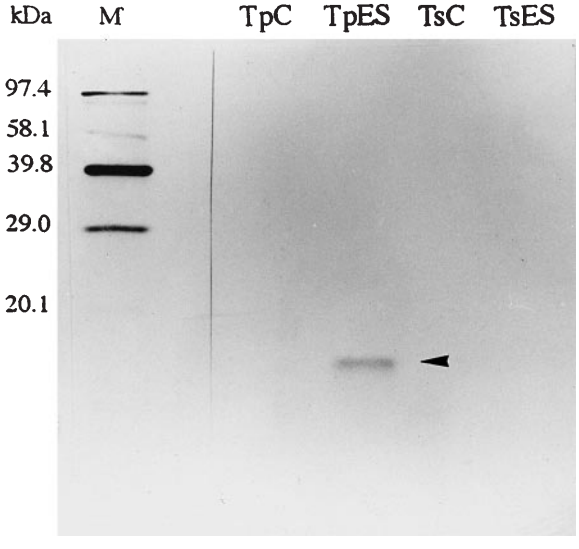

Fig. 7 Immunoblots of crude worm extracts (*C*) and excretory/secretory products (*ES*) of *T. pseudospiralis* (*Tp*) and *T. spiralis* (*Ts*) using the anti-fusion protein serum as developing antibody. Note the presence of a 15-kDa band (*arrowhead*) in the TpES lane

Immunocytolocalization

When the anti-fusion protein serum was used as developing antibody, positive reactions were observed in some sections of the stichosome of *T. pseudospiralis* (Fig. 8a). Muscles adjacent to the worms were also weakly stained. However, negative reactions were observed when the anti-fusion protein serum was applied to sections of *T. spiralis* (Fig. 8b), or when normal rabbit serum and anti-bacterial lysate serum were used as developing antibodies.

Discussion

In this study, a recombinant antigen which possesses an active epitope specific to *T. pseudospiralis* was successfully produced. The antigen did not cross-react with the antisera against *T. spiralis* and various heterologous nematodes. It can therefore be used for the differential diagnosis of trichinellosis.

The cloned protein is likely to be a secretory molecule. The immunoblotting result shows that when anti-fusion protein serum was used as developing antibody, a band was only observed in the ES products sample. The result of the immunocytolocalization study also confirms that the antigen is a specific molecule of *T. pseudospiralis* because its epitope was not detected in *T. spiralis*. Since positive reactions were observed only in some regions of the stichosome of *T. pseudospiralis*, the secretion of this antigen is probably restricted to a specific cell type, e.g., α or β. However, further studies are required to elucidate the biological functions and origin of the specific antigen.

The cDNA isolated from *T. pseudospiralis,* consisting of 606 bp, shares no sequence homology with those of the *T. spiralis* antigens reported previously. The reading frame extends from the start codon (methionine) at nucleotides 97–99 to a stop codon at nucleotides 505–507. It begins with a 5′ non-translated region of 96 bp, followed by a core translated sequence of about 408 bp and ends with a 3′ poly(A)$^+$ tail. The ATG (nucleotide position 97–99) reading frame was chosen because translation using two other frames resulted in early terminations, thus producing only short peptides (less than 30 amino acids). Moreover, immunoblotting (using anti-fusion protein as probe) identified a specific 15-kDa protein in the ES products of *T. pseudospiralis*, whose size matches with this frame.

The amino acid sequence of the cDNA-derived polypeptide was analyzed using the PSORT program. BLAST searches revealed a 100% homology over a stretch of 20 nucleotides between the cDNA and several nucleotide sequences in GenBank, e.g., *B. burgdorfei* (GenBank AE001123, AE001158), *O. volvulus* (Gen-

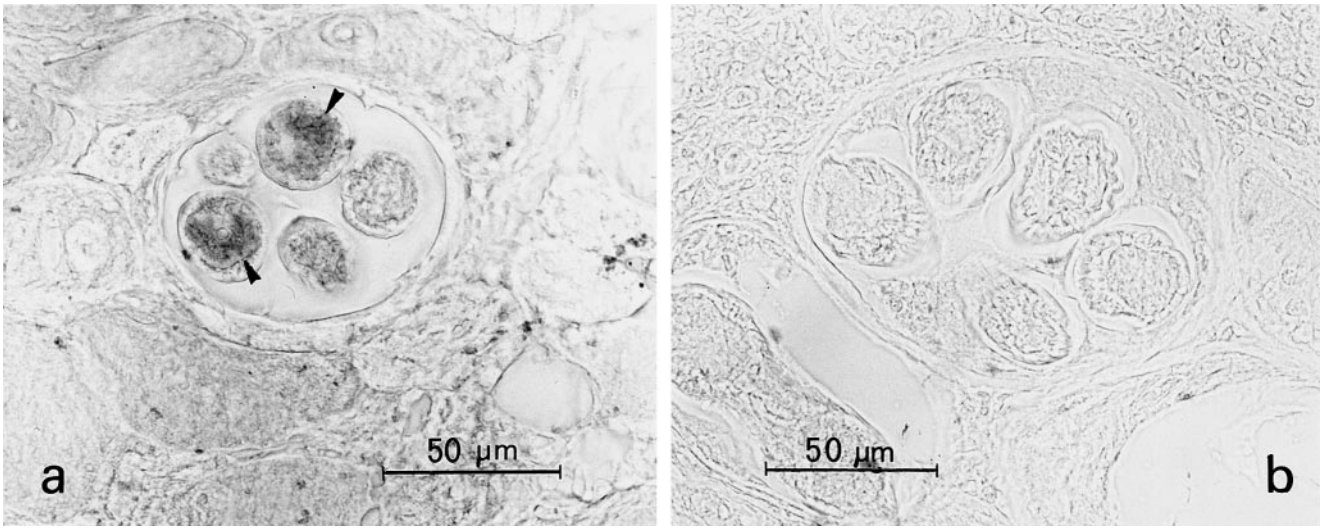

Fig. 8 Immunocytolocalization showing a positive reaction (*arrowheads*) in stichocytes of *T. pseudospiralis* (**a**), and a negative reaction in stichocytes of *T. spiralis* (**b**). Shown are cross-sections of worms in mouse diaphragm. Anti-fusion protein serum was used as developing antibody. The antiserum was diluted 1:64, conjugate 1:320

Bank U00693), and *C. elegans* (GenBank U58754, AF016415, U60059). However, no conclusion can be drawn on the biological significance of this cDNA, which appears to be novel.

Genomic hybridization shows that the cDNA insert from *T. pseudospiralis* is highly specific, and does not cross-hybridize with DNA fragments from *T. spiralis*, mouse, or rat. Using the cDNA as the hybridization probe, a single band was detected in the endonuclease digests. This indicates that the genome of *T. pseudospiralis* contains only one copy of the corresponding gene (Gurr et al. 1991). The 46- and 53-kDa antigens of *T. spiralis* are encoded by a single-copy and a multiple-copy gene, respectively (Sugane and Matsuura 1990; Zarlenga and Gamble 1990). However, the 43-kDa antigens are co-expressed by several related genes (Vassilatis et al. 1992).

The biological significance of the low gene number is not known. One copy of the gene may produce sufficient products required for a given function. This can be achieved either at the transcriptional or translational level. In silkworm, during cocoon formation, both machineries seem to operate hyperactively for the fibroin gene (Suzuki et al. 1972). Only one copy of the gene is present in the silkworm genome, but it yields 10^5 mRNAs, translating into 10^{10} fibroin proteins in 4 days.

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