



Echinococcus granulosus tropomyosin isoforms: From gene structure to expression analysis[☆]

Gabriela Alvite¹, Adriana Esteves^{*}

Biochemistry Section, Cellular and Molecular Biology Department, Faculty of Sciences, University of the Republic, Iguá 4225, Montevideo, Uruguay

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ABSTRACT

Tropomyosins (Trps) constitute a family of actin filament-binding proteins found in all eukaryotic cells. In muscle cells, they play a central role in contraction by regulating calcium-sensitive interaction of actin and myosin. In non-muscle cells, tropomyosins regulate actin filament organization and dynamics. *Trps* genes exhibit extensive cell type-specific isoform diversity generated by alternative splicing. Here, we report the characterization of tropomyosin gene transcribed sequences from the parasitic platyhelminth *Echinococcus granulosus*. Using RT-PCR approach we isolated three isoforms (*egtrpA*, *egtrpB* and *egtrpC*), which display significant homologies to known tropomyosins of different phylogenetic origin. The corresponding gene, *egtrp* (5656 bp), contains eight introns and nine exons. Southern blot hybridization studies showed that *egtrp* is present as single copy locus in *E. granulosus*. We demonstrated that *egtrp* expresses three different transcripts which differ in alternatively spliced exon 4 and intron VI. Interestingly, intron VI suffers intron retention and contains an internal stop codon in frame. Three major bands are also detected by Western blot analysis using a specific anti-rEgTrp antiserum. Immune-localization and in situ hybridization studies showed that *egtrp* transcription and translation is mostly localized at the protoscoleces suckers. This is the first report of alternative splicing in this parasite.

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1. Introduction

Tropomyosin (Trp) is an alpha-helical, parallel, two-chain coiled coil protein which binds along the length of actin filaments in both muscle and non-muscle cells. In muscle, Trps are responsible for mediating contraction via regulation of the actin–myosin interaction. Non-muscle cells, including yeast, also contain Trps; a role in stabilizing actin filaments by modulating the interaction with proteins that are responsible for the regulation of actin dynamics has been proposed (Smillie, 1979; Lees-Miller and Helfman, 1991; Perry, 2001). The amino-terminal region of muscle tropomyosins is highly conserved (Coulton et al., 2008). The structure of the N-terminus is crucial for most aspects of tropomyosin function (Heald and Hitchcock-Degregori, 1988; Kluwe et al., 1995; Palm et al., 2003), and acetylation of Trp at its N-terminus is essential (Heald and Hitchcock-Degregori, 1988; Kluwe et al., 1995; Palm et al., 2003; Monteiro et al., 1994). Tropomyosins are expressed in developmental and cell specific patterns. Certain isoforms may be found in more than one cell type or tissue; however, other Trp isoforms are more specific, in particular,

those expressed in skeletal and smooth muscle (Ruiz-Opazo and Nadal-Ginard, 1987; Wicczorek et al., 1988; Lees-Miller et al., 1990).

In mammals, Trps are derived from four highly conserved genes, which, because of alternative splicing, can give rise to over 40 isoforms. Alternative splicing is a widespread mechanism that increases protein diversity and regulates gene expression in higher eukaryotes. This process generates several mRNAs from a single gene, leading to the synthesis of several proteins with distinct biological functions, different intracellular localizations, or different stabilities (Stamm et al., 2005). From numerous studies, it appears that the regulation of the alternative splicing results from a complex interplay between multiple *trans*-acting factors and *cis*-acting sequences that facilitates or prevents the recruitment of splicing factors by the splicing machinery (Black, 2003; Smith and Valcarcel, 2000).

The selective repertoire, including the number and type of Trps isoforms expressed in different cell types, is strictly regulated by yet-unknown mechanisms (Schevzov et al., 2005). Studies using both mRNA and protein analysis have demonstrated widespread regulation of Trp isoform expression during development and between different cell types (Gunning et al., 2005). Qualitative and quantitative variation in the Trp content of actin filaments has the potential to contribute to specialized actin function in different cell types. Indeed, isoform-specific changes in tropomyosin isoform expression have been commonly associated with cancer (Hendricks and Weintraub, 1981; Matsumura et al., 1983; Cooper et al., 1985; Leavitt et al., 1987; Franzen et al., 1996; Wang et al., 1996).

[☆] Note: Nucleotide sequence data reported in this paper are available in the GenBank™: AF011923.3 (EgTrpA mRNA), EU370233 (EgTrpB mRNA), EU370234 (EgTrpC mRNA), EU370232 (*egtrp* gene).

^{*} Corresponding author.

E-mail addresses: gabal@fcien.edu.uy (G. Alvite), aesteves@fcien.edu.uy (A. Esteves).
¹ Facultad de Ciencias, Sección Bioquímica, Iguá 4225, P3 Anexo Norte, CP 11400, Montevideo, Uruguay. Tel.: +598 2 5252095; fax: +598 2 5258617.

In a previous work Esteves et al. (2003b) have isolated a clone containing 531 bp of the *Echinococcus granulosus* tropomyosin gene coding region lacking the 5' terminal end. The corresponding protein was localized in the suckers and in the subtegumental region of the protoscolex.

Echinococcus species are the smallest tapeworms of medical significance and as other tapeworms require two mammalian hosts, including man, for the completion of their life cycle. *E. granulosus* is universally distributed representing in many countries a major public health problem. Its life cycle begins when eggs are shed with the feces of the definitive host containing the embryo (onchosphere). Once a suitable intermediate host ingests the eggs, they hatch and the onchosphere is released, entering the circulation. At the final site of infection the larval stage may progress to form a hydatid cyst. The cyst produces thousands of protoscoleces, which can progress to the adult form when the cyst is ingested by the definitive host (Thompson, 1995).

In the present work, we report the isolation of the *egtrp* gene and three isoforms generated from this single copy gene by alternative splicing. We elucidate the exon–intron pattern and analyze putative splicing signals. Furthermore, we examine the tropomyosins tissue expression at mRNA and protein level. To our knowledge, this study represents the first analysis of alternative splicing in *E. granulosus*.

2. Materials and methods

2.1. Organism

Fertile hydatid cysts were collected from the lungs of naturally infected cattle in Uruguay. The hydatid cyst fluid and protoscoleces were aseptically aspirated. Once settled by gravity, the protoscoleces were extensively washed in phosphate-buffered saline (PBS) to remove dead protoscolex debris. They were observed under a light microscope for viability (flame cell and vital dye exclusion).

2.2. Nucleic acid isolation, cloning procedures and sequencing

Chromosomal DNA was isolated from protoscoleces as described (McManus et al., 1985). RNA was isolated from protoscoleces using Tri Reagent (Sigma, USA) according to the manufacturer's instructions.

Some PCR products were cloned employing the pGEM-T Easy vector system (Promega, USA) and sequenced using specific primers on an ABI prism 377 DNA sequencer (Applied Biosystem) (CTAG Service, Faculty of Sciences, Uruguay). Other PCR products were sequenced directly from an agarose electrophoresis eluted gel band by the Service mentioned above. Eluted DNA was purified using a "GFX Gel Band Purification Kit" (Amersham Biosciences) before sequencing.

2.3. PCR and RT-PCR amplification

Reverse transcription was performed using Superscript II RNase H⁻ reverse transcriptase (Invitrogen, Life technologies) with 250 ng of random primer and 4.6 µg of total RNA, according to the manufacturer instructions. In addition, a specific cDNA double strand enriched in *egtrp* 3' ends was generated using a modification of SMARTTM method (BD Biosciences/Clontech, Basingstoke, UK). Briefly, 1 µg of total RNA was mixed with 10 pmol of CDS primer: 5'-AAGCAGTGGTAAC-AACGACAGTACT₃₀NN-3' (BD Biosciences/Clontech, Basingstoke, UK), 10 pmols of a specific *egtrp* forward primer (primer f): 5'-CTTGGCAGTGACCGAGGTC-3' and water to 4 µl. The mix was incubated during 2 min at 70 °C. In parallel, a second mix was prepared with 2 µl of First-Strand buffer 5×, 1 µl DTT 20 mM, 1 µl dNTPs 10 mM, 200 U of Superscript II RNase H⁻ reverse transcriptase (Invitrogen, Life technologies) and 1 µl of RNaseOUT (40 U/µl) (Invitrogen, Life Technologies). After that, both mixtures were mixed and incubated 37 min at 42 °C, 35 min at 50 °C, 70 min at 42 °C and 7 min at 70 °C. Also this technique was utilized to generate enriched 5'

ends cDNA, using in this case SMARTII primer (Clontech): 5'-AAGCAGTGGTAACAAACGACAGTACGCGGG-3', and q primer: 5'-CCCAAGCTTTTCAGAAGGAAGTGAG-3'. The reaction products were kept at -20 °C until use and 1 µl was used for each PCR reaction.

PCR reactions were performed using a Perkin-Elmer thermocycler and a final volume of 25 µl. 10 pmol of each primer, dNTPs 0.8 mM, 1× buffer (Amersham or Invitrogen), 1.5 mM magnesium chloride, 1.25 U Taq DNA polymerase (Amersham or Invitrogen) were used. To amplify large PCR products an AccuTaq LA DNA polymerase (Sigma) was employed following manufacturer instructions. Thirty-five amplification cycles of three steps were performed: 94 °C (45 s), 54–58 °C (45 s) (depending on primer annealing temperatures), 72 °C (60 s). Before the cycles there was a denaturalization step at 94 °C (5 min) and after them there was an elongation final step at 72 °C (10 min). In every PCR reaction a negative control was included. The presence of contaminant genomic DNA (gDNA) was controlled through the amplification of a known gene (*egfabp1*) having an intron (Esteves et al., 2003a) because the genomic PCR product is larger than cDNA product. PCR products were fractionated by 2% agarose gel electrophoresis. The bands were excised from the gel and purified using a GFX gel band purification kit (Amersham Biosciences). For genomic DNA amplifications 25 to 50 ng of protoscoleces gDNA was used as template and the same PCR and DNA purification protocols were used. A large primer battery was used to obtain the introns of the *egtrp* gene and isolate tropomyosin isoforms (Table 1).

2.4. Sequence analysis

Nucleotide sequences were analyzed using Chromas v. 1.43 software (<http://www.technelysium.com.au/chromas.html>) and they were manually corrected when it was necessary. Sequences were submitted to BLASTX 2.2.15 analysis (Altschul et al., 1997) against the complete GenBank, European Molecular Biology Laboratory (EMBL), DNA Bank of Japan (DDBJ) and Protein Data Bank (PDB) databases (nonredundant protein sequences), including all organisms. DNA and protein sequences alignments were performed using CLUSTALW algorithm under default conditions: DNA weight matrix IUB; protein weight matrix Gonnet PAM 250, score plot scale=5; residue exception cut-off=5; minimum length of segments=1 (Higgins et al., 1994). NSPLICE version 0.9 (<http://www.fruitfly.org>) (Reese et al., 1997) algorithms was also employed.

2.5. Southern blot analysis

For Southern blot, chromosomal *E. granulosus* DNA was isolated from parasite material as described before (McManus et al., 1985).

Table 1

List of primer used to amplify *E. granulosus* tropomyosin gene and isoforms

Primer name	Sequence (5→3')	Annealing region
A	CGCGGATCCATGGATTCCATCAAAAAG	Exon 1 (sense)
J	CGGTGCTACTTCAGTCTG	Exon 2 (antisense)
B	GATGAATGACTGGCTCTC	Exon 2 (sense)
K	CGCAGCTTCTCGGTTC	Exon 2 (antisense)
C	GAGTCTCTCCAGGAAGCG	Exon 2 (sense)
L	GACGGCCACTAGACTGTTT	Exon 3 (antisense)
D	GCTATGACCCGTCGCAITC	Exon 3 (sense)
LI	TAGTTGGGCGATTCGTTTCATC	Exon 4 (antisense)
E	GAGACCAAGTCTATTCTGACG	Exon 4 (sense)
M	GACTCCGCTCGTCCAAGTCG	Exon 5 (antisense)
F	CTTGGCAGTGACCGAGGTC	Exon 5 (sense)
G	CGAAGTCTCCGAGCAAGAG	Exon 6A (sense)
P	TGGAGCTTGGACACTTGCG	Exon 7 (antisense)
H	CAGCGTGCTGCCGAA	Exon 7 (sense)
T	GTGGTCCCGCTAGGC	Intron VIII (sense)
S	CTTGGAGGATCGGTTG	Intron V (sense)
O	GGCATTGTTACTTCTTG	Intron VI (antisense)
Q	CCCAAGCTTTCAGAAGGAAGTGAG	Exon 8 (antisense)

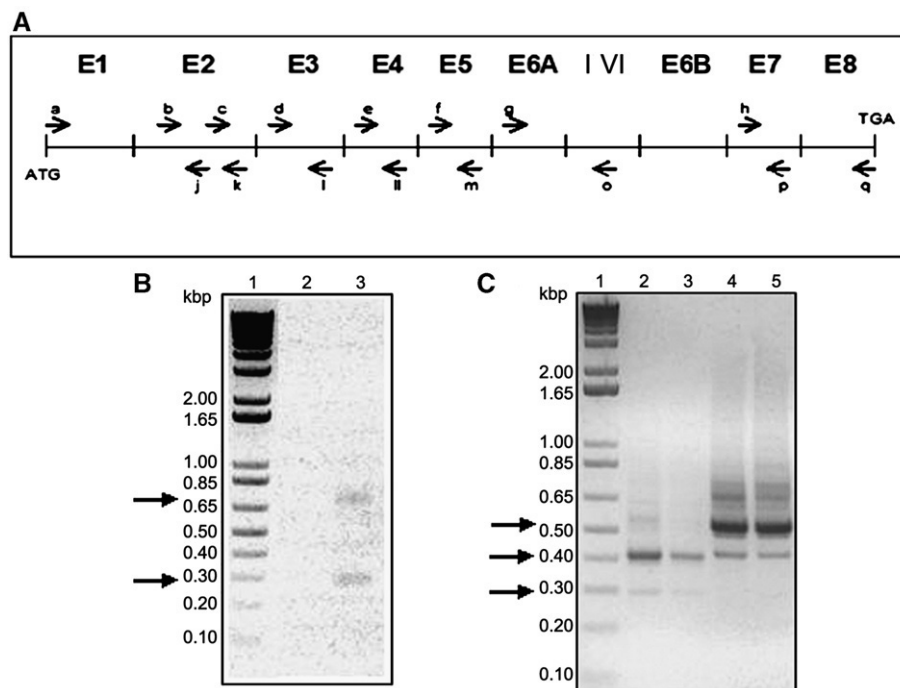


Fig. 1. Tropomyosin isoforms identification. A) Scheme of *egtrp* exons and intron VI with the primers used in the screening of *egtrp* putative isoforms and to determine the *egtrp* intron positions. The arrows represent the primers used in PCR reactions; localization, direction and primer name are shown. B) Electrophoresis of RT-PCR products using primer g and q. Lane 1: molecular weight marker 1 kb Plus DNA ladder (Invitrogen); lane 2: PCR negative control; lane 3: RT-PCR products. C) Electrophoresis of RT-PCR products using primer combination b-m, and a-m. Lane 1: molecular weight marker 1 kb Plus DNA ladder (Invitrogen); lane 2: PCR products using cDNA generated with random primers and primer b and m; lane 3: PCR products using enriched cDNA and primer b and m; lane 4: PCR products using cDNA generated with random primers and primer a and m; lane 5: PCR products using enriched cDNA and primer a and m. The left arrows indicate the sequenced PCR products.

Sixty micrograms of chromosomal DNA was then digested to completion with BamHI, EcoRI and PstI restriction enzymes (Amersham Biosciences) according to the manufacturer's instructions. Separation of the resulting fragments, transfer to nylon membrane, hybridization and washing were subsequently performed as described (Sambrook and Russell, 2001). As a probe for Southern blot hybridization, a PCR fragment was amplified from chromosomal *E. granulosus* DNA using the primers c and l (see Table 1) which spans the region between exon 2 and exon 3 (983 bp) of *egtrp*. The PCR product was purified using GFX gel band purification kit (Amersham Biosciences). After denaturalization, it was digoxigenin labelled using the DIG High Prime DNA labelling kit according to the manufacture's protocol (Roche Applied Science). The signal was detected using an alkaline phosphatase conjugated anti-digoxigenin antibody (Roche Applied Science) to bind the probe and NBT/BCIP reagents provided by Detection Starter Kit I following manufacturer instructions for color develop (Roche Applied Science).

2.6. Western blot analysis

A protoscolec lysate was prepared by homogenization in Tris-HCl 50 mM, pH 8.5, NaCl 0.15 M, Triton X-100 1% and phenylmethylsulfonyl fluoride 900 μ M. Complete destruction of protoscolec was checked by visual inspection under light microscope. The lysate was centrifugated for 5 min, at 8000 g and 4 °C. The supernatant was dosed for protein concentration by BCA protein assay kit (Pierce). Fifty micrograms of parasite proteins per well were separated on a 12% SDS-polyacrylamide gel and electrotransferred to a PVDF Hybond-P membrane (Amersham Biosciences). The membrane was incubated with rabbit antiserum raised against the recombinant *E. granulosus* tropomyosin lacking the N-terminal end (rEgTrp) (Esteves et al., 2003b). A goat anti-rabbit IgG conjugated with alkaline phosphatase (Sigma) was used as secondary antibody. The bands were visualized using NBT and BCIP reagents (Sigma).

2.7. In toto immune-localization analysis

To localize EgTrps expression in toto immune-localization studies were performed on fixed protoscolec using an anti-rEgTrp (Esteves et al., 2003b) rabbit antiserum diluted 1/1000 and following the technique previously described by Rinaldi et al. (2008). The material was mounted with 50% glycerol for light microscope observation and it was viewed using Olympus BX40 light microscope; contrast and brightness were adjusted using GIMP 2.4.0 free software.

2.8. In situ hybridization studies

A DNA fragment spanning *egtrpA* mRNA sequence (AF011923) until stop codon was cloned in the pGEM-T-Easy vector (Promega) and an antisense digoxigenin-labelled riboprobe was generated. Histological processing and probe hybridization were performed as previously described (Britos et al., 2007). The control sections were treated with the homeobox region of *eghox5* gene as antisense probe which presents a ubiquitous expression in protoscolec (Lalanne, 2003). Sections were viewed using Olympus BX40 light

Table 2
PCR reactions conditions used to identify the intronic sequences

Primer combination	Annealing temperature (°C)	Amplified region
a-j	57	Exon 1–intron I–exon 2
c-l	56	Exon 2–intron II–exon 3
d-ll	56	Exon 3–intron III–exon 4
e-m	56	Exon 4–intron IV–exon 5
g-p	56	Exon 6A–intron VI–exon 6B–intron VII–exon 7
f-p	55	Exon 5–intron V–exon 6A–intron VI–exon 6B–intron VII–exon 7
h-q	57	Exon 7–intron VIII–exon 8

microscope, contrast and brightness were adjusted using GIMP 2.4.0 free software.

3. Results

3.1. Characterization of transcribed *egtrp* sequences

Using a RT-PCR strategy with a sense primer designed with the first 18 nucleotides of *Echinococcus multilocularis* tropomyosin (AJ314792) (primer a) and an antisense primer designed from the known *egtrp* sequence (AF011923.2) (primer q), a DNA fragment of 855 bp was amplified and cloned, referred as *egtrpA* isoform. The extended sequence contains one single open reading frame which encodes a 284 amino acid protein. The first six amino acids overlap the designed *E. multilocularis* primer; however, it is likely that these 18 base pairs being shared between both genes because of the high nucleotide identity percentage of these tropomyosin sequences (99.2%) (data not shown). The initial BLASTX search (default conditions) at the National Center for Biotechnology Information (NCBI) showed that EgTrpA amino acidic sequence has the highest similarity (100%) with *E. multilocularis* tropomyosin (TPM_ECHMU, CAC85552). Less similarity was detected with tropomyosins from *Schistosoma japonicum* (SjcTM, Q26519) (87%), *Caenorhabditis elegans* (CeTM1, BAA07540) (72%), *Onchocerca volvulus* (Ov-tmy-1, AAC28900.1) (72%), *Drosophila melanogaster* (DmTm1, NM_169636.1) (72%), *Bos taurus* (BtTm1, NM_001013590.2) (69%) and *Gallus gallus* (GgTm1, NM_205401.1) (67%). A high conservation degree within tropomyosin protein family was revealed. In addition, the region of head to tail overlap of adjacent tropomyosins in the N and C-terminal ends, and the two troponin T-binding regions were found (McLachlan and Steward, 1975; Pearlstone and Smillie, 1983; Philips et al., 1986).

Considering the existence of other *egtrp* isoforms besides *egtrpA*, we performed RT-PCR by combination of a large set of primers (Table 1 and Fig. 1A), expecting more than one specific PCR product in any reaction if more than one isoform were expressed. All RT-PCR products obtained were sequenced. The primers were designed from *egtrpA* sequence sweeping the entire sequence. For each reaction cDNA generated with random primers or specific primers was used as template and the annealing temperature was between 54 and 58 °C depending on the primers used. A unique and specific PCR product was generated using each of the following primer combinations: a–k, a–l, a–q, b–p, b–q, c–p, c–q, d–p, d–q, e–q, f–q and g–p; while two PCR products were obtained with primer combinations g–q, and a–m (Table 1 and Fig. 1). The sequences of the fragments obtained in the last two reactions showed an insertion region and a skipped region regarding to the identified tropomyosin *egtrpA* respectively, suggesting that new isoforms were detected which hereafter were named as *egtrpB* and *egtrpC*. When one PCR product was obtained its sequence 100% overlapped with *egtrpA* sequence. After intron position was revealed (see section 3.2) we could identify 9 exons that were named accordingly (E1, E2, E3, E4, E5, E6A, E6B, E7, E8). Fig. 1B shows PCR products obtained with primer combination g–q and 56 °C as annealing temperature. The smallest fragment (244 bp) was coincident with *egtrpA* sequence and the largest one (640 pb) showed and insertion of 396 pb. Upstream and downstream sequences from this insertion overlapped with *egtrpA*. This insertion, 76 bp downstream the 5' end of the fragment was named intron VI since it is a case of intron retention (see section 3.2) (Galante et al., 2004) and it belongs to *egtrpB* isoform. The surrounded intron VI exons were named exons 6A and 6B. In order to search the intron VI upstream sequence, RT-PCR was performed using an intron VI specific primer (primer o) in combination with a primer overlapping exon 1 (primer a) and 52 °C as annealing temperature. The sequence of the unique PCR product indicated that exons 1, 2, 3, 4, 5, and 6A are upstream of intron VI.

Two PCR products of 551 and 431 bp in size were also generated with primer combination a–m (Fig. 1C) and an annealing temperature

of 55 °C. The largest DNA fragment was coincident with the known *egtrpA* sequence and the smallest one had the same sequence without exon 4. The isoform lacking exon 4 was named *egtrpC*. This was verified amplifying with primer combination b–m (Fig. 1C).

Bringing together these results, three isoforms were found referred as *egtrpA* (exons 1, 2, 3, 4, 5, 6A, 6B, 7 and 8), *B* (exons 1, 2, 3, 4, 5, 6A, intron VI, exons 6B, 7, and 8), and *C* (exons 1, 2, 3, and 5). *egtrpB* sequence analyses revealed a stop codon TGA in intron VI 54 bp from the intron VI 5' end, so the remaining 3' sequence is untranslated. This sequence neither possesses an open reading frame nor potential SECIS (selenocysteine insertion sequence) elements (<http://genome.unl.edu/SECISearch.html>). Taken together this information intron VI codifies for the carboxyl terminal end of EgtrpB. Although this strategy used several primer combinations, we could not identify the 3' *egtrpC* region.

3.2. Characterization of the *egtrp* genomic locus

A PCR approach was also used to search for *egtrp* introns. A combination of a large set of primers was used to amplify different regions of the gene. Table 2 summarizes *egtrp* PCR amplifications conditions. A perfect DNA sequences overlap was achieved obtaining a chromosomal sequence of 5656 bp (5' primer not included). The coding region was contained within 9 exons (E1, E2, E3, E4, E5, E6A, E6B, E7, E8) and a portion of intron VI with lengths ranged from 63 to 450 bp. The gene contains 8 introns (I, II, III, IV, V, VI, VII, VIII) with lengths ranged from 224 to 1169 bases (Figs. 1A and 2).

Bioinformatic analysis revealed several *cis* acting signals involved in RNA processing. Typical polypyrimidine tract in the 3' intron region was detected. Using NSPLICE predictor software, consensus sequences, including GT–AG splice junctions, were identified. In addition, a putative branch site sequence (UACUAC) similar to the yeast consensus branch site sequence (UACUAAC) was localized in introns II and VIII. Finally, several conserved hexamer motifs (Kabat et al., 2006) were found in introns III, V, VI, VII and VIII (Fig. 2). These short intronic sequences are putative alternative splicing regulatory motifs conserved in mammals and nematodes and some of them were experimentally confirmed (Kabat et al., 2006; Jin et al., 2003; Huh and Hynes, 1994; Hedjran et al., 1997; Min et al., 1997).

Since vertebrate tropomyosins are coded by four genes, we investigated the situation for *egtrp* in *E. granulosus* by Southern blot analyses (Fig. 3). Chromosomal DNA was digested with several restriction enzymes and hybridized with a probe that spanned exons 2–3 of *egtrp* (Fig. 4). In all cases, one single prominent hybridization fragment was obtained indicating that *egtrp* is a single copy gene in *E. granulosus*.

To summarize these results, a diagram was developed showing *E. granulosus* tropomyosin splicing process (Fig. 4). *egtrpA*, *egtrpB* and *egtrpC* mRNA arise from *egtrp* gene by alternative splicing.

3.3. Western blot analysis

In order to analyze tropomyosins expression at protein level we performed Western blot assays using an anti-rEgTrp antiserum. Three protein bands (I, II, III) were showed in *E. granulosus* total protein extract (Fig. 5) whose apparent molecular masses did not coincide with the theoretical molecular masses of tropomyosin isoforms.

However, an altered electrophoretic mobility behavior of these proteins was detected and it could explain these differences. A 4500 Da mass difference was observed between the apparent mass of rEgTrp (27.0 kDa) (Fig. 5) and the MALDI-TOF mass spectrometry determination (22.5 kDa). Taking into account this difference we suggest that EgTrpA with a theoretical molecular mass of 33 kDa could be band II (37.5 kDa). EgTrpB with a theoretical molecular mass of 26.5 kDa could correspond to band III (31.0 kDa). Finally, the largest band (I) of 41.5 kDa could be EgTrpC or an unidentified isoform. We

----- ATT ATG ATG
 9 GCA ATG AAA TTG GAG AAA GAA AAT GCA TTG GAA AA
 44 gtaagtcattttattgcagttagtttgattgaacttctatgcgctccagtagacgctgaggggc
 108 gaaaatattaccttcttaacactagcgaatttcagtgagagactacgaccgccttagtcttcaa
 172 aagagaatatatcatccaatttcagaaagaatcgtagcaacatctgagcttgctcagtttaggaa
 236 ggtatctttacaatgaaccggtttaagctttcgatagagtgggtctccacacgcctaaacaatg
 300 atttgttaaataggttttggactgctttaaaaagcacaaaatgactattttttgtggtatgaa
 364 atccaataagcattgcgtattaggttttacttaagcatattataaattgggtggctaaattaca
 428 acgcttggtggtggttattacggcaacgatctaagctcaagatttaaatattccatcaagaaatc
 492 gaaattcaccaagaaattacatgactaaaaattccgaattacttcaccgcttatcaaattgagc
 556 gaataagcttaaaagcttgctgatgtagagatcagatacattttattgtaaatctacaacatta
 620 ggaaaagaaatagtataccaatacataagtggttatgaagtgataggaatgcacgaattaagg
 684 gcataatattcagcaggtggttttgcgacatttaaaaggcatccataaggaatctagtggattcag
 748 tcgcgcatatctttaattggtgcaatggatagatgatgatgttgatgatcggcacacctttt
 812 tgag G GCC ATA AAT CTC GAA AAT CAA CTC AAG GAG AAG GCC AAA GAT
 860 TTT GAG AAG AAA GAA GAA GAG ATG AAT GAC TGG CTC TCT AAA GTG AAG
 908 AAC ATT CAG ACT GAA GTA GAC ACC GTA CAA GAG TCT CTC CAG GAA GCG
 956 ATT AGC AAA CTC GAA GAA ACC GAG AAG CGT GCG ACA AAC
 995 gtgagttgattgtgcttagaataaccgtgtagaggcacctagagagctcacttgcgactaccac
 1059 gttacttattgctatggaataagtcactctattttcgtaaaatatgtttagtccacatcaaaat
 1123 ggtctattttaaaataatcacgagtttggtattaagacgggttgctcagtaaaattttagaactt
 1187 ggattgtacttttctaaatgttgaaactcattccaataacaatgaaaaatcggaattacttt
 1251 aataagttctaaagtgcataattggtatggttagtggtgcacatgagacgtgaatgcacggtagt
 1315 tgtcaaaaagattccgcgcccccaactcaaacacgggctttggattaatgttcaaaatctatgta
 1379 aacctggaaagaatacagtatgccttaataattataatgcttagtaaaaacgacattggtggcga
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 1699 catgacgattttggagtcgagtccttttttaattgtagtttagtagggctgccccaaaattcgga
 1763 ttatagtgttttaacttcttcttctccgtag GCC GAA GCC GAA GTT GCT GCT ATG
 1819 ACC CGT CGC ATT CGG CTT TTG GAG GAA GAT TTT GAA CAG TCT AGT GGC
 1867 CGT CTT ACT GAA ACA TCT ACT AAG CTT GAC GAT GCT AGC AAA GCT GCT
 1915 GAG GAG AGC GAA gtattgcctgtgctattgagtgcttcataattgtaatctgctgcatat
 1974 ttgcatgctttgttatgtcatctgcatgtatttagtgacgctggcggttaataatcaacaag
 2038 gcgctgatgagatgaaaattactgagttagaacgctctattgaggctgcatccttggtactca
 2102 ggaatctgtgtggaaatatgaggaggtattttgtggctttaagccatttgcatctctctgctgt
 2166 cacctgtcattaagctgtggtctgtctatagcgtgaaagaatatagcagtttaattcaataat
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 2286 ACC AGG TCT ATT TCT GAC GAT GAA CGA ATG GCC CAA CTA GAA GAA CAG
 2334 GTA AAG GAG GAG TAC ATC GCT GAA GAT GCC GAA CGG AAA TAC GAT
 2382 GAA gtcaagtagacttatttttctcattttctggaaatcaatctgctaggattgggtgtcttaaa
 2446 tgtgcggttttcttgattttataagtcctccgactcatgttagtaaatatattttacgcctgtct
 2510 taatcattttttgcaattccaggggactagaccacattagattctgtgtccgggttctccta
 2574 ctgtggaccacattattgaaatattatgtcgtag GCT GCT CGC CGC TTG GCA GTG
 2630 ACC GAG GTC GAC TTG GAG CGA GCG GAG TCT CGT TTG GAA ACC TCG GAG
 2678 Adgtaacctcgaaggcaacgcaacccacttgacgggtctttttaccgctctcctgcacgcgcatt
 2742 tgtttttctctattatcggttcagcacgttgacgaatcttaataagcatgctgtctctttctc
 2806 ctactctttggcattgaagacaatgaagaggattattgctgctcagcaaaactttatgcaccg
 2870 agaggagaaggcacaacaattgaggtgtgaggttgccaaaaattgtagcagagacgaatttcag
 2934 acccggttttgatgcattagtttacttttcacgtttgacgctggccaaaactgccgtagtagcc
 2998 attatacttaagaaattgtctatcgtcgagggtatggagaatttaaaaggaatttgctctgcca
 3062 acatttggtacttccggttttagctgcgcaaaaacttgaggatcggttggtgctgtcgtcgtg
 3126 caaaattgaaagccaaagaaattgcacacgataaggtgatgcgtgcttgttctttttgtctta
 3190 tgtctgccttgaaatgcattgataccaaatcggtccccgaccttagtttacacaaaactcaagca
 3254 gcagtaatgactttttag C AAA ATC GTT GAA CTT GAG GAA GAA CTG CGC ATT
 3305 GTT GGT AAC AAC ATG AAA TCT CTC GAA GTC TCC GAG CAA GAG gda ata
 3353 gtt caa tta gtg act agc gat aca aga agt aac aat gcc tat aca tga
 3401 tttcaagcttttaggacgtggcgaaatttcttatgaacatagaataaggatttgcatacattatt
 3465 ggggtcacaccatgtgcatgttcgaatacagctcgatttcatgcgtactgcgtggtctatatttc
 3529 cactttgattgttgtaattgggtgtttttgtagtacctggagcgttatggtaactcttatta

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3593 cctgatcactacaggacaagattacgttattatgcgttggcctatattgtaaatagggaacct
3657 aaaccatgttctgagttccacttgttgcaactgataattgtcgtcttcgagttccttttggtt
3721 gtgtctcctttggaatcgtac TCT CTT CAG CGC GAA GAA AGT TAC GAA GAA
3773 ACC ATT CGA GAT TTG ACA GAG CGC TTG AAG ACG
3806 gtaagactgctgtcgtccgtaaaaggatgcaatgacttgtttactcattctgaatgagtggtgc
3870 ataattcttgttttttttttctaacggctgatgttttcttttttgcagatgcatgcatgtgca
3934 cgtaacaatggctttcatacaccgtgtagaacgtcgaactggaagaaatgtatttgaaacgtc
3998 ttaatgtcttacgggaacaagtgaagggaagtgaagtattcaaagcttttatagaattatgacca
4062 ggctgctagaactacaccgagaggagtttgataagagattttaggacataaaggacacgatgt
4126 agttaaggcttcccttactgtaaatgggtttgtttcaaaagacgtggcttggccagctttcgacg
4190 tgggtgtgtatttcgcacacaagatctgcattttattagagtctttgaccgctctctaaatgcatg
4254 catactgtggcatgctgctctcagtatctatacatgaagcactagaacttttgatcagtggtca
4318 atcattactaatacag GCA GAG CAG CGT GCT GCC GAA GCT GAA CGC CAA GTG
4370 TCC AAG CTC CAA AAT GAA GTT GAC CGC CTT GAA G
4404 gtttgaattttattactctgctaactgtgcttgttattcctatctgcatgtttgcctgcgcac
4468 gtaccgttctgtctgcagtcctctccaaggccgagtgagagcacaagatgcggaatccgtgtc
4532 caagcgatatgaactagaagtacaacgtgttaaagggttagtttcgggggtgtctctttcgaaatg
4596 ttgaatcatgtgtagtaattatatacgatgcagttccttgattccgtttgatatacattttta
4660 cacacgtagagtaataacgcacaccaagtttgaaaataataaccgtgtgaaaaaaattggccttg
4724 ctgctgttccccctattttaaacctactttttgtagacactcttttctcgagagaacaggatcttta
4788 cgcttcttcgcaaggaaatcgattctgcactggacgagtagcatttcgatttaaatggacctgttg
4852 aactagtgtcttattccttctgattctgacgtcgcgtgatcctccatttctttaactgccgcc
4916 tgcattctagcatgccctactttaaaacttcttgatttgcattttctttgtggtgccgctaggcatc
4980 attcaatagtatttcggatggtgcttattttaaccataatttcattgcctgcttccattttgtgc
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5108 cgaacgccttcagtttatggcattgttcaattgggtagaatagcaatgcattgcagttggttg
5172 ggaaaagacgacacagatttaagttagtttagaagtagattcaattttatcttcgtaaatgggt
5236 gcattctgtgtatgtgtgtggttaaaggcgttctgtgacgtggaattaaactctgagggggcct
5300 gaacgtctatcggtattttacgcaactggagcttgcaggtcttcttagcttgagtagtggggcg
5364 tagcgatgtcacgcagctggttcgtgtctaaagcttggcagataacgcagcctcaaacgcgtag
5428 tgtctcgagcgttttcgggtctcacctgtgtgagaacgagggctgggatggcatctgcatgaagt
5492 cgttattttgaacataagatctttaaggcagctcttgcgtttggtttactacttcagaatttaata
5556 tgccttttccccctcag AT GAG CTA CTA TCC GAG AAG GAA CGT TAC CGA GCC
5608 ATC AGC GGA GAA CTG GAT ACT ACC TTC GCG GAG CTC ACT TCC TTC TGA
5656 -----

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Fig. 2. *egtrp* gene nucleotide sequence. The exons and introns are shown in capital and lowercase letters respectively. In bold letters: stop codons (TGA) and putative branch sites; in squares: donor and acceptor intron sites; underlined: splicing consensus sequence; in italic letters: polypyrimidine tract; in shaded gray: conserved regulatory hexamer motifs; dotted line: unknown sequence. Numbers on the left indicate nucleotide position (bp). In brackets, position limiting the exons (E) and introns (I): E1 (1–44); I1 (45–817); E2 (818–995); I2 (996–1795); E3 (1796–1927); I3 (1928–2265); E4 (2266–2385); I4 (2386–2609); E5 (2610–2680); I5 (2681–3271); E6A (3272–3347); I6 (3348–3743); E6B (3744–3806); I7 (3807–4334); E7 (4335–4404); I8 (4405–5573); E8 (5574–5656).

cannot infer the EgTrpC theoretical molecular mass since its C-terminal region could not be identified.

3.4. In toto immune-localization analysis

To further study protein expression, we investigated tropomyosins tissue distribution by two complementary techniques, in toto immune-localization and in situ hybridization. In toto immune-localization assays of evaginated protoscoleces (Fig. 6A) revealed an intense staining in apical region corresponding to rostellum and suckers (scolex) and in caudal region where the “stalk zone” is found. The body or scolex basal soma showed a faint staining. In the invaginated protoscolex a clear staining was observed in the caudal region containing the remaining stalk region (Fig. 6B). No signal was observed in the apical region. However, a low staining level was observed in the apical tegument, in the operculum zone. Control sections were unstained (Fig. 6C).

3.5. In situ hybridization studies

An antisense digoxigenin-labelled riboprobe generated from the coding sequence of *egtrpA* was employed to analyze protoscolex *E.*

granulosus tropomyosin expression. The most intense stained was observed in the suckers while a homogenous low-level labelling was showed in the parenchymal region (Fig. 6D). Small stained spots were observed in germinal layer which could correspond to developing brood capsules. No signal was observed in the laminated layer. The control section using an *eghox5* digoxigenin-labelled riboprobe showed a ubiquitous expression (Fig. 6E).

4. Discussion

Three *E. granulosus* tropomyosin isoforms named EgTrp A, B, and C, with high protein sequence similarity scores are reported. BLAST searching and multiple sequence alignment confirm that these proteins belong to the tropomyosin family, indicating a high degree of conservation along the zoological scale. The existence of other isoforms is not discarded. The presence of two or more highly similar tropomyosin isoforms is not surprising; *Caenorhabditis elegans* (Kagawa et al., 1995; Anyanful et al., 2001) and *Drosophila melanogaster* tropomyosins (Hanke and Storti, 1988) are good examples among invertebrates.

Southern blot hybridization experiment indicates that *egtrp* is a single copy gene in *E. granulosus*. This is supported by the results of

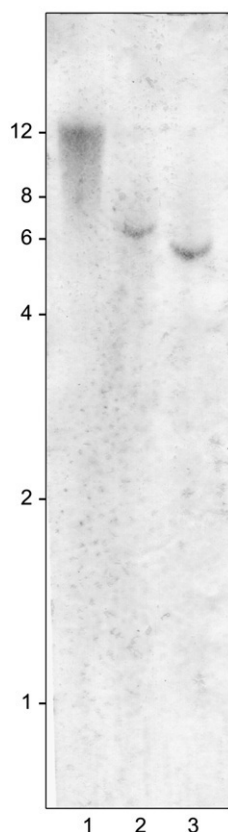


Fig. 3. Southern blot hybridization. Genomic *E. granulosus* DNA was digested to completion with BamHI (lane 1), EcoRI (lane 2) and PstI (lane 3), separated on 0.8% agarose gel, transferred to nylon membrane and probed at a hybridization temperature of 58 °C with a digoxigenin labelled probe. DNA marker sizes in kbp are indicated to the left.

PCRs from genomic DNA using a combination of a large set of primers, which exclusively generate one PCR product. Hence, the situation in *E. granulosus* seems to closely resemble that of *C. elegans*, where *tmy-1* is the only tropomyosin gene (Anyanful et al., 2001) and differs from that of *D. melanogaster* (Hanke and Storti, 1988) and vertebrates (Perry, 2001) where closely related tropomyosins are encoded by different genes.

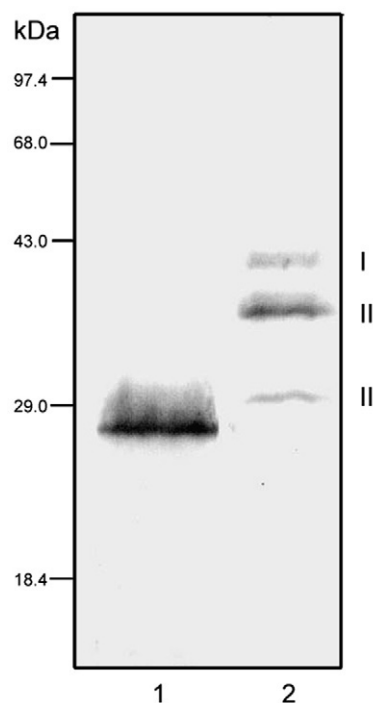


Fig. 5. Expression of EgTrp isoforms in *Echinococcus granulosus* larval stage. Western blot analysis using an antiserum raised against rEgTrp diluted 1:1000 and an alkaline phosphatase-conjugated anti-rabbit IgG diluted 1:30,000 as secondary antibody. Lane 1 contains 2.5 µg of purified recombinant EgTrp and lane 2 contains 50 µg of proteins from protoscolex lysate.

Many tropomyosin genes alternatively splice the exons encoding the last amino acid residues. Isoforms with different carboxy terminal ends were observed in *D. melanogaster Tml* alternative spliced gene. This gene gives rise to two tropomyosin isoforms, an embryonic and a thoracic one, which only differ in 27 amino acid residues from the carboxyl terminal end. The thoracic isoform contains exons 1, 2, 3, 4 and 5, while in the embryonic isoform the exon 4 skips. Stop codons in exons 4 and 5 determines that these exons code for the last 27 residues of each isoform respectively (Basi et al., 1984). The carboxyl terminal end of tropomyosin molecules contains one of the two troponin T-binding regions (McLachlan and Steward, 1975; Pearlstone and Smillie, 1983; Philips et al., 1986) and is also the region of head to

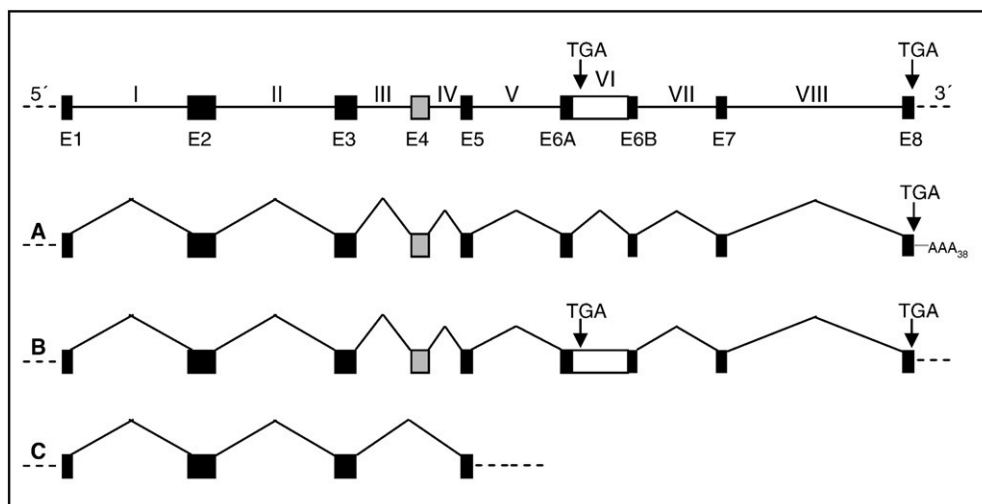


Fig. 4. *E. granulosus* tropomyosin gene and isoforms scheme. The upper map represents the *egtrp* gene and the diagrams A, B, and C correspond to *egtrpA*, *egtrpB* and *egtrpC* mRNAs. The black, gray and white rectangles indicate the exons, the alternative skipping exon and the retained intron respectively. The connection lines represent introns and the unknown sequence is marked with a dotted line. The EgTrpA polIA end is shown.

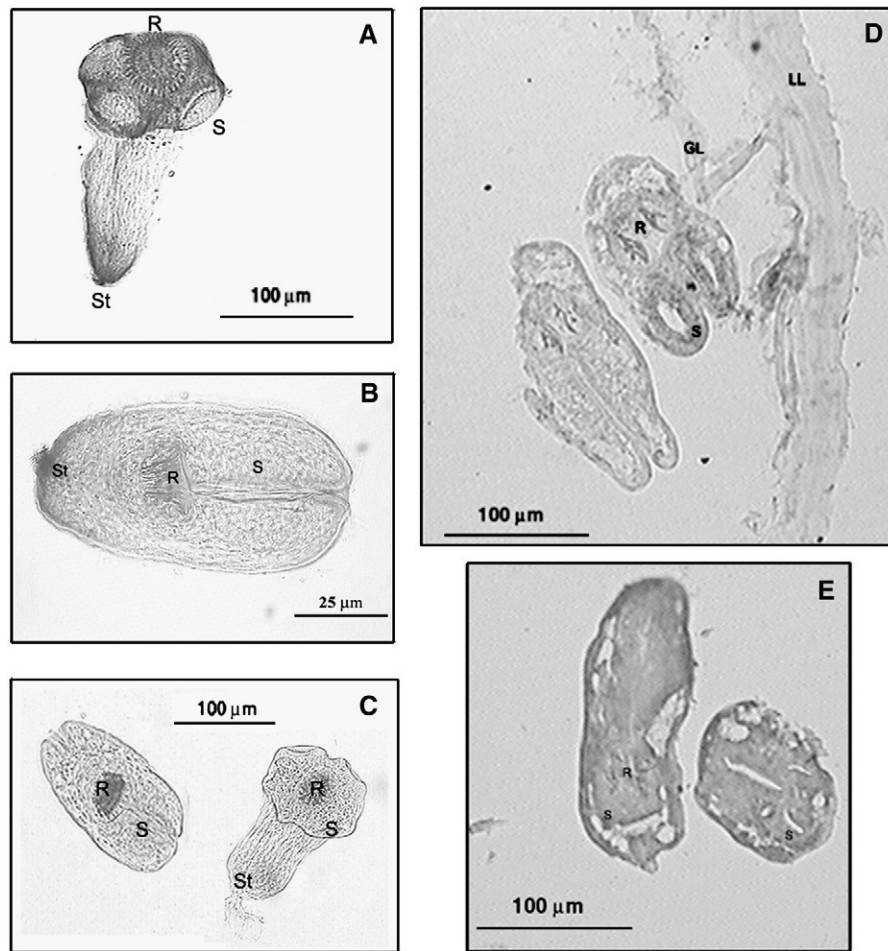


Fig. 6. Immune-localization and in situ hybridization studies of EgTrp isoforms in *Echinococcus granulosus* larval stage. PSs incubated with an antiserum raised against rEgTrp as primary antibody and an alkaline phosphatase-conjugated anti-rabbit IgG as secondary antibody (panels A and B) or with the secondary antibody alone (panel C). Protoscoleces sections hybridized with digoxigenin-labelled antisense RNA probes: D) *egtrpA*; E) *eghox5* (control); and developed using an alkaline phosphatase-conjugated anti-digoxigenin antibody. The following structures are indicated: GL, germinal layer; LL, laminated layer; R, rostellum; S, sucker; St, stalk region.

tail overlap of adjacent tropomyosin. Thus, the differences in the carboxyl terminal end between EgTrpA and EgTrpB isoforms are likely to have significant effects on the structure and function of the different isoforms (Forry-Schaudies and Hughes, 1991). EgTrpB isoform not only lacks the last troponin T-binding and head to tail overlap regions, but also the first troponin T-binding regions is incomplete due to the exon 6B insertion. Actin-linked regulatory system of striated muscle is mediated through the interactions of the troponin complex with F-actin and tropomyosin (Pearlstone and Smillie, 1983). It was proposed that the amino terminus of the molecule must play a constant role in the polymerization of the molecule, and the functional specialization of the molecule to meet different physiological needs most likely comes about by modulation at the carboxy terminus (Basi and Storti, 1986). These data supports the idea that EgTrpA could be a muscular isoform while EgTrpB could be a non-muscle one.

Vertebrate tropomyosin are classified into two groups, the high molecular weight (HMW) group containing 284–281 amino acids and the low molecular weight (LMW) group containing 245–251 amino acids (Perry, 2001). The two major tropomyosin isoforms present in yeast have even lower molecular weights, containing 199 and 161 amino acids residues respectively (Drees et al., 1995). EgTrpA and EgTrpB seem to be similar to HMW and LMW vertebrate group respectively.

The sequence analysis shows that all identified introns contain donor GT and acceptor AG sites in the exon–intron boundaries that

characterize this type of processing (Breathnach and Chambon, 1981). With the exception of the intron IV donor site, all donor sites are at a consensus context. The intronic regions contain the typical polypyrimidine tracts of different extension, upstream from the AG site. Particularly in the introns II and VIII, acceptor sites are within a consensus sequence. The choice of splice sites is determined by interplay between *cis*-acting elements within the pre-mRNA and *trans*-acting regulatory factors (Black, 2003; Grabowski and Black, 2001; Cáceres and Kornblihtt, 2002; Matlin et al., 2005). For example, polypyrimidine tract binding protein, PTB/hnRNPI, is involved in the repression of a wide range of tissue-specific exons (Black, 2003; Grabowski and Black, 2001; Cáceres and Kornblihtt, 2002; Matlin et al., 2005; Wagner and García-Blanco, 2001). It was reported that RNA-binding motif protein 4, RBM4, can activate the selection of skeletal muscle-specific exons of vertebrate α -TM isoform, possibly via binding to intronic pyrimidine-rich elements. By contrast, the splicing regulator PTB excluded these exons; moreover, RBM4 antagonized this PTB-mediated exon exclusion likely by competing with PTB for binding to a CU-rich element (Lin and Tarn, 2005). Conserved alternative splicing regulatory motifs are found in introns surrounding alternative spliced *egtrp* exon 4 confirming the existence of exon 4 alternative skip. It is interesting that the existence of several hexameric motifs in exon 7 surrounding introns (VII and VIII) suggesting that alternative regulatory factors could be implicated in exon 7 or 8 skipping. Many of these high-scoring nematode hexamer motifs correspond to known mammalian

splicing regulatory sequences, such as TGCATG, indicating that the alternative splicing regulating mechanism is highly conserved in metazoans (Kabat et al., 2006). *egtrp* intron VI suffers a type of alternative splicing called intron retention. While exon skipping seems to be the most frequent alternative splicing type (Modrek et al., 2001), intron retention is certainly the least studied especially because these variants are believed to be largely derived from unspliced or partially spliced pre-mRNAs. It was suggested that a significant fraction of the intron retention events is not spurious and probably reflects biological significance (Galante et al., 2004; Rio, 1991; Le Hir et al., 2002).

A different splicing event has been reported in *E. granulosus* where alternative thioredoxin glutathione reductase mRNAs arise from *EgTGR* trans-splicing (Agorio et al., 2003). Both splicing processes represent generalized mechanisms that increase the diversity of gene expression.

In addition, we boarded the study of putative tropomyosin isoforms in *E. granulosus* detecting isoforms at protein level. From a Western blot experiment, three protein bands are marked from a protoscoleces total protein extract, whose apparent molecular masses do not coincide with those deducted from their coding sequences isolated by RT-PCR. Discrepancy between the theoretical and the apparent molecular mass has been reported previously (Blackshear, 1993) and can be attributed to amino acids composition and protein structure.

Tropomyosins expression study is also addressed by whole mount immune-localization and in situ hybridization. The suckers are mainly formed by muscle cells and are involved in adhesion to the small intestine walls of the final host, who cling heavily on the villi base (Thompson, 1995). The sucker signal is consistent with muscle tropomyosin isoforms presence, while the weak and wide-spread signal could correspond to non-muscle isoforms. The location of the signal at caudal level could also be associated with muscular structures. PSs join germinal layer through the stalk and it is then reabsorbed (Thompson, 1995), often sketches remain at caudal level. We have seen through in vivo optical microscopy that the stalk has movement (data not shown), and we assume that this area should be rich in muscle cells. There is no signal in whole mount immune-localization at suckers level (apical region) when PSs are invaginated suggesting that the antiserum does not penetrate properly at the apical tegument level because of its particular microtriches composition (Antoniu and Tselentis, 1993). To deepen this study, it would be interesting to perform an electronic immunohistochemistry analysis to characterize *EgTrps* intracellular location and verify their interactions with other proteins such as actin.

Tropomyosins expression at protein level is coincident with its expression at mRNA level, according to in situ hybridization assays with a very strong signal in the suckers and a milder and widespread expression in the rest of the PS. The latter signal may belong to non-muscle isoforms. This indicates that tropomyosins transcription and translation take place in the same region of the parasite. Expression studies could be deepened through in situ hybridization assays using adequate probes to the known isoforms to a better discrimination of location of the expression.

This report is an important contribution to *E. granulosus* biology considering that helminth muscle proteins are considered potent antigens that may be vaccine candidates (Soisson et al., 1992; Martin et al., 1997; Yang et al., 1999; Flanigan et al., 1989; Nara et al., 1994; Nanduri and Kazura, 1989; Li et al., 1993; Taylor et al., 1996; Jenkins et al., 1998; Fu et al., 1999; Fraize et al., 2005; Petavy et al., 2008). In addition, the characterization of *E. granulosus* tropomyosin isoforms could facilitate the identification of key molecules involved in basic mechanisms, such as muscle contraction and alternative splicing. Until now, the existence of alternative splicing in this parasite has not been reported, thus constituting a new field of study.

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