Cloning and analysis of a *Trichinella britovi* gene encoding a cytoplasmic heat shock protein of 72 kDa

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SUMMARY

A gene encoding a protein of 646 amino acid residues with a molecular mass of 71·3 kDa showing homology to the cytoplasmic form of the 70 kDa heat shock protein was cloned and sequenced from the nematode parasite *Trichinella britovi* (Tb). The gene was expressed *in vitro* as a protein of 71 kDa that was immunoprecipitated by a *Trichinella*-infected rabbit serum. Monospecific polyclonal antibodies raised against the recombinant Tb Hsp70 expressed in *Escherichia coli*, recognized a protein of 70 kDa by Western blot analysis of Tb soluble antigen (muscular stage). Tb Hsp70 was located in the nuclei of the muscle larvae as determined by the indirect immunofluorescent pattern on cross-sections of the worm. The expression of this protein was not detected in adult worm nuclei suggesting a differential expression of Hsp70 between the 2 stages of *Trichinella*.

Key words: *Trichinella britovi*, cDNA, cytoplasmic Hsp70, antigenicity, immunolocalization.

INTRODUCTION

Trichinella is unique among nematode parasites since during its life-cycle, a free-living stage is not present.† Also species of this parasite have a worldwide distribution and they can infect a wide range of hosts. The last report on the taxonomy of *Trichinella* genus (Pozio *et al*. 1992) included 8 taxa. Among them, *Trichinella britovi* (Tb) is the etiological agent of sylvatic trichinellosis which is widespread in temperate areas. Infection of mammals and some birds occurs when meat containing the 1st-stage larvae is ingested. *Trichinella* larvae can develop in striated skeletal muscles and induces the formation of the 'nurse cells' (Despommier, 1998). It has been shown that molecules from the muscle larvae (ML) directly interact with myocyte nuclei (Lee *et al*. 1991; Ko *et al*. 1994). These interactions involve changes in genomic expression of myocyte nuclei by molecules which participate in translocation and in regulation such as heat shock proteins (Hsp). These proteins, known to be among the most highly conserved proteins present in all organisms, are considered as molecular chaperones (Ulrich Hartl, 1995; Hendrick & Ulrich Hartl, 1995) during synthesis, folding, assembling, transport and degradation of proteins. These proteins also modulate the effects of environmental stress (Macario, 1995). In particular the expression of the 70 kDa heat shock protein (Hsp70) is involved directly or indirectly in the development of several organisms. For example, knocked out *hsf-1* gene (heat shock transcription factor which regulates the expression of *hsp* genes) caused disregulation of development, reproduction and responses to physiological and pathophysiological stress (Boscheinen, 1997). On the other hand parasite Hsp seem to play a major role in the adaptation of the parasites to the mammalian host and also in parasite differentiation, infectivity or virulence, and, they function (especially Hsp70) as prominent antigenic proteins (Polla, 1991; Maresca & Carratù, 1992).

Recently, several proteins induced by heat were identified after radio-isotope labelling *Trichinella* ML (Ko & Fan, 1996) but no *Trichinella* Hsp has been well characterized. Their purification using conventional methods is time consuming and also difficult to achieve. So, an alternative strategy to study Hsp in *Trichinella* is to clone and express the *hsp* genes. Based on this, the present report describes the cloning and characterization of Tb cytoplasmic form of Hsp70 to elucidate its antigenicity and physiological function. Antigenicity of Hsp70 was

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analysed using sera from a Tb-infected rabbit. An anti-Tb Hsp70 polyclonal serum was raised to determine Hsp70 expression and localization in adult and ML stages.

MATERIALS AND METHODS

Parasite and antigenic preparations

Tb (M/VUL/FR/87/ISS137) ML were recovered from infected OF1 mice by acid-pepsin digestion (Kohler, 1981) of striated muscles 47 days post-infection (p.i.) and incubated in RPMI 1640 (Gibco BRL) culture medium (Murrell *et al*. 1986). The adult stage was collected from the mice gut 4 days p.i. (1500 ML/mouse). Soluble antigen was prepared from Tb ML or *Escherichia coli* resuspended in NaCl solution (1 g/l). The suspension was submitted to 3 cycles of freezing–thawing. The larval or bacterial fragments were then sonicated 3 times for 5 min at 4 °C, and the supernatants were collected after centrifugation at 30000 ***g*** for 1 h at 4 °C. Excretory–Secretory (E–S) antigen was prepared as described by Gamble *et al*. (1988).

Purification of Tb mRNA and construction of a cDNA library

Purified *Trichinella* ML were incubated for 20 h at 37 °C, then total RNA was isolated from ML according to the method described by Chomczynski & Sacchi (1987). ML were suspended in guanidium thiocyanate 4 M, sodium citrate 25 mM, pH 7, sarcosyl 0·5 %, 2-β mercaptoethanol 0·1 M before being disrupted in a Dounce Potter homogenizer. Selection of poly A+ RNA was achieved using a column of oligo (dT)-cellulose (Bioprobe) and a λZap II cDNA library was constructed according to the manufacturer's instructions (Stratagene).

Isolation of a Tb hsp70 clone

Degenerate oligonucleotide primers were synthesized by Eurogentec (Primer A (forward): 5′-CAR GCN ACN AAR GAY GCN GG-3′/Primer B (reverse): 5′-GC NAC NGC YTC RTC NGG RTT-3′). PCR amplification was carried out with Tb ML cDNA using the oligonucleotide primers A and B as described by Galley, Singh & Gupta (1992). After purification, the amplified fragment was labelled with ECC kit (Amersham) and used as a probe to screen $1{\cdot}5 \times 10^5$ recombinant phages of Tb cDNA library. Positive phage clones were selected and plaques purified 3 times by hybridization at each step to check the presence of the insert.

DNA sequencing

pBluescript plasmids containing cDNA were derived from the λZap phages using the *in vivo* excision procedure previously described by Short *et al*. (1988). Two independent clones, designed pBS33 and pBS21 were selected and the sonicated cloned inserts were subcloned into the *Sma*I site of M13mp18. DNA sequences were performed using the sequenase version 2.0 kit (Amersham, UK) by the dideoxy chain termination technique (Sanger, Nicklen & Coulson, 1977). Sequence data were analysed and assembled with PCgene (Intelligenetics, CA, Version 6.6, 1995) computer program.

Southern blot analysis

Total cellular DNA was isolated from Tb ML applying the Genomic DNA Preparation Kit (Qiagen) according to the manufacturer's specifications. Two μg of DNA were then digested with one of several restriction enzymes before being resolved on a 1 % agarose gel. DNA was then transferred to 0·45 μm nitrocellulose filter with a vacuum blotter (Appligene). The filter was hybridized for 12 h at 55 °C with [^{32}P] random primed labelled *Msc*I/*Sac*I fragment from clone 33 in hybridization buffer (6 × SSC, 5 × Denhart's solution, 0·1 % SDS), washed twice at a final stringency of 0·1 × SSC, 0·1 % SDS at 55 °C for 30 min and exposed to X-ray films for 24 h at 70 °C.

Hsp70 comparison analysis

The amino acid sequence of Tb Hsp70 was entered into an extensive alignment of 200 cytoplasmic Hsp70 homologues using the MUST package (Philippe, 1993); 609 unambiguously aligned positions were used. Phylogenetic trees were constructed using maximum likelihood, maximum parsimony and distance methods with the programs PROTML (Adachi & Hasegawa, 1992) version 2.2, PAUP (Swofford, 1993) version 3.1 and NJ in the MUST package (Philippe, 1993) version 1.0, respectively. The distances were computed using the substitutions model of Kimura (1983). Maximum parsimony trees were obtained by 100 random addition heuristic search replicates and maximum likelihood trees by the quick add OTUs search, using the JTT model of amino acid substitution and retaining the 1000 top ranking trees (options -jf -q -n 1000). Bootstrap proportions were calculated by analysis of 200 replicates for maximum parsimony analysis and 1000 for neighbour joining analysis (Saitou & Nei, 1987). For maximum likelihood analysis, bootstrap proportions were computed by using the RELL method (Kishino, Miyata & Hasegawa, 1990) due to computing time limitations.

In vitro *translation and radio-immunoprecipitation*

Cell-free translation of mRNA synthesized *in vitro* from pBS33 (Ampliscribe Kit, Tebu) was carried out with rabbit reticulocyte lysates (Promega). The

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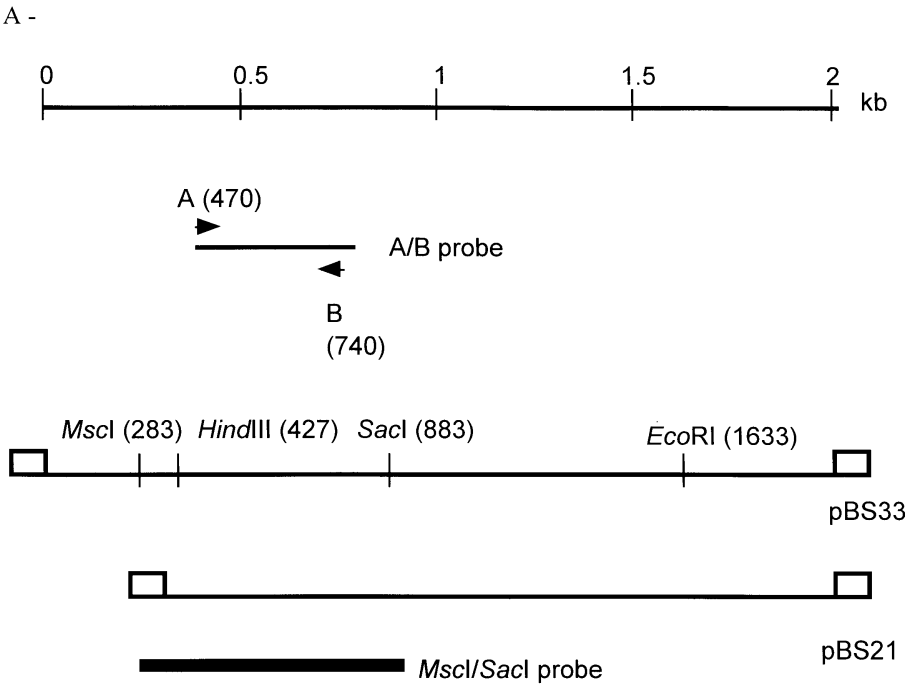

Fig. 1. (A) Map of pBS33 and pBS21 inserts, with relative position of *Msc*I/*Sac*I and A/B probes. The numbers given in parentheses indicate the position in respect of the first nucleotide of the nucleotide sequence Y13114 in the EMBL data base. (B) Southern blot analysis of the *hsp70* gene of Tb. Samples of 10 μg of *Hin*dIII or *Eco*RI-digested Tb genomic DNA were hybridized with [^{32}P]-labelled *Msc*I/*Sac*I probe. Molecular weights are in kb.

proteins were radio-isotope labelled with [^{35}S]methionine (> 37 Tbeq/mmol, Amersham). Immunoprecipitations were then carried out as described previously (Vautherot, Laporte & Boireau, 1992).

Expression of GST fusion protein in E. coli

The *Hin*dIII–*Eco*RI fragment of pBS33 was ligated in frame to the *Bam*HI/*Eco*RI cleaved pGEX-5 vector (Pharmacia). *Hin*dIII and *Bam*HI extremities became compatible sites after being blunt ended with Klenow fragment (Boerhinger). The resulting vector pGEX-5Hsp70 was introduced in DH5α *Escherichia coli* using Cellject Basic Electroporator (Equibio). Screening of transformants and small-scale expression of GST fusion protein was done according to the method described by Smith & Johnson (1988). The insoluble fraction, containing the recombinant Tb Hsp70 (ΔHsp70), was suspended in phosphate-buffered saline (PBS 1X).

Preparation of immune sera against Tb E–S antigen and ΔHsp70 protein

Tb E–S antigen or the ΔHsp70 protein were mixed with an equal volume of complete Freund's adjuvant and used to immunize intraperitoneally OF1 mice; a booster was given 2 weeks later and serum samples were collected 1 week after the last injection.

A New Zealand rabbit was infected with 5000 Tb ML. The animal was bled once a week for 6 months; the serum was then tested by indirect immunofluorescent antibody test on ML cryostat sections.

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     -GCACGAGCTTGTGGGTTGTGAGTAATTTTTCTGCTTCGTTGCACG ATG TCG AAG AAT GCT ATT GGA ATT GAT 72
                                                     M   S   K   N   A   I   G   I   D9
TTG GGT ACT ACG TAC AGT TGT GTT GGC GTG TTC CAG CAT GGT AAA GTT GAA ATT ATT GCC AAC GAT 138
 L   G   T   T   Y   S   C   V   G   V   F   Q   H   G   K   V   E   I   I   A   N   D31
CAA GGG AAT CGT ACC ACG CCA AGT TAT GTT GCT TTC ACC GAT ACC GAA CGA CTG ATT GGA GAT GCG 204
 Q   G   N   R   T   T   P   S   Y   V   A   F   T   D   T   E   R   L   I   G   D   A53
CAA AAA AAT CAA GTT GCG TTG AAT CCT CAT AAT ACA GTT TTT GAT GCG AAG CGA CTC ATC GGT CGT 270
 Q   K   N   Q   V   A   L   N   P   H   N   T   V   F   D   A   K   R   L   I   G   R75
CGA TTT GAT GAT GCT GCG GTG CAG TCT GAT ATG AAA CAT TGG CCA TTC AAA ATT ATC AAC GAT GGA 336
 R   F   D   D   A   A   V   Q   S   D   M   K   H   W   P   F   K   I   I   N   D   G97
TCG AAA CCT AAA ATT CAA GTC GAA TAC AAA GGC GAG TCG AAG TCA TTT ACA CCA GAA GAA ATC AGT 402
 S   K   P   K   I   Q   V   E   Y   K   G   E   S   K   S   F   T   P   E   E   I   S119
GCT ATG GTG TTA GTG AAG ATG AAG GAA ACT GCC GAA GCT TAT TTG GGA AAA ACT GTT AAG GAT GCC 468
 A   M   V   L   V   K   M   K   E   T   A   E   A   Y   L   G   K   T   V   K   D   A141
GTA ATT ACT GTT CCG GCA TAT TTT AAC GAT TCT CAG CGT CAG GCG ACC AAA GAC GCT GGA ACG ATT 534
 V   I   T   V   P   A   Y   F   N   D   S   Q   R   Q   A   T   K   D   A   G   T   I163
TCT GGT TTG AAT GTT TTG CGT ATC ATC AAC GAA CCA ACA GCG GCT GCA ATT GCT TAT GGA CTT GAT 600
 S   G   L   N   V   L   R   I   I   N   E   P   T   A   A   A   I   A   Y   G   L   D185
AGA AAG GGT GGC GGT GAA CGA AAT GTT CTG ATA TTT GAC CTT GGT GGT GGT ACT TTT GAT GTG TCT 666
 R   K   G   G   G   E   R   N   V   L   I   F   D   L   G   G   G   T   F   D   V   S207
ATT TTG ACC ATC GAA GAT GGC ATT TTT GAG GTC AAG TCT ACC GCT GGT GAC ACT CAT TTG GTG GGT 732
 I   L   T   I   E   D   G   I   F   E   V   K   S   T   A   G   D   T   H   L   G   G229
GAG GAC TTC GAC AAT CGC ATG GTA AAT CAT TTC GTG GCC GAG TTT AAG CGC AAG AAC AAG AAG GAC 798
 E   D   F   D   N   R   M   V   N   H   F   V   A   E   F   K   R   K   N   K   K   D251
ATG TCA TCT AAC CCA CGC GCA TTG CGT CGC TTG CGC ACA GCT TGT GAA CGA GCG AAG CGC ACG TTG 864
 M   S   S   N   P   R   A   L   R   R   L   R   T   A   C   E   R   A   K   R   T   L273
AGT AGC TCT ACA CAG GCG AGC ATT GAA ATT GAT AGC CTC TAC GAA GGC ATT GAT TTC TAC ACC ACT 930
 S   S   S   T   Q   A   S   I   E   I   D   S   L   Y   E   G   I   D   F   Y   T   T295
ATT ACA AGA GCT CGC TTC GAA GAG TTG AAT GCT GAC TTG TTC CGT TCT ACT TTG GAA CCA GTG GAA 996
 I   T   R   A   R   F   E   E   L   N   A   D   L   F   R   S   T   L   E   P   V   E317
AAA GCT CTT CGT GAT GCG AAA TTA GAC AAA GCA GTG ATT CAT GAA GTT GTT TTG GTG GGC GGT TCT 1062
 K   A   L   R   D   A   K   L   D   K   A   V   I   H   E   V   V   L   V   G   G   S339
ACT CGC ATT CCT AAA GTT CAG AAG TTG TTG CAA GAT TTT TTT AAT GGG AAA GAA CTT AAC AAA AGC 1128
 T   R   I   P   K   V   Q   K   L   L   Q   D   F   F   N   G   K   E   L   N   K   S361
ATC AAT CCC GAC GAA GCT GTT GCC TAT GGA GCA GCT GTT CAA GCG GCA ATT TTG TCC GGC GAA AAG 1194
 I   N   P   D   E   A   V   A   Y   G   A   A   V   Q   A   A   I   L   S   G   E   K383
CAC GAA GCA GTT CAA GAT CTG TGT CTT CTT GAT GTG ACA CCT CTT TCG TTG GGT ATT GAA ACC GCA 1260
 H   E   A   V   Q   D   L   L   L   L   D   V   T   P   L   S   L   G   I   E   T   A405
GAG GGT GTA ATG ACA GCG CTA ATT AAG CGC AAC ACT ACC ATC CCT ACA AAA GTA TCG CAA GTG TTC 1326
 G   G   V   M   T   A   L   I   K   R   N   T   T   I   P   T   K   V   S   Q   V   F427
ACT ACT TAC TCG GAC AAT CAG CCT GGG GTA TTG ATT CAA GTG TAC GAA GGA GAG CGT GCA ATG ACT 1392
 T   T   Y   S   D   N   Q   P   G   V   L   I   Q   V   Y   E   G   E   R   A   M   T449
AAA GAC AAT AAT TTG CTT GGA AAA TTT GAG TTG ACT GGA ATC CCA CCT GCT CCA CGT GGT GTT CCA 1458
 K   D   N   N   L   L   G   K   F   E   L   T   G   I   P   P   A   P   R   G   V   P471
CAA ATT GAA GTT ACT TTT GAC ATC GAT GCC AAC GGC ATT TTG AAC GTT TCT GCC GTT GAC AAA AGT 1524
 Q   I   E   V   T   F   D   I   D   A   N   G   I   L   N   V   S   A   V   D   K   S493
ACC GGC CGT CAA AAC AAA ATC ACC ATC ACT AAT GAC AAA GGT CGG CTT AGC AAA GAG GAC ATC GAT 1590
 T   G   R   Q   N   K   I   T   I   T   N   D   K   G   R   L   S   K   E   D   I   D515
CGT ATG GTG CGA GAA GCC GAC CAA TAT AAA CAG GAA GAT GAA AAG CAG AGA GAC CGA ATT CAA GCG 1656
 R   M   V   R   E   A   D   Q   Y   K   Q   E   D   E   K   Q   R   D   R   I   Q   A537
AAA AAT GGT CTG GAA AGT TAT GCT TTT AAT GTT AAA TCA ACA ATT GAA GAT GAG AAA TTG AAA GAT 1722
 K   N   G   L   E   S   Y   A   F   N   V   K   S   T   I   E   D   E   K   L   K   D559
AAA ATT CCT GAA TCA GAT CGT AAG GCG GTG TTG AAC AAA TGT GAA GAA GTT TTG CGC TGG CTG GAA 1788
 K   I   P   E   S   D   R   K   A   V   L   N   K   C   E   E   V   L   R   W   L   E581
ACA AAT CAG TTG GCT GAA AAA GAT GAA TTT GAG CAC AAG CAG AAA GAC CTG GAA TCG CTG TGC AAT 1854
 T   N   Q   L   A   E   K   D   E   F   E   H   K   Q   K   D   L   E   S   L   C   N603
CCA ATA TGC AAG TGT ATC AGA ATG CGT GTG AAT GCC AGT GGT ATG CCG AAT TTG CAG TTC CTG CAG 1920
 P   I   C   K   C   I   R   M   R   V   N   A   S   G   M   P   N   L   Q   V   D   L 625
GTG GAC CTC CAG GTG GCG TCG TCT CGT GGT GGT GGT GGT GGT CCT ACT ATT GAA GAA GTT GAT TAA 1986
 Q   V   A   S   G   G   G   S   R   G   G   G   G   G   P   T   I   E   E   V   D646
AAAAGCATATTGTGATGATGATGATTACCTAGTCAACGCGCGTATTATATATACTACAATATTCTGGGCGTAGTTCTAATAAATTAT
ATAAAATGAAAAAAAAAAAAAAAAAAAAAAAAAAA 2107
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Fig. 2. Nucleotide sequence of the gene encoding the Hsp70 protein and predicted amino acid sequence deduced from the cDNA inserts (Fig. 1A). A putative proteolytic cleavage site is present between amino acids 401 and 402. The putative ATP binding site is underlined. The 3 Hsp70 signatures are dotted underlined, putative calmodulin binding site is in italics and dotted underlined and, finally, the consensus nuclear targeting signal is double underlined. The last 4 amino acids EEVD are specific to cytoplasmic protein.

Serum samples were obtained from non-infected animals for use as a negative control. A monoclonal antibody named G19 (Mattei *et al*. 1989) raised against *Plasmodium falciparum* Hsp70 conserved epitope was used as a positive control.

Solid-phase competitive ELISA for binding of mouse anti-Tb Hsp70 sera

Wells in microtitre plates (Probind plates, Falcon) were coated with 5–0·04 μg of Tb soluble antigen or

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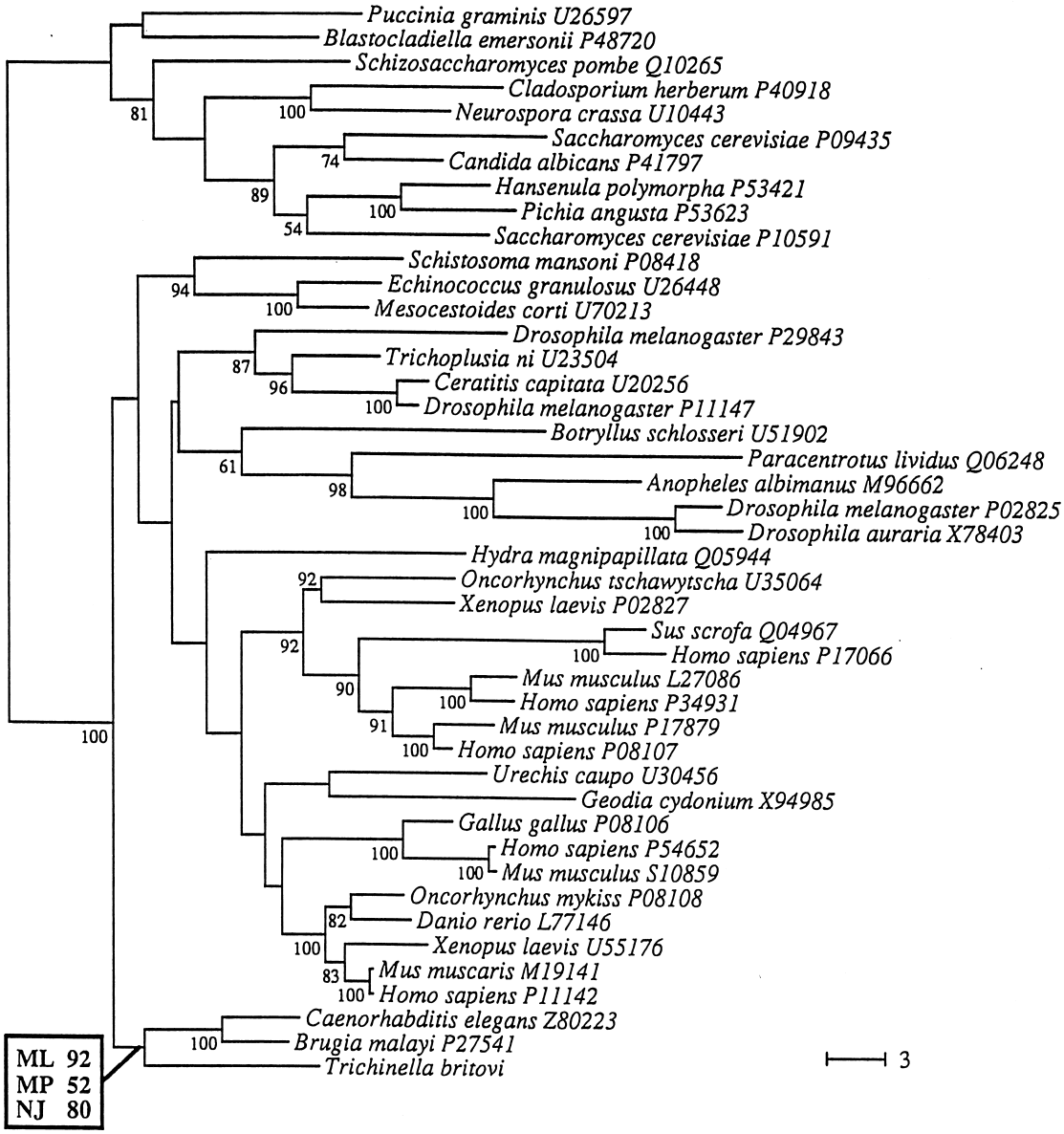

Fig. 3. Phylogenetic tree based on amino acid comparison of Tb cytoplasmic Hsp70 sequence with 43 other cytoplasmic Hsp70 sequences. The outgroup is represented by fungi. The tree was constructed using the maximum likelihood method employing the JTT model of amino acid substitution (ln L = −14851·9). The reliability of nodes is given as bootstrap values for each node (when BP > 50 %), and those obtained with maximum parsimony and neighbour joining methods are given only for the node of special interest.

E. coli antigen and incubated for 12 h at 4 °C. Plates were washed 4 times in PBS containing 0·05 % Tween 20 and unreacted sites were blocked by incubation for 1 h in a 3 % solution of BSA. After washing, the wells were incubated with 100 μl of mouse anti-Tb *Δ*hsp70 for 1 h. The pre-incubated sera were then titrated by a standardized ELISA (Calamel & Lambert, 1988). The percentage inhibition was determined using the following formula: Titration of pre-incubated serum/Titration of non-preincubated serum × 100.

Polyacrylamide gel electrophoresis and Western blotting

Soluble or E–S antigens were separated by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) under reducing conditions (Laemmli, 1970). Transfer of proteins from polyacrylamide gels onto nitrocellulose membranes for immunodetection of parasite antigens was performed as described (Towbin, Staehelin & Gordon, 1979). The bound alkaline phosphatase was detected using nitroblue-tetrazolium/bromochloroindoyl-phosphate (NBT/BCIP, Gibco-BRL) according to the manufacturer's instructions.

Indirect immunofluorescent antibody test (IIF)

Indirect immunofluorescent antibody test was performed on sections of muscle larvae or adult stage obtained in the cryostat as previously described (Boireau *et al.* 1997). Nuclear counterstaining was

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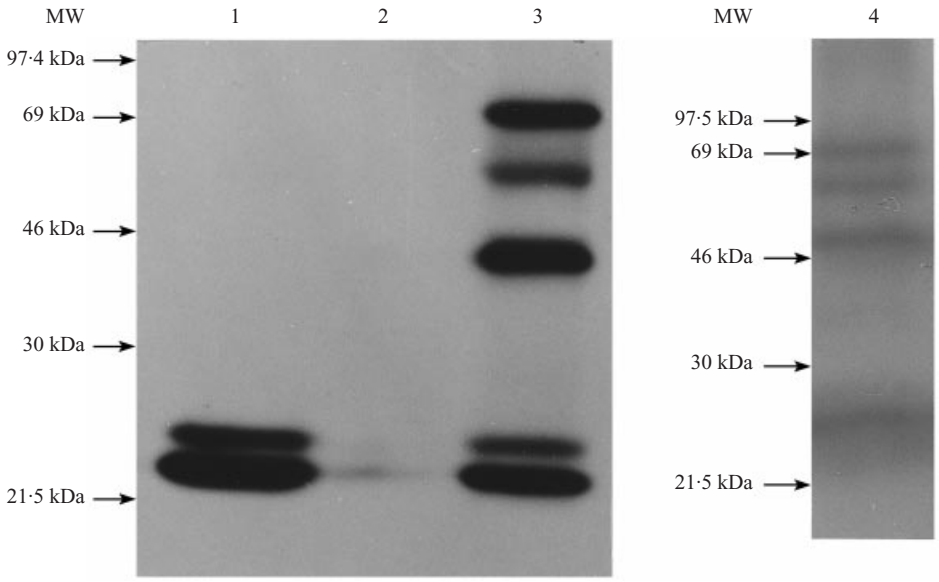

Fig. 4. Immunoprecipitation of the *in vitro* translation products generated by pBS33 with various sera. Lane 1: monoclonal antibody raised against *Plasmodium falciparum*; lane 2: non-infected rabbit serum; lane 3: Tb-infected rabbit serum. After dissociation, immunoprecipitates were separated on a 10% polyacrylamide gel. Lane 4: cell-free translation of mRNA synthesized *in vitro* from pBS33 using a rabbit reticulocyte lysate. The protein molecular weight markers (in kDa) are indicated on the left of each gel.

performed by incubating cryostat sections with Hoechst dye Nr 33 258 (Bisbenzimide, Sigma) for 45 min before being washed and visualized with an epifluorescence microscope.

RESULTS

Isolation of cDNA clones, sequencing and analysis of the deduced amino acid sequence

Tb mRNA was extracted directly after the purification of ML or after the larvae had been incubated at 37 °C for 20 h. Using the latter incubation period the ratio of purified mRNA increased 5 times. Approximately 5 μg of poly(A) mRNA were purified and used to construct a cDNA library consisting of 2×10^6 primary recombinant bacteriophages. Reverse transcription carried out on mRNA of Tb followed by PCR using degenerated oligonucleotide primers A and B (Galley *et al*. 1992) derived from the 5′ and 3′ conserved sequences of *hsp70* genes resulted in the synthesis of a 650 bp Hsp70 probe (A/B) (Fig. 1A). Samples of $1{\cdot}5 \times 10^5$ bacteriophages of the cDNA library were screened with A/B probe. Two phage clones (pBS21 and pBS33) remained positive through 3 successive rounds of screenings and were found to have nearly identical restriction sites. The nucleotide sequence of the 2 cDNA clones confirmed the preliminary analysis that pBS21 (1·6 kb) overlapped the 3′ end of pBS33 (2·1 kb). The largest clone had a total of 2107 bp with the ATG start codon at position 46 and an in frame stop codon 1938 bases downstream. The consensus cDNA sequence terminated with a poly A tail of 26 bp and was characterized by an AT content of 57%. The main open reading frame encoded a 646 amino acid protein with a calculated molecular mass of 71·3 kDa and an isoelectric point of 5·6 (Fig. 2). Several Hsp70 motifs were identified on the deduced amino acid sequence: a putative ATP and calmodulin binding sites, 3 Hsp70 signatures, a conserved nuclear targeting signal, and the 4 last amino acids EEVD identifying cytoplasmic Hsp70 (Fig. 2).

To confirm the origin of the selected clones, multiple sequence alignment of the deduced amino acid sequence was performed with other known Hsp70. The amino acid sequence showed lower similarity for mitochondrial or endoplasmic protein than cytoplasmic Hsp70. The comparison with cytoplasmic Hsp70 revealed between 65 and 80% identity which confirmed that cloned *Trichinella* sequence was the cytoplasmic form of Hsp70. To define more precisely its evolutionary relationship, a detailed analysis was carried out with the standard method and considering all the metazoan cytoplasmic Hsp70. The tree (Fig. 3) was rooted on 10 fungal sequences. As expected, this analysis showed with a relatively good statistical support (bootstrap value between 52 and 92%, depending on the tree reconstitution method used) that *Trichinella* is closely related to 2 other nematodes which *hsp70* genes have been sequenced (*Brugia* and *Caenorhabditis*). The copy number of *hsp70* gene in *Trichinella* genus was investigated by a Southern blot analysis performed with the internal *Msc*I/*Sac*I probe (Fig. 1A). Two main fragments hybridized when using genomic DNA digested by *Eco*RI or *Hin*dIII (Fig. 1B) suggesting that the *Trichinella* genome has at least 2 genes belonging to *hsp70* family in absence of any intron in the region overlapped by the probe. This analysis was confirmed by the recent

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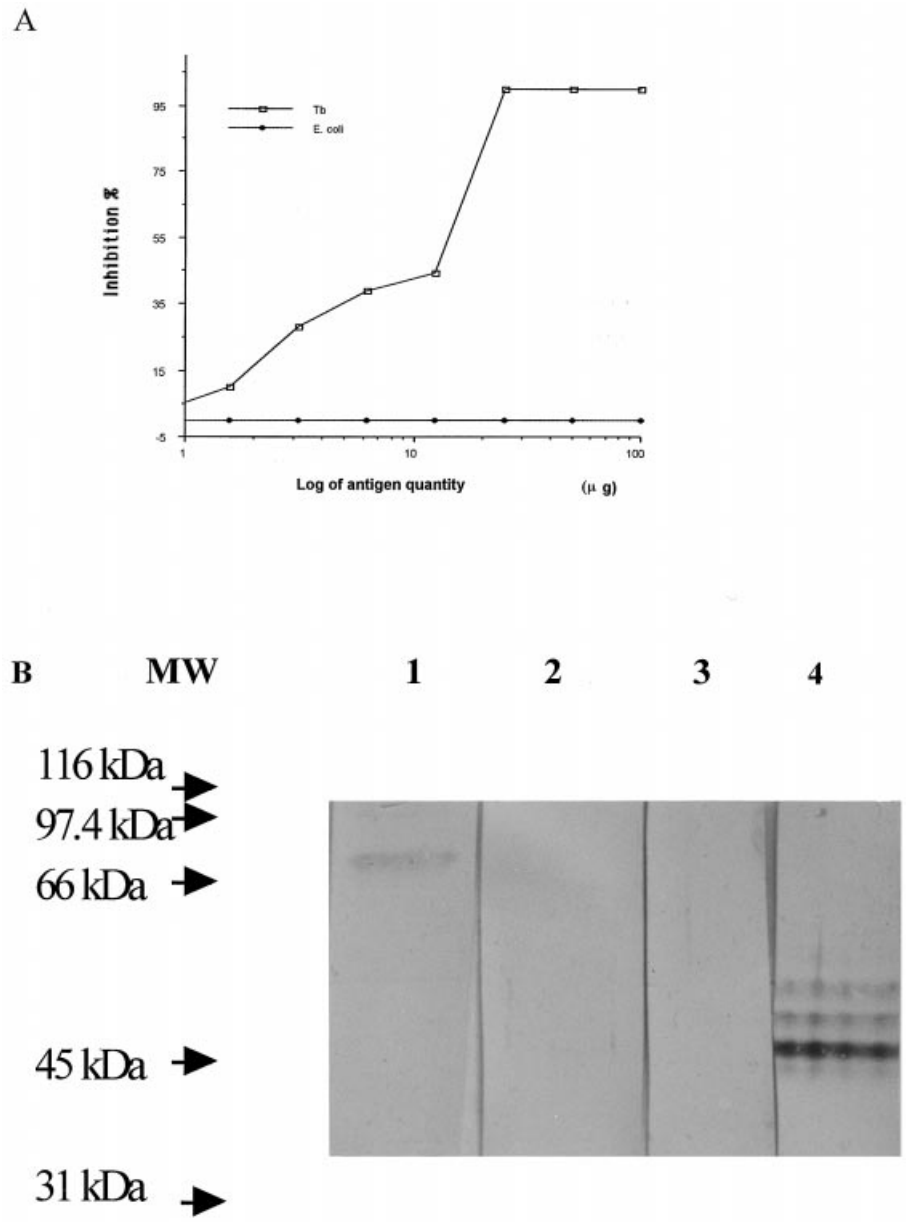

Fig. 5. (A) Monospecificity of the anti-Tb *Δ*Hsp70 serum was tested by incubating the anti-Tb *Δ*Hsp70 serum with either Tb soluble antigen or bacterial antigen. The residual anti-*Δ*Hsp70 antibodies were titrated by standard ELISA. Monospecificity was detected by comparing the inhibition of serum titration after incubation with Tb or bacterial antigen preparations. Inhibition percentage = (pre-incubated serum titration/non-preincubated serum titration) $\times 100$. (B) Immunoblot of 2 antigenic preparations of Tb muscle larvae with an anti-*Δ*Hsp70 serum. Soluble antigen (20 μg) (lanes 1 and 2), E–S antigen (20 μg) (lanes 3 and 4) were separated by SDS–PAGE (10 %) gel, transferred to nitrocellulose filter and immunolabelled with the primary antiserum. The membrane was then immunolabelled with phosphatase-conjugated goat anti-mouse antibody and developed. Lanes 1 and 3 were incubated with anti-Tb *Δ*Hsp70 serum, lanes 2 and 4 were respectively incubated with non-infected mouse serum and anti-E–S serum. The protein molecular weight markers (in kDa) are indicated on the left gel.

cloning and sequencing of a constitutive cytoplasmic hsp70 gene of *Trichinella* (data not shown) that share 96 % nucleotide identify with the ORF sequence of pBS33 clone.

Antigenicity of Tb Hsp70

The antigenicity of Tb Hsp70 was analysed by immunoprecipitation of the *in vitro* translated *hsp70* mRNA using serum of infected rabbits. The RNA transcripts synthesized from pBS33 clone were translated in rabbit reticulocyte lysates and the products were separated by SDS–PAGE into 4 main polypeptides with molecular mass of 71, 60, 50 and 26 kDa (Fig. 4). A polypeptide of the expected size (71 kDa) was immunoprecipitated together with other smaller polypeptides by serum of a Tb infected rabbit (28 p.i.) (Fig. 4, lane 3). Most of these peptides corresponded to products obtained due to an arrest of *in vitro* elongation of transcription or translation

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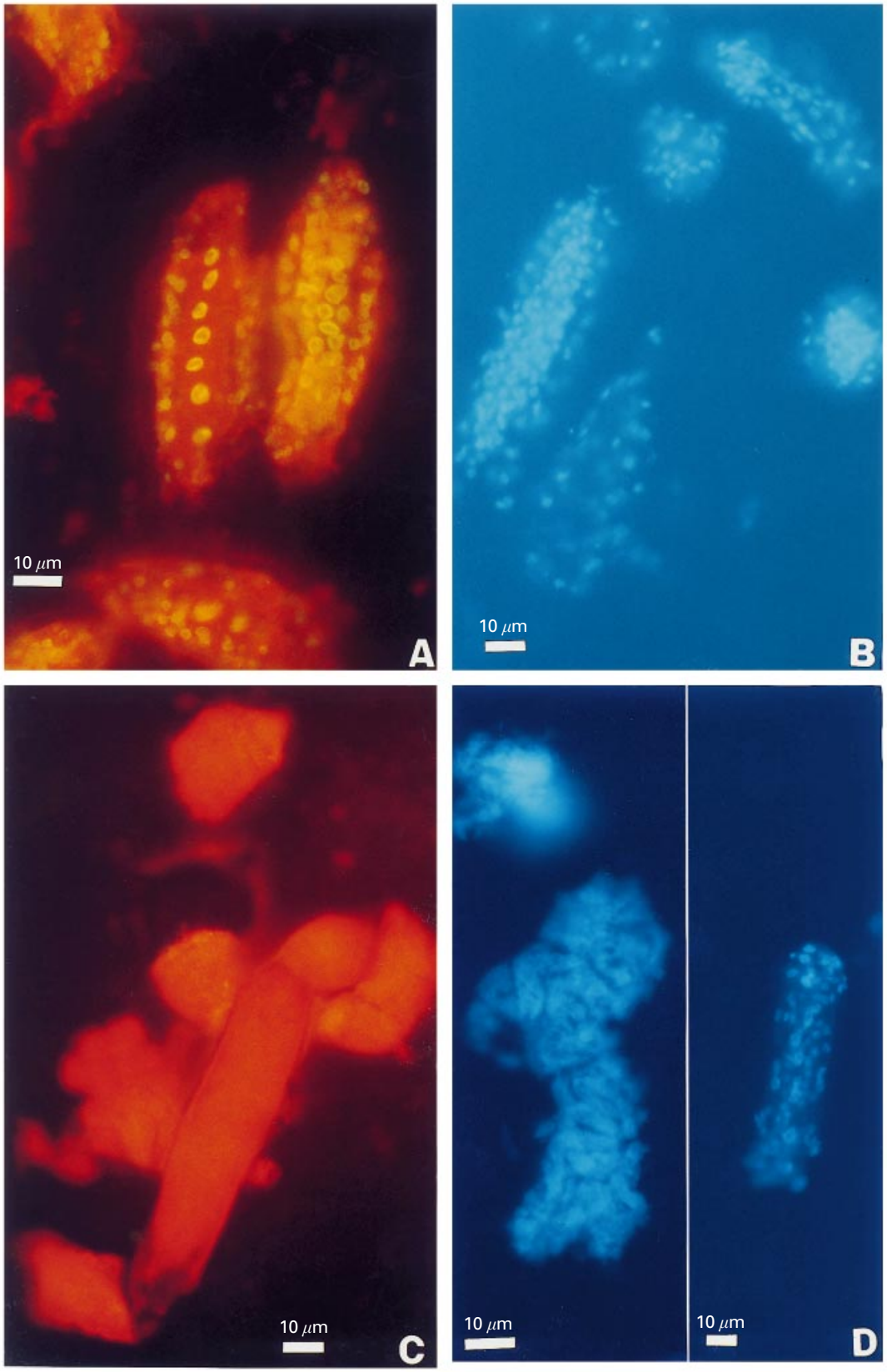

Fig. 6. Immunolocalization of Hsp70 on cryostat sections of Tb muscle larvae or adult stages. (A) Indirect immunofluorescence with anti-Tb *Δ*Hsp70 serum on ML cryostat sections. Stichocyte nuclei are characterized by a line of large dots in the centre of the section. Muscle cell nuclei are localized in the periphery of the larvae. Cord cell nuclei are visualized as a sparse line of dots just interior from the muscle cells. The genital primordium cell nuclei are composed of a multitude of dots pressed close together on the whole *Trichinella* posterior sections. (B) Staining of larval cell nuclei visible by Hoechst nuclear counterstaining. (C) Immunostaining of adult worms with anti-Tb *Δ*Hsp70: immunofluorescence was not detected. The red colour is induced by the Evans Blue counterstaining and corresponds to the background. (D) Staining of adult cell nuclei visible by Hoescht nuclear counterstaining. A female with embryos can be recognized on the left part of the picture.

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since a monoclonal antibody G19 raised against a conserved *Plasmodium falciparum* Hsp70 epitope immunoprecipitated the 2 bands with a lower molecular mass (Fig. 4, lane 1) and a control serum (non-infected rabbit serum) did not recognize any of these polypeptides.

Detection of Hsp70 in soluble extracts of Tb and localization of this protein on cryostat sections of ML and adult stages of Tb

To detect and localize the Hsp70 of Tb, a monospecific serum against Tb Hsp70 was prepared. For this part of pBS33 cDNA insert (from 266 to 1596 bp) was subcloned into the expression vector pGEX-5 in frame fusion to the *Schistosoma japonicum* glutathione S-transferase (GST) gene. Expression of GST-fusion protein was monitored by SDS–PAGE and immunoblotting using the anti-Tb rabbit serum (day 28 p.i.). Bacteria transformed with the recombinant plasmid produced as expected for GST-fused protein an insoluble fusion protein of 75 kDa that was recognized by the infected rabbit sera from day 14 p.i. up to day 180 p.i. (data not shown). The concentrated recombinant protein was used to immunize mice to raise a specific serum against the parasitic Hsp70. The specificity of the collected serum was tested by a competitive ELISA. The mouse serum was incubated with various concentrations of Tb or *E. coli* soluble antigens. After incubation, the depleted serum was then titrated by standard ELISA using the soluble antigen of Tb ML. When the serum was pre-incubated with more than 25 μg of Tb soluble antigen, a 100% inhibition was observed in the filtration of the serum. The percentage of inhibition diminished when the serum was pre-incubated with decreasing amounts of soluble antigen. No inhibition was observed when the serum was pre-incubated with *E. coli* antigen (Fig. 5A).

The anti-Tb ΔHsp70 serum was used to detect the presence of Hsp70 in the soluble or E–S antigens of ML. The serum recognized a 72 kDa protein in the soluble antigenic preparations (Fig. 5 lane 1) whereas no protein was detected by the same serum in E–S product (Fig. 5 lane 3).

Immunolocalization of Hsp70 was then performed by IIF test on cryostat sections of ML (obtained by pepsin HCl digestions at 37 or 44 °C) and adult worms extracted at 37 °C (Fig. 6). As shown in Fig. 6A, dispersed clear positive staining was observed in round subcellular structures that appeared throughout the whole larva independently of the temperature used in the purification of ML. A similar counterstaining pattern was obtained with Hoechst dye (Fig. 6B), suggesting the Tb Hsp70 was located inside nuclei of several larval cells including stichocytes, muscle cells, cord cells and primordium genital cells. In contrast, the anti-b ΔHsp70 serum was unable to detect any proteins in all adult sections (Fig. 6C) even though the adult cell nuclei were easily detectable with Hoescht dye counterstaining (Fig. 6D) or with a chromatin-specific monoclonal antibody (data not shown). No reactivity of the monospecific serum was observed in the external part of ML cuticle as determined by confocal microscopy analysis (data not shown).

DISCUSSION

Cloning of a Trichinella *cytoplasmic* Hsp70 *gene*

Although the presence of Hsp in *Trichinella* E–S and somatic products has already been described (Ko & Fan, 1996), cloning of *Trichinella hsp* genes has not been published. In this study an *hsp70* homologous fragment from ML cDNA was amplified and used to screen a Tb λZap library. Two phage clones encoding a cytoplasmic form of the Hsp70 family were isolated and sequenced *Trichinella hsp70* belongs to a multiple gene family as has been previously described for other organisms (Günter & Walter, 1994; Bock & Langer, 1993). The guanine–cytosine (GC) content of *Trichinella hsp70* gene (43%) seems to be similar to the one described for other cloned *Trichinella* genes (Su, Prestwood & McGraw, 1990; Sugane & Matsuura, 1990) but is higher than the average GC percentage of the overall genome (35%) characterized by Hammond & Bianco, (1992). Results from the deduced amino acid sequence showed characteristic Hsp70 universal signatures (Rensing & Maier, 1994; Stevenson & Calderwood, 1990; Searle, McCrossan & Smith, 1993). The size of the deduced amino acid sequence and the presence of the last 4 amino acids EEVD suggest that the cloned Hsp70 sequence is an inducible cytoplasmic Hsp70. This initial analysis was confirmed by the strongest identity at the amino acid level between Tb Hsp70 sequence and the cytoplasmic Hsp70 sequences of other organisms. During normal physiological conditions this protein is found in the cytoplasm whereas under a heat shock or other stresses, it concentrates within nuclei. The nucleo/cytoplasmic stress 70 kDa proteins are characterized by a bipartite motif on their primary sequence (Dingwall & Laskey, 1991) that serves as a nuclear localization signal. This motif was indeed identified at 245 amino acids from the N-terminus part of the amino acid sequence of Tb Hsp70 protein and explained the localization of this protein in the cell nuclei of Tb larvae.

A detailed comparison of amino acid sequence of Tb Hsp70 with those of Hsp70 proteins available in current databank revealed strongly conserved regions in the N-terminal part of the protein. The high level of amino acid conservation was as expected reduced towards the C-termini of the protein. The sequence data on the Hsp70 family provide a useful

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tool for investigating narrow phylogenic relationships since this is the most conserved protein known up to date (Gupta & Singh, 1992). Hsp70 has proven to be a reliable phylogenetic marker within bacteria (Falah & Gupta, 1994) as well as for eukaryotes (Gupta *et al*. 1997). The phylogenetic analysis of Tb Hsp70 showed that this parasite is undoubtedly located within the metazoan phyla, in a sister group together with other nematodes. A note of caution has to be taken since several paralogues can exist within a given species (i.e. 5 in mammals). However, it is probable that gene duplications may have occurred late during the evolution of Metazoan. For example, the 3 human genes (p17066, p24931, p08107) appeared after the divergence of mammals and amphibians. Since the monophyly of most of the metazoan phyla represented by several species is clearly recovered, the Hsp70 can be considered as a reliable marker for phylogeny of Metazaon. It can therefore be proposed that comparison of Hsp70 sequences of all species of *Trichinella* may allow a better determination of the phylogeny in *Trichinella* genus.

Trichinella *Hsp70 is an antigenic protein*

The Tb cytoplasmic Hsp70 is only present in soluble somatic products of the parasite as has been previously described (Ko & Fan, 1996). This is noteworthy since Hsp70 is the first characterized *Trichinella* antigen that did not belong to the E/S fraction (Su *et al*. 1990; Zarlenga & Gamble, 1990; Bock & Langer, 1993). The antigenicity of Hsp70 was shown by immunoprecipitation of the *in vitro* translated polypeptides using a serum of Tb infected rabbit (day 30 p.i.). This result confirmed that Hsp70 is a prominent immunogen containing numerous T and B epitopes (Kaufman *et al*. 1987). Since Hsp70 is one of the most conserved molecules amongst all the organisms, the presence of antibodies raised against Tb Hsp70 in the serum of infected animals could have dramatic consequences for autoimmune reactions particularly in humans. However, even though Hsp expression could be associated with autoimmune diseases, their direct role still remains a matter of controversy (Heufelder *et al*. 1992; Erkeller-Yueksel *et al*. 1992). Despite this marked identity in the sequences of the parasite and the host Hsp70, the parasitic Hsp70 has sufficiently diverted to elicit a strong and specific immune response (Hedstrom *et al*. 1988; Selkirk *et al*. 1989; Rothstein *et al*. 1989; Skeiky *et al*. 1995; Gomez, Allendoerfer & Deepe, 1995). Moreover the epitopes recognized by the host's sera are often located in the variable C-terminal part of Hsp70 protein (Behr *et al*. 1992; Amorim *et al*. 1996). Since a similar antibody reactivity from sera of Tb-infected rabbits collected from days 14 to 180 p.i. was observed by a Western blot analysis using either the recombinant Tb *Δ*Hsp70 or the soluble *Trichinella* antigen, it is suggested that such recombinant protein could be considered in a future ELISA test. Nevertheless it remains to be tested whether this recombinant protein can also offer advantages over other diagnostic antigens in terms of cost and early detection of Tb infections.

Changes on the expression of Hsp70 between parasite stages

Tb Hsp70 was localized in the nuclei of ML cells whereas it was not detected in the adult stage; suggesting that there is a different functional activity of Hsp70 among these two stages. Indeed, Hsp can be localized in discrete cellular compartments according to their specific chaperone functions (Lindquist, 1986; Ohtuska & Lazlo, 1992; Soltys & Gupta, 1996). After heat shock or ultra-violet irradiation the inducible Hsp70 translocates into the nucleus then back to the cytosol during recovery (Lazlo, Wright & Roti, 1992; Neri *et al*. 1995). The nuclear versus cytosolic localization is required by the Hsp70 to solubilize aggregated nuclear polypeptides, to 'chaperone' either DNA or DNA-associated proteins misfolded by stress. Finally, nuclear Hsp70 protects the transcriptional process (Beck & De Maio, 1994; Perin-Misini, Kantengawa & Polla, 1994; Neri *et al*. 1995). The nuclear localization of Hsp70 on cryostat sections of ML may reflect the chaperoning role played by this protein. In this case Hsp70 may transport transcriptional factors as has been demonstrated in other organisms in which Hsp70 acts during targeting and translocation phases of nuclear transport, possibly as a molecular chaperone to promote the stability of transcriptional factors (Mandell & Feldherr, 1990; Ellis & Van der Vries, 1991; Shi & Thomas, 1992; Moreau *et al*. 1994). Based on this it can therefore be suggested that low pH and/or gastric enzymes, induce into stress the encapsulated larvae which change from the quiescent form to adult worms within a few hours. This exposure to gastric content would stimulate genes which are controlled by stress such as *hsp*. Indeed the observation that transcription of mRNA increased 5 times in the larva during the 20 h following the acid-pepsin digestion suggest an induction of genes by stress. The strong inducible heat shock promoter could then contribute to rapid changes which are characterized during moulting in the *Trichinella* genus. Therefore it can be suggested that the induced protein will then be transferred to the nuclei where Hsp70 stimulates the transport of transcriptional factors, which in turn directly regulate gene expression and trigger off changes from ML to adult stage.

However, the significance of the presence of Hsp70 in the nuclei of purified ML remains a subject for further investigations. Particularly, it will be relevant

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to focus now on the expression of parasite *hsp70* gene in the nurse cell and to study its genetic variability in *Trichinella* genus.

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