Trichinella spiralis thymidylate synthase: cDNA cloning and sequencing, and developmental pattern of mRNA expression

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(*Received 23 July 2003; revised 29 August 2003; accepted 2 September 2003*)

SUMMARY

The persistent expression of thymidylate synthase activity has previously been demonstrated not only in adult forms, but also in non-developing muscle larvae of *Trichinella spiralis* and *T. pseudospiralis*, pointing to an unusual pattern of cell cycle regulation, and prompting further studies on the developmental pattern of *T. spiralis* thymidylate synthase gene expression. The enzyme cDNA was cloned and sequenced†, allowing the characterization of a single open reading frame of 307 amino acids coding for a putative protein of 35 582 Da molecular weight. The amino acid sequence of the parasite enzyme was analysed, the consensus phylogenetic tree built and its stability assessed. The aa sequence identity with thymidylate synthase was confirmed by the enzymatic activity of the recombinant protein expressed in *E. coli*. As compared with the enzyme purified from muscle larvae, it showed apparently similar V_{max} value, but higher K_m^{app} values describing interactions with dUMP (28·8 μM *vs.* 3·9 μM) and (6*RS*, α*S*)-$N^{5,10}$-methylenetetrahydrofolate (383 μM *vs.* 54·7 μM). With the coding region used as a probe, thymidylate synthase mRNA levels, relative to 18S rRNA, were found to be similar in muscle larvae, adult forms and newborn larvae, in agreement with muscle larvae cells being arrested in the cell cycle.

Key words: thymidylate synthase, histone H.3-like, *Trichinella spiralis*, mRNA, gene expression, cell cycle.

INTRODUCTION

Thymidylate synthase (EC 2.1.1.45) catalyses the reaction of reductive transfer of the methylene group from $N^{5,10}$-methylenetetrahydrofolate onto dUMP with concomitant production of dTMP and dihydrofolate. This reaction, being the sole cellular source of *de novo* synthesis of dTMP (Carreras & Santi, 1995), is a target in chemotheraphy (Rustum *et al*. 1997). Thymidylate synthase specific activity rises in exponentially growing cells, and is not detected in quiescent ones (Pestalozzi *et al*. 1995). Although the high level of this enzyme is believed to be associated with cell proliferation, it has also been found in non-proliferating systems, e.g. unfertilized eggs of certain invertebrates (Carpenter, 1973; Rode & Szymanowska, 1976; Yasumasu *et al*. 1979), in accord with oocytes being arrested in the cell cycle (Sagata, 1996), hence not quiescent (*cf*. Rode *et al*. 2000).

In non-growing muscle larvae of the parasitic nematode *T. spiralis*, we have found surprisingly high thymidylate synthase specific activity (Dąbrowska *et al*. 1996; Rode *et al*. 2000), persisting for at least 2 years after infection. As muscle fibres infected with *T. spiralis* become cell cycle-activated and, following DNA replication, arrested in G_2/M phase of the cell cycle (Jasmer, 1995), and assuming that parasite cells also respond to the same signals and are blocked in the cell cycle, high thymidylate synthase activity in non-growing *T. spiralis* muscle larvae may be a consequence of cell cycle arrest (Rode *et al*. 2000).

In order to learn more about the *T. spiralis* thymidylate synthase gene expression pattern, its cDNA was cloned and sequenced, and the coding region used as a probe to compare thymidylate synthase mRNA levels in muscle larvae, adult forms and newborn larvae. The sequence identity was confirmed by purification and characterization of the recombinant protein expressed in *E. coli*. The amino acid sequence of the parasite enzyme was analysed, the consensus phylogenetic tree built and its stability assessed.

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† Nucleotide sequence data reported in this paper are available in the GenBank™, EMBL and DDBJ databases under the Accession number AF162691.

Parasitology (2004), **128**, 209–221. © 2004 Cambridge University Press
DOI: 10.1017/S0031182003004426 Printed in the United Kingdom

https://doi.org/10.1017/S0031182003004426 Published online by Cambridge University Press

A

oligo(dT)$_{22}$: 5'-AAAAAGCTTTTTTTTTTTTTTTTTTTT-3'
RT1: 5'-CGTCGACTTTCCGGTTCTTCACGAATC-3'
SYN2: 5'-AAAAGCTTGCRTARCTNGCDATRTTRAA-3'
SYN3: 5'-GGGGAATTCTTYCARTGGCGNCAYTTYGG-3'
SYN4: 5'-GGGGAATTCTTYCARTGGAGRCAYTTYGG-3'
SYN7: 5'-AAAAGCTTCTGTTGTAATTGATCGTAGCC-3'
SYN8: 5'-GGGGAATTCGAAGTGCAGATATGGGATTG-3'
SYN9: 5'-AAAAGCTTAATCGGCCGTCATCGATGAATAC-3'
SYN10: 5'-GTCAAATGTATCAACGAAG-3'
SYNEX1: 5'-GGGGATCCATATGACAGAAACTGTTCACAAATTAG-3'
SYNEX2: 5'-AAAAGCTTACACAGCCATAGGCATTGATA-3'

B

LMPCR1: 5'-GCGGTGACCCGGG**AGATCTGAATTC**-3'
LMPCR2: 3'- **TCTAGACTTAAG**-5'

LMPCR1: 5'-GCGGTGACCCGGGAGATCTGAATTC-3'
LMPCRKR: 5'-GCGGTGACCCGGGAGATCTG-3'

C

MSPPOW: 5'-GAAGCAATGTATGCCGGAGAGCTCACTCTC**ACCAACGTCGAC**-3'
BGLMALY: 3'-**TGGTTGCAGCTG**-5'

MSPPOW: 5'-GAAGCAATGTATGCCGGAGAGCTCACTCTCACCAACGTCGAC-3'
MSPPB: 5'-GAAGCAATGTATGCCGGAG-3'
POWIE: 5'-CTCACTCTCACCAACGTCGA

Fig. 1. (A) RT-PCR primer sequences. Y indicates C or T; R indicates A or G; D indicates A or G or T; N indicates A or C or G or T. (B) Sequence alignment of oligonucleotide adaptors (LMPCR1/LMPCR2) and oligonucleotide adaptor LMPCR1 with adaptor primer LMPCRKR, used for *Trichinella spiralis* thymidylate synthase cDNA carboxy region amplification. Complementary sequences are in bold, identical sequences are underlined. (C) Sequence alignment of oligonucleotide adaptors (MSPPOW/BGLMALY) and oligonucleotide adaptor MSPPOW with adaptor primers (MSPPB and MSPPOW), used for *T. spiralis* thymidylate synthase cDNA amino region amplification. Description as in (B).

MATERIALS AND METHODS

Biological material

Trichinella spiralis human isolate H2 strain from the Instituto Superiore di Sanita, Rome, Italy, ref. No. MHOM/PO/88/ISS67 (T1) was maintained in adult Swiss mice. Muscle larvae were obtained from mouse carcases with the use of pepsin-HCl digestion (Gould, 1970). Adult forms and newborn larvae were isolated according to the method described by Wranicz *et al*. (1998).

RNA purification

Total RNA was isolated from *T. spiralis* muscle larvae, adult forms and newborn larvae using TRIZOL ultra-pure reagent from GibcoBRL. Poly(A)$^+$RNAs were prepared from *T. spiralis* muscle larvae RNA using PolyATract mRNA Isolation Systems kit (Promega, Madison).

cDNA synthesis

In order to obtain the first strand of cDNA, polymerization was performed on poly(A)$^+$RNA using MMLV reverse transcriptase (Promega). Reverse transcription was done in 2 rounds of 1 h incubation at 42 °C, using a degenerate SYN2 primer (Fig. 1A), and with the full open reading frame (ORF) or the 3′ end of coding sequence 2 rounds of 1 h incubation were carried out with reverse transcriptase at 37 °C, using oligo(dT)$_{22}$ primer. In order to obtain ds cDNA for amplification of the 3′ end of the coding region, the second cDNA strand was synthesized in a single polymerization step, using 50 pmol of SYN10 primer (Fig. 1A), 1 μl of thermostable DNA polymerase mixture from Expand Long Template PCR System kit (Boehringer, Mannheim) and buffer 1 from the same kit, with initial denaturation (1 min at 94 °C) followed by annealing (2 min at 46 °C) and polymerization (2 min at 68 °C). After phenol/chloroform extraction and ethanol precipitation in the presence of glycogen (20 μg/ml), ds cDNA ends were blunt ended using 2·5 units of Klenow fragment of *E. coli* DNA polymerase I (Promega), the latter followed by phenol/chloroform treatment and ethanol precipitation. For the 5′ end of the coding region, amplification ds cDNA was done with poly(A)$^+$RNA using Universal RiboClone cDNA Synthesis System

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(Promega) and 2 rounds of 1 h reverse transcription at 42 °C, primed by RT1 primer (Fig. 1A). The resulting ds cDNA was then phenol/chloroform-extracted and ethanol-precipitated in the presence of glycogen (20 μg/ml).

Ligation of adaptor oligonucleotides

Two pairs of unphosphorylated adaptor oligonucleotides, LMPCR1/LMPCR2 and MSPPOW/BGLMALY (Fig. 1B, C) were used for ligation with ds cDNA. Prior to ligation, ds cDNA was phosphorylated using T4 polynucleotide kinase (Promega) then phenol/chloroform-extracted and ethanol-precipitated in the presence of glycogen (20 μg/ml). A 1/10 part of the resulting preparation was ligated overnight at 16 °C with 20 pmol of each unphosphorylated adaptor oligonucleotide using T4 DNA ligase (USB, Cleveland). A 1/50 part of ligation mixture was used for PCR analysis (Płucienniczak *et al*. 1999).

PCR

The enzymatic reaction was performed in a final volume of 50 μl using degenerate primers (each 5 μM) or sequence-specific or adaptor primers (each 1 μM), dNTPs (each 0·2 mM), and either Taq DNA polymerase from Terpol (Sieradz) or Pfu DNA polymerase from Promega or thermostable DNA polymerase mixture from Expand Long Template PCR System kit from Boehringer. The enzyme was always added at 80 °C after an initial denaturation step (3 min at 95 °C). The final extension step was carried out for 15 min. For particular primer combinations the remaining reaction conditions were as follows: (i) SYN2 and SYN3 or SYN4, 41 cycles: 30 sec at 95 °C, 45 sec at 65 °C and 45 sec at 72 °C with 2 units of Taq DNA polymerase, (ii) SYN8 and LMPCRKR, 30 cycles: 30 sec at 94 °C, 30 sec at 58 °C and 60 sec at 68 °C with 1 μl of thermostable DNA polymerase mixture from Expand Long Template PCR System kit (Boehringer), (iii) SYN7 and MSPPB, 30 cycles: 30 sec at 94 °C, 30 sec at 54 °C and 60 sec at 68 °C with thermostable DNA polymerase mixture as above, (iv) SYN9 and POWIE, as in (iii) except for the annealing temperature being 58 °C, (v) SYNEX1 and SYNEX2, 35 cycles: 30 sec at 95 °C, 30 sec at 57 °C and 60 sec at 74 °C with 1·25 units of Pfu DNA polymerase. PCR fragments were separated and isolated from polyacrylamide gel as previously described (Dybczyński & Płucienniczak, 1988).

Cloning and sequencing

The isolated PCR fragments were digested with the appropriate restriction endonucleases (GibcoBRL), and then purified using Qiaquick-spin PCR Purification Kit (Qiagen) and cloned in pBluescript SK(+) plasmid (Stratagene) propagated in *E. coli* 'DH5αF' strain. Sequencing was performed with Taq Track Sequencing Systems kit (Promega).

Histone probe

This probe resulted from the partial sequencing of adult newborn larvae cDNA library (P. Boireau and M. Liu, manuscript in preparation). Strong identity (90%) of the selected clone (610 bp) with histone H3.3 nucletode sequence of *Trichinella pseudopsiralis* and other histone H3 nucleotide sequences indicated it belonged to the H3-like gene family (data not shown). This probe was used to follow the expression of mRNA for a constitutive protein of the *Trichinella* nuclei.

Sequence analysis

The amino acid sequence of *T. spiralis* thymidylate synthase was analysed with the use of the National Institutes of Health software, ENTREZ and BLAST (Altshul *et al*. 1997), then the amino acid sequences of various eukaryotic thymidylate synthases were aligned and the neighbour-joining tree was constructed with Clustal X programme (Thompson *et al*. 1994, 1997), using the default options. Three short regions of the alignment, which could not be unambiguously aligned, were omitted from the analysis. The alignment is available from the corresponding author upon request. The consensus phylogenetic tree was built and its stability assessed by posterior probabilities using Bayesian inference (Huelsenbeck *et al*. 2001) and general time reversible model (GTR + I + G) with the rates of amino acid substitutions, amino acid frequencies, gamma distribution shape and proportion of invariant sites estimated by Markov Chains MonteCarlo method, as implemented in MrBayes 2.01 programme (Huelsenbeck & Ronquist, 2001).

Northern blot analysis

Total RNA was size-fractionated on 1·2% agarose-formaldehyde gels then subjected to Northern transfer onto Hybond-N^+ nylon membranes (Amersham) and hybridized with one of the following specific probes (i) 921 bp long cDNA fragment, corresponding to *T. spiralis* thymidylate synthase coding region (*cf*. Results), (ii) 600 bp long cDNA fragment corresponding to a part (452 bp) of the *T. spiralis* histone H3.3 coding region or (iii) a reference probe – 612 bp long *T. spiralis* rDNA fragment (Kuratli *et al*. 1999). All procedures were performed according to Sambrook, Fritsch & Maniatis (1989), with hybridization and autoradiography of each blot carried out 3 times in a sequence, each time with a different probe, at 68 °C in the presence of 10% dextran sulphate. The probes were radioactively labelled with

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the use of Prime-a-Gene Labeling system (Promega). Autoradiographic results were assessed on personal densitometer (Molecular Dynamics) using the ImageQuant program.

T. spiralis *thymidylate synthase expression vector*

The cDNA corresponding to the enzyme total coding region was cloned into pBluescript SK(+) plasmid (its sequence was confirmed) and subcloned into PIGDM4stop expression vector, propagated in *E. coli* HMS174 strain (Novagen). PIGDM4stop expression vector is a derivative of the pPIGDM1 plasmid (Mikiewicz *et al*. 1997), containing an ampicillin resistance gene (inserted into the putative *rom* gene), T7 phage promoter, transcription terminator and rare arginine tRNA genes ($tRNA^{Arg(AGG/AGA)}$).

T. spiralis *thymidylate synthase expression system*

The recombinant PIGDM4stop/*T. spiralis* thymidylate synthase expression vector was transformed by PEG treatment (Chung & Miller, 1988) into *E. coli* BL21(DE3)pLysS strain (Novagen). The procedure for the enzyme expression induction in *E. coli* BL21(DE3)pLysS strain was performed as recommended in the Novagen pET System Manual. For large-scale expression the trypton phosphate medium was used (Cieśla *et al*. 1995).

Recombinant enzyme purification and electrophoretic analysis

The previously described methods (Dąbrowska *et al*. 1996) were used, with the enzyme activity assayed spectrophotometrically (Wahba & Friedkin, 1961). The activity unit was defined as the enzyme amount required to convert 1 μmol of (6*RS*, α*S*)-$N^{5,10}$-methylenetetrahydrofolic acid to dihydrofolic acid per 1 min at 37 °C.

Kinetic studies

K_m values for interaction of the recombinant enzyme with both substrates, dUMP and $N^{5,10}$-methylenetetrahydrofolate, were determined as earlier reported (Dąbrowska *et al*. 1996).

RESULTS

Thymidylate synthase cloning was achieved in 3 steps. The core nucleotidic sequence was amplified using degerated primers, based on a region of amino acid sequence highly conserved among known thymidylate synthases (Carreras & Santi, 1995). The 3′ and 5′ ends of the coding sequence, and the corresponding untranslated regions, were obtained separately using RT-PCR but their amplification required an increase in reaction specificity at both RT and PCR steps. Therefore reverse transcription was primed with sequence-specific primers, based on the core sequence, and the PCR step was done under conditions allowing either a PCR suppression effect (Chenchik *et al*. 1996; Lukyanov *et al*. 1997) or both PCR suppression and nested PCR effects (the latter only with the 5′ end). Ligation of unphosphorylated adaptor oligonucleotides to ds cDNA (LMPCR1 in case of the 3′ end and MSPPOW in case of the 5′ end), allowed (i) PCR suppression to take place if occasionally, in the first PCR cycle, unspecific template elongation occurred and (ii) to learn the sequences of the two untranslated regions (Chenchik *et al*. 1996).

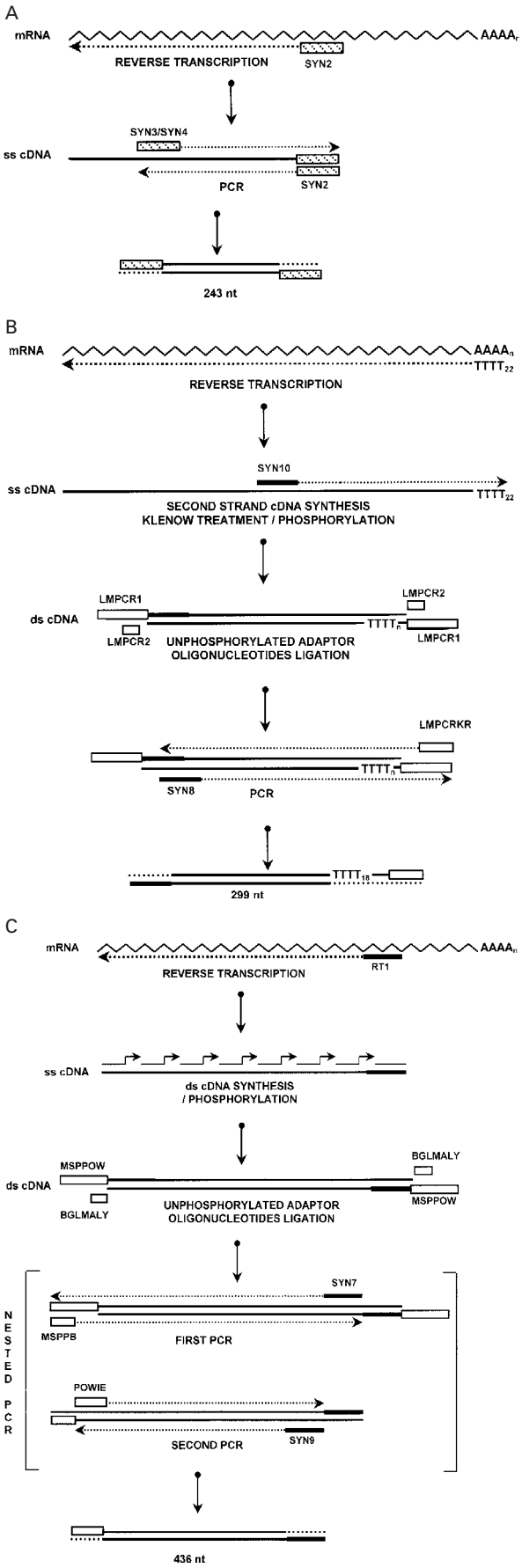
Core sequence of T. spiralis *thymidylate synthase coding region*

Based on two different 7-amino-acid stretches, degenerate sense (SYN3 and SYN4) and antisense (SYN2) primers were designed (Fig. 1A; for reciprocal orientation *cf*. Fig. 3). In order to amplify the desired cDNA, the SYN2-primed reverse transcription (attempts to prime it by $oligo(dT)_{22}$ failed), followed by PCR primed by the SYN2 and either SYN3 or SYN4 (Fig. 2A), were applied. Two fragments (each *ca* 288 bp long) resulted, each from reaction with a different primer combination: SYN2/SYN3 or SYN2/SYN4. Following digestion with *Hin*d III and *Eco* RI endonucleases, cloning and sequencing, both were estimated to be of the same length (243 nt) and sequence, and the corresponding amino acid sequence highly resembled those of thymidylate synthases of other species. As expected (*cf*. primer design; Fig. 3), it encompassed the enzyme active site. The amino acid sequence of the latter was VALPPCH, in agreement with a high level of conservation of the corresponding sequences among other thymidylate synthases (Carreras & Santi, 1995).

Sequence of the carboxy part of the coding region and 3′ untranslated region of T. spiralis *thymidylate synthase*

In order to clone the 3′ part, with respect to the 243 nt-long core sequence of the enzyme cDNA, a modified RACE strategy (Frohman, Dush & Martin, 1988) was employed (Fig. 2B). Two sense primers, SYN8 and SYN10 (Figs 1 and 3) were designed, based on the identified core region sequence. Reverse transcription with $oligo(dT)_{22}$ primer, the second cDNA strand synthesis with SYN10 primer and subsequent ligation with LMPCR1 and LMPCR2 unphosphorylated adaptor oligonucleotides, were performed as described in the Materials and Methods section. Adaptor-ligated cDNA was used for amplification with SYN8 and LMPCRKR (Fig. 1) primers. The resulting DNA fragment, *ca* 380 nt-long, was digested with *Eco* RI endonuclease, cloned

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and sequenced. It was found to contain a further part (267 nt) of the coding region of the enzyme and 32 nt of the 3′ untranslated region together with TGA stop codon. A polyadenylation signal, AATAAA, starts at the 14th position after the stop codon. The 3′ untranslated region is followed by 18 A residues.

Sequence of the amino part of the coding region and 5′ untranslated region of T. spiralis *thymidylate synthase*

The 243 nt-long core region of the cDNA served for antisense primer design. Each of the resulting primers, RT1, SYN7 and SYN9, was in this precedence order internal to the preceding one (Figs 1 and 3). The whole amplification procedure is depicted in Fig. 2C. Reverse transcription primed by the RT1 was followed by the second cDNA strand synthesis and ligation with unphosphorylated adaptor oligonucleotides MSPPOW and BGLMALY. On the adaptor-ligated cDNA as a template, nested PCR was performed. MSPPB (Fig. 1C) and SYN7 (Fig. 1A) primers were used in the first PCR round, then POWIE (Fig. 2C) and SYN9 (Fig. 1A) primers were used for reamplification. The resulting fragment, *ca* 470 nt-long, was digested with *Hin*d III and *Sal* I endonucleases, cloned and sequenced. It was found to contain 25 nt of the 5′ untranslated and remaining 411 nt of the enzyme coding region sequence. Amino acids 69–71 (TTK) of the coding region, constitute $N^{5,10}$-methylentetrahydrofolate binding site, highly conserved among various thymidylate synthases (Carreras & Santi, 1995).

T. spiralis *recombinant thymidylate synthase expression, purification and kinetic properties*

The enzyme was expressed in *E. coli* and purified as described in the Materials and Methods section. Thymidylate synthase specific activities, assayed in the absence of Mg^{2+} ions in crude extracts of *E. coli* BL21(DE3)pLysS cells untransformed and transformed with the PIGDM4stop/*T. spiralis* thymidylate synthase expression vector, were 0·20 munit/mg protein (mean result of 2 determinations in separate cultures, with the results differing from the mean by less than 1%) and 8·9 munit/mg protein (mean result of 2 determinations in separate cultures, with the results differing from the mean by 7%), respectively. The resulting enzyme preparation (purified with 16% yield) was apparently homogeneous (Fig. 4) and the specific activity of the richest fraction was

Fig. 2. Schematic representation of the three-step method of *Trichinella spiralis* thymidylate synthase cDNA amplification. (A) the core region amplification (B) the 3′ region amplification (C) the 5′ region amplification. ▭ Degenerate primers, ····· sequences not included in the given nt length, ▬ specific primers, ▭ adaptor oligonucleotides or adaptor primers.

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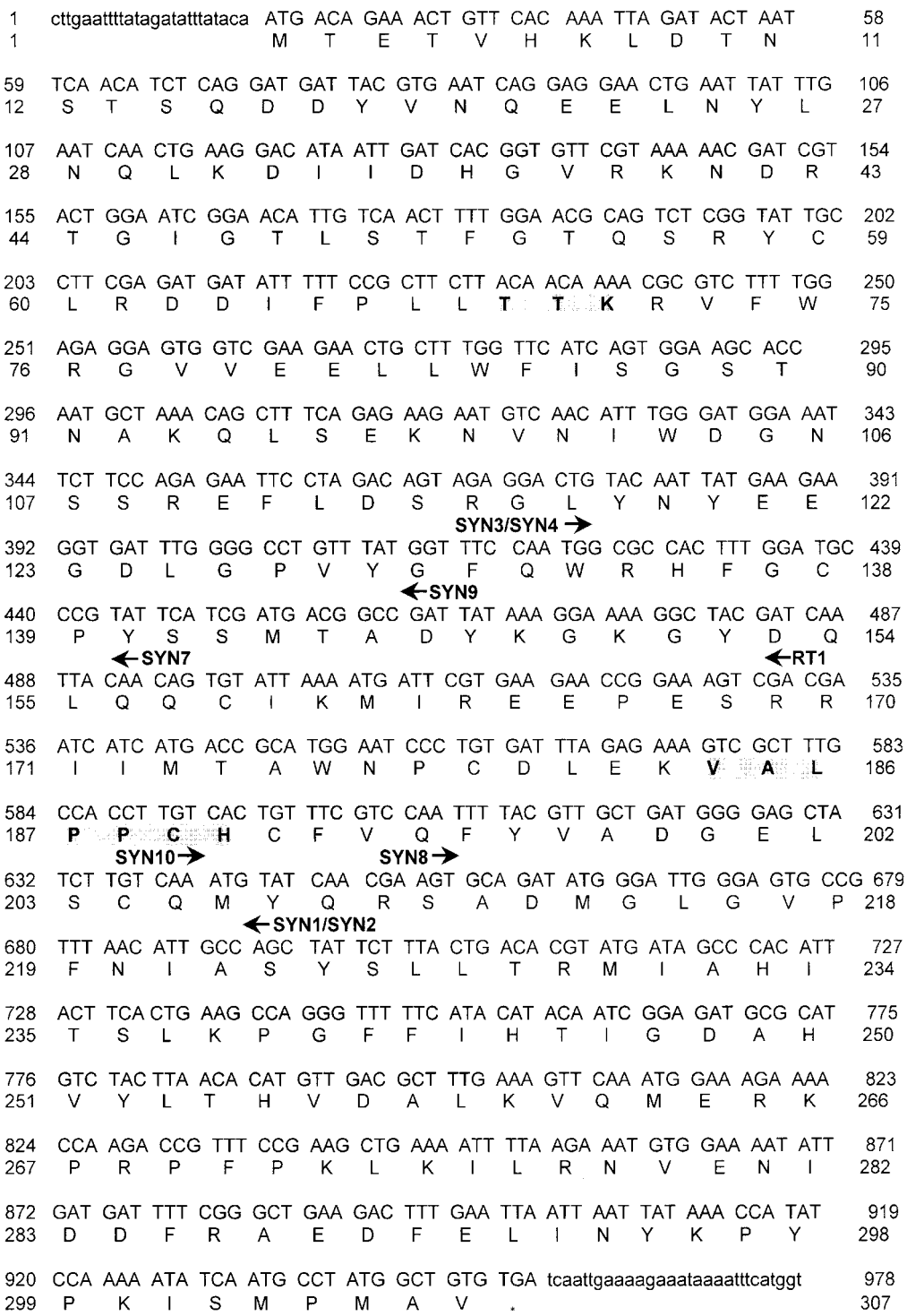


Fig. 3. Nucleotide and deduced amino acid sequence of *Trichinella spiralis* thymidylate synthase cDNA. The orientations of primers used for cloning of its three fragments are marked above. $N^{5,10}$-methylenetetrahydrofolate and dUMP binding sites are marked in bold.

0·25 unit/mg protein, 5·5-fold higher than that of the enzyme purified from *T. spiralis* muscle larvae (Dąbrowska *et al.* 1996). As the latter preparation was only $\leqslant 30\%$ pure, its further purification to homogeneity would presumably result in a similar specific activity, hence also V_{max} value, to that of the homogeneous recombinant enzyme. Comparison of interactions of both enzyme preparations with the substrates in a parallel experiment showed distinct differences, the apparent K_m values for dUMP and $N^{5,10}$-methylenetetrahydrofolate, estimated from double reciprocal plots of reaction velocity *vs.* dUMP or (6*RS*, α*S*)-$N^{5,10}$-methylenetetrahydrofolate concentration, being higher with the recombinant (28·8 μM and 382·7 μM, respectively) than muscle larvae (3·1 μM and 54·7 μM, respectively) enzyme. In accordance, the real K_m values (expressed as mean $\pm$ SEM) for the recombinant enzyme interaction with

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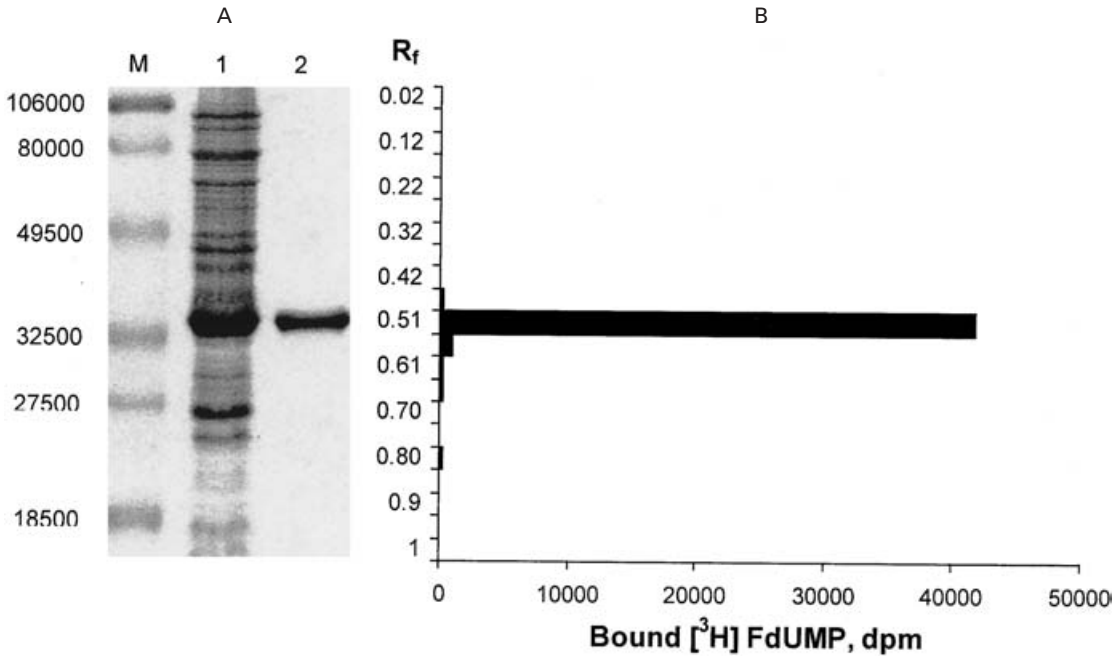


Fig. 4. (A) SDS-PAGE of crude extract of *E. coli* BL21(DE3)pLysS strain overexpressing *Trichinella spiralis* thymidylate synthase (lane 1: 25 μg of protein) and the purified enzyme (lane 2: 3·2 μg of protein). M: molecular weight markers with their molecular weights in Da. (B) Distribution of radioactivity following SDS-PAGE of purified recombinant *T. spiralis* thymidylate synthase (5 μg) pre-incubated with [^{3}H] FdUMP in the presence of $N^{5,10}$-methylenetetrahydrofolate as previously described (Dąbrowska *et al.* 1996).

dUMP and (6*RS*, α*S*)-$N^{5,10}$-methylenetetrahydrofolate were 20·1 $\pm$ 3·5 (N = 3) and 102·3 $\pm$ 22·2 (N = 3), respectively (*cf.* Dąbrowska *et al.* 1996).

Expression of thymidylate synthase mRNA, histone H3-like mRNA and 18S rRNA in muscle larvae, adult forms and newborn larvae of T. spiralis

The results of Northern blot analyses of *T. spiralis* total RNA are presented in Fig. 6. Thymidylate synthase mRNA, relative to 18S rRNA, level was found to be maintained at a constant level in *T. spiralis* muscle larvae, adult forms and newborn larvae (Fig. 5A). In contrast, *T. spiralis* histone *H3-like* mRNA level, also relative to 18S rRNA, was different at those three developmental stages (Fig. 5B), with the highest level observed in newborn larvae and the lowest in non-growing muscle larvae.

Sequence comparison between the enzyme from T. spiralis *and other thymidylate synthases*

Similarities found between the sequences, at nucleotide and amino acid levels, are summarized in Table 1. There are only short fragments in *T. spiralis* enzyme cDNA, ranging from 20 to 56 nucleotides (coding amino acids 131–137 and 212–225), that display high levels of sequence similarity with the corresponding fragments of nucleotide sequences coding different mammalian, plant and viral thymidylate synthases or the enzyme from another nematode *A. suum*. No such nucleotide sequence similarities were found in relation to the enzyme from the free-living nematode *C. elegans*. In agreement with thymidylate synthase being long known as a protein highly conserved in evolution (Carreras & Santi, 1995), the *T. spiralis* enzyme also showed high overall amino acid homologies with other thymidylate synthases (Table 1). The percentage of identical and similar positions is slightly higher in relation to the enzyme from mammals and plants than from another nematode, *A. suum*. Also the phylogenetic tree, based on the neighbour-joining method, locates *T. spiralis* enzyme in one clade with Viridiplantae (green plants) but the bootstrap value is too low for the observed affiliation to be significant (data not shown). Phylogenetic analysis based on a more advanced model of amino acid sequences evolution implemented in MrBayes programme indicated thymidylate synthases not only of *T. spiralis*, but also of *A. suum* and *C. elegans*, to belong to the clade of metazoan enzymes (Fig. 6). The protein sequences of the three nematode species form a paraphyletic assemblage with the sequences of *A. suum* and *C. elegans*, compared with *T. spiralis*, being closer to mammalian homologues. Such a placement suggests a relatively high level of divergence of the *T. spiralis* enzyme, although with the low percentage of probability (85%).

DISCUSSION

The *T. spiralis* thymidylate synthase sequence, obtained from 3 individually amplified cDNA

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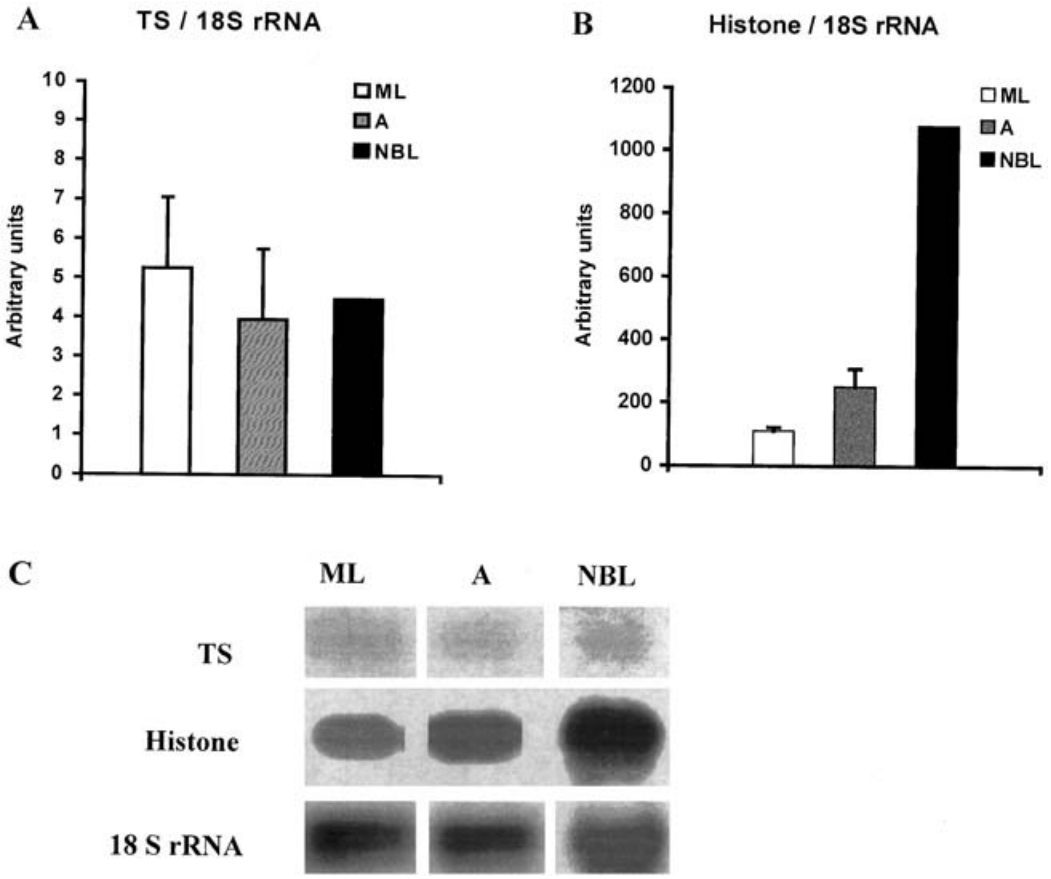


Fig. 5. (A) Thymidylate synthase mRNA level in *Trichinella spiralis* muscle larvae (ML), adult forms (A) and newborn larvae (NBL) related to 18S rRNA level. (B) Histone H.3-like mRNA level in *T. spiralis* muscle larvae (ML), adult forms (A) and newborn larvae (NBL) related to 18S rRNA level. (C) Thymidylate synthase, histone H.3-like and 18S rRNA bands in *T. spiralis* muscle larvae (ML), adult forms (A) and newborn larvae (NBL). Densitometric results (mean ± SEM) are expressed as arbitrary units.

fragments, is 978 nt-long and consists of 25 nt-long 5′ untranslated region, 921 nt-long coding region and 32 nt-long 3′ untranslated region. The 3′ untranslated region is followed by a poly(A)$_{18}$ tail. Polyadenylation signal AATAAA begins at the 14th position after the TGA stop codon. The enzyme coding region corresponds to a 307 amino acid-long polypeptide chain of molecular weight 35 582 Da. The latter value is in good agreement with the experimentally determined molecular weight (M_r = 35 000) of the purified enzyme (Dąbrowska *et al.* 1996). No significant similarities of the nucleotide sequence were found to thymidylate synthases of other species. Short regions sharing a considerable percentage of identical positions correspond to amino acid regions (131–137 and 212–225 numbering for *T. spiralis* enzyme), highly conserved among various species enzymes, but not to other highly conserved regions, important for catalytic activity of the enzyme, i.e. folate (amino acids 69–71) or dUMP (amino acids 184–190) binding sites. The overall *T. spiralis* enzyme amino acid sequence is highly homologous to other thymidylate synthases, with the highest variability found for the enzyme 5′ end. Interestingly, the active site (dUMP binding site) has a unique composition (amino acids 184–190: VALPPCH) differing by valine in the first position and substituting, conserved in a vast majority of species, alanine (Carreras & Santi, 1995). Compared with *T. spiralis* thymidylate synthase active site, *C. elegans* enzyme active site, differing from the most conservative version by valine substituting alanine, next to methionine, contains 2 different amino acids (M and V corresponding to *T. spiralis* 184V and 185A, respectively). In this respect, it is worthwhile mentioning deoxyribonuclease II with differences at the purported active site histidine residue, recently found in *T. spiralis* and *T. pseudospiralis* (MacLea, Krieser & Eastman, 2003).

Expression of the recombinant protein in *E. coli* cells confirmed the sequence identity, and considerable differences in affinities for substrates found with highly purified preparations of the recombinant and muscle larvae thymidylate synthases pointed to possible influence of post-translational modification(s) on enzyme properties. However, a possibility of an influence of enzymes contaminating the muscle larvae enzyme preparation should not be overlooked. It should be mentioned that, although *E. coli* BL21 (DE3)pLysS cells express their own thymidylate

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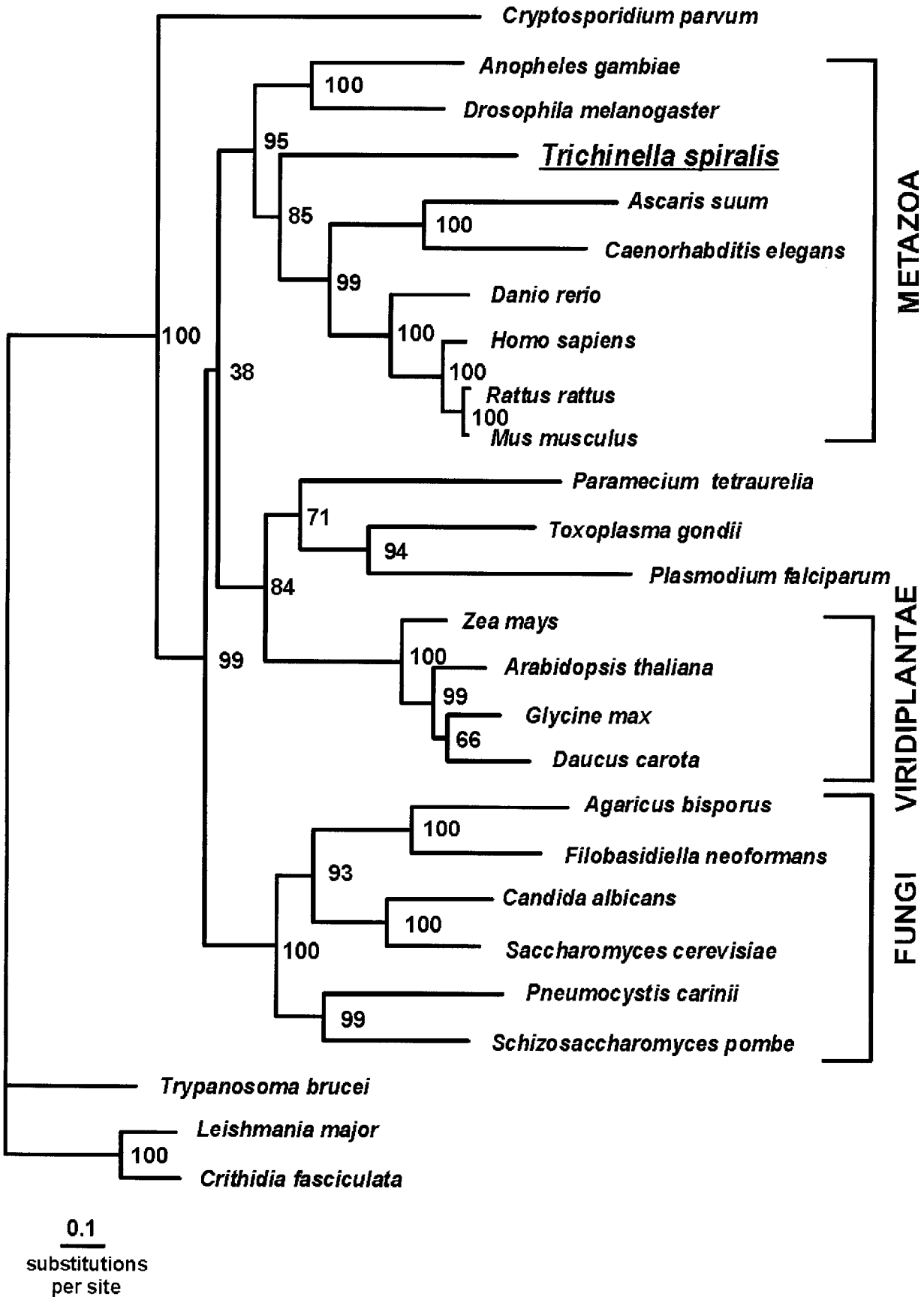


Fig. 6. Phylogenetic tree of eukaryotic thymidylate synthases. The tree was constructed with MrBayes programme run for 1 000 000 generations, using GTR + G + I model of amino acid substitution, and rooted by TreeView programme (Page RDM 1996) using *Euglenozoa* as an outgroup. The numbers at nodes are percentages of posterior probabilities of particular branches.

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Table 1. Thymidylate synthase nucleotide and amino acid sequence comparisons between *Trichinella spiralis* and other species (chosen representative comparisons), performed with the use of BLAST

(Amino acid numbering is that of *T. spiralis* enzyme. Number of amino acids does not agree with the corresponding nucleotide sequence length when the latter contains incomplete codon(s).)

	Amino acid sequence		cDNA sequence	
Species	Identities (%)	Similarities (%)	Length[1]/ % identity	Coded amino acids
Homo sapiens[2]	68	79	—[3]	—
Rattus norvegicus[4]	67	79	40/87	212–225
Mus musculus[5]	67	78	40/87	212–225
Arabidopsis thaliana[6]	67	77	56/83	204–222
Zea mays[7]	66	77	29/96	79–87
			31/93	200–208
Equine herpesvirus 2[8]	65	77	—	—
Herpesvirus saimiri 2[9]	65	77	42/90	212–225
Herpesvirus ateles 2[10]	65	75	42/88	212–225
Kaposi's sarcoma associated herpesvirus[11]	64	77	38/92	213–224
Glycine max[12]	64	75	44/86	123–136
			44/86	200–213
Drosophila melanogaster[13]	63	77	—	—
Danio rerio[14]	63	75	—	—
Daucus carota[15]	63	73	50/86	121–136
			28/92	200–208
Human herpesvirus 3[16]	61	77	—	—
Ascaris suum[17]	61	72	20/100	212–217
Caenorhabditis elegans[18]	60	75	—	—
Trypanosoma brucei[19]	60	74	—	—
Saccharomyces cerevisiae[20]	60	73	32/90	212–221
Toxoplasma gondii[21]	60	72	—	—
Leishmania amazonensis[22]	58	71	—	—
Pneumocystis carinii[23]	57	70	—	—
Candida albicans[24]	57	68	—	—
Agaricus bisporus[25]	56	71	—	—
Filobasidiella neoformans[26]	55	67	—	—
Paramecium tetraurelia[27]	54	72	—	—
Schizosaccharomyces pombe[28]	54	68	—	—
Crithidia fasciculata[29]	54	69	—	—
Plasmodium falciparum[30]	53	70	—	—
Cryptosporidium parvum[31]	53	67	—	—
Escherichia coli[32]	50	65	—	—
Chilo iridescent virus[33]	51	67	33/90	211–220
Lactobacillus casei[34]	43	56	—	—

[1] Bases; [2]Takeishi *et al*. 1985; [3]Not found; [4]Cieśla *et al*. 1995; [5]Perryman *et al*. 1986; [6]Lazar *et al*. 1993; [7]NCBI database accession no. **AF073488**; [8]Telford *et al*. 1995; [9]Honess *et al*. 1986; [10]Richter *et al*. 1988; [11]Russo *et al*. 1996; [12]Wang *et al*. 1995; [13]NCBI database accession no. **AAC27622**; [14]NCBI database accession no. **AY005804**; [15]Luo *et al*. 1993; [16]Davison & Scott, 1986; [17]NCBI database accession no. **AF09672**; [18]NCBI database accession no. **AF09673**; [19]Gamarro *et al*. 1995; [20]Taylor *et al*. 1987; [21]Roos, 1993; [22]Nelson *et al*. 1990; [23]Edman *et al*. 1989; [24]Singer *et al*. 1989; [25]NCBI database accession no. **CAB96042**; [26]Livi *et al*. 1994; [27]Schlichtherle *et al*. 1996; [28]NCBI database accession no. **CAB52423**; [29]Hughes *et al*. 1989; [30]Snewin *et al*. 1989; [31]Vasquez *et al*. 1996; [32]Belfort *et al*. 1983; [33]NCBI database accession no. **AF059506**; [34]Pinter *et al*. 1988.

synthase, the present results indicate that activity of this enzyme form could participate in the recombinant enzyme preparation to the extent of only 2·2%.

Recent observations of persistent expression of thymidylate synthase activity not only in adult forms, but also in non-developing muscle larvae of *T. spiralis and T. pseudospiralis* (Rode *et al*. 2000), pointed to an unusual pattern of cell cycle regulation and prompted us to further investigate the developmental pattern of *T. spiralis* thymidylate synthase gene expression by studying the corresponding mRNA levels. In parallel, *T. spiralis* histone H.3-like mRNA levels were determined and all results related to18S rRNA expression levels, used as a reference. No differences between thymidylate synthase mRNA levels, related to 18S rRNA, were found in the muscle larvae, adults and newborn larvae. On the other hand, *T. spiralis* histone H3-like mRNA levels, studied in the same way, were different at these three developmental stages, with the highest and

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lowest levels found in fast growing newborn larvae and non-develping muscle larvae, respectively. Thus the two genes appear to be differently expressed, with thymidylate synthase mRNA level being constant, independent of the occurrence of proliferation (adults with female worms filled with proliferating embryos *vs*. newborns and non-proliferating newborns and muscle larvae) or development (quickly growing newborns *vs*. non-growing and non-developing muscle larvae).

The steady-state level of thymidylate synthase mRNA, very low in quiescent (G_0) mammalian cells, is known to increase 10 to 20-fold as such cells progress to the S phase (Jenh, Geyer & Johnson, 1985; Ayusawa *et al*. 1986; Gribaudo *et al*. 2002) and decrease again with differentiation (Horie, Nozawa & Takeishi, 1992). When compared in a variety of cells and tissues, the amount of thymidylate synthase mRNA varies, reflecting differences in cell proliferation rates (Lee, Shen & Johnson, 1999). The foregoing, persistent expression of thymidylate synthase mRNA at a constant level in all *T. spiralis* developmental forms studied, including developmentally arrested muscle larvae, points to an unusual pattern of cell cycle regulation and is in agreement with our previous hypothesis assuming muscle larvae cells to be arrested in the cell cycle. In contrast, low histone H3-like expression in muscle larvae, relative to newborn larvae and adults, could be expected, in view of previously described downregulation of *Caenorhabditis elegans* histone gene family in developmentally arrested dauer larvae relative to actively growing stages (Dalley & Golomb, 1992). Interestingly, a gene homologous to histone H3 has been found to be heat-induced in *T. spiralis* muscle larvae (Mak, Su & Ko, 2001).

The *T. spiralis* thymidylate synthase amino acid sequence seems to be more similar to the corresponding sequences of mammals and plants then other nematodes. Also the phylogenetic tree based on the neighbour-joining method and a simple model of amino acid replacement implemented in Clustal X programme (Thompson *et al*. 1994, 1997) locates the *T. spiralis* enzyme in one clade with Viridiplantae, but the bootstrap value is too low to be certain that the observed affiliation is true (not shown). The more advanced, general time reversible model of amino acid sequence evolution resulted in the tree grouping all animal sequences together, although with the *Trichinella* enzyme branching off before the other two nematode enzymes. Whether evolution of the *Trichinella* sequence was influenced by intracellular parasitism (*cf*. Stephens *et al*. 1998), remains to be established.

This work was supported by the Polish State Committee for Scientific Research, grants 4P05F03011p02 and 6 P04C 021 18.

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