

# Developmental arrest in *Caenorhabditis elegans* dauer larvae causes high expression of enzymes involved in thymidylate biosynthesis, similar to that found in *Trichinella* muscle larvae

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## SUMMARY

Crude extract specific activities of thymidylate synthase, dUTPase, thymidine kinase and dihydrofolate reductase were high during the development of *Caenorhabditis elegans*, the dauer larva activities being similar to those previously determined in *Trichinella spiralis* and *T. pseudospiralis* muscle larvae (with the exception of thymidine kinase, not detected in *Trichinella*). High thymidylate synthase expression in developmentally arrested larvae, demonstrated also at the mRNA and protein levels, is in agreement with a global cell cycle arrest of dauer larvae and indicates this unusual cell cycle regulation pattern can be shared by developmentally arrested larvae of *C. elegans* and the two *Trichinella* species. Hence, the phenomenon may be characteristic for developmentally arrested larvae of different nematodes, rather than specific for the parasitic *Trichinella* muscle larvae. Endogenous *C. elegans* thymidylate synthase was purified and its molecular properties compared with those of the recombinant protein, expression of the latter in *E. coli* cells confirming the NCBI database sequence identity.

**Key words:** thymidylate synthase, dUTPase, thymidine kinase, dihydrofolate reductase, *Caenorhabditis elegans*, *Trichinella* spp., developmental arrest, dauer larva, muscle larva, cell cycle.

## INTRODUCTION

Enzymes involved in thymidylate (dTMP) biosynthesis, thymidylate synthase (EC 2.1.1.45), catalysing the N<sup>5,10</sup>-methylenetetrahydrofolate-dependent C(5) methylation of 2'-deoxyuridylate (dUMP), dihydrofolate reductase (EC 1.5.1.3), catalysing dihydrofolate reduction to tetrahydrofolate, dUTPase (EC 3.6.1.23), hydrolysing dUTP to dUMP, and thymidine kinase (EC 2.7.1.75), responsible for thymidine salvage *via* phosphorylation, are expressed in association with cell proliferation (Kit, 1976; Johnson, Fuhrman & Wiedemann, 1978; Vilpo, 1983; Blakley, 1984; Santi & Danenberg, 1984; Hokari *et al.* 1987; Békési *et al.* 2004). Therefore high specific activities of thymidylate synthase, dUTPase and dihydrofolate reductase, found in non-growing muscle larvae of *Trichinella spiralis* (Dąbrowska *et al.* 1996; Rode *et al.* 2000), a parasitic nematode causing trichinellosis, a serious disease involving colonization of striated skeletal muscle tissue by the parasite larvae (Despommier,

1998), were surprising. As thymidylate synthase induction in cells had been shown (Pestalozzi *et al.* 1995) to be due to leaving quiescence (cell cycle G<sub>0</sub> phase), high thymidylate synthase expression in developmentally arrested muscle larvae, demonstrated also at the mRNA level (Dąbrowska *et al.* 2004), was hypothesized to be a consequence of cell cycle arrest (Rode *et al.* 2000; Dąbrowska *et al.* 2004).

Of obvious interest was whether the phenomenon, observed in *T. spiralis* muscle larvae and potentially exploitable as a target in anti-parasitic chemotherapy, is specific for intracellular parasitic life or is common for developmentally arrested life-cycle stages of other, including free-living, nematodes. In order to test the latter possibility, specific activity developmental pattern of enzymes involved in thymidylate biosynthesis was studied for *Caenorhabditis elegans*. The life-cycle of this free-living nematode, being a model for parasitic nematodes (Bürglin, Labos & Blaxter, 1998), also involves a developmentally arrested stage, dauer larva, corresponding to *T. spiralis* muscle larva (Bürglin *et al.* 1998). The results indicate the enzyme expression pattern in developmentally arrested larvae to be characteristic also for *C. elegans*, providing new evidence for previously hypothesized state of a global cell cycle arrest in dauer larvae (Euling & Ambros, 1996;

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Hong, Roy & Ambros, 1998). They also point to a possibility that this unusual regulatory pattern is specific for developmentally arrested life-cycle forms of other free-living/parasitic nematodes and indicate a convenient model for further studies of the underlying mechanism.

## MATERIALS AND METHODS

### Materials

Protease Inhibitor Cocktail Tablets (Complete, Mini) were purchased from Roche Diagnostics GmbH (Mannheim, Germany), (6*RS*, $\alpha$ *S*)tetrahydrofolic acid, thymidine, dUMP and FdUMP from Sigma (St Louis, MO), DEAE-cellulose (DE-52) from Whatman (Maidstone, UK), [5-<sup>3</sup>H] dUMP (15 Ci/mmol) and [methyl-<sup>3</sup>H] thymidine (64.7 Ci/mmol) from Moravsek Biochemicals (Brea, CA) and other reagents were of analytical grade. pBluescript SK<sup>-</sup> vector and *E. coli* DHF $\alpha$ F' strain were from Stratagene, TX61<sup>-</sup>, a derivative of the *E. coli* BL21(DE3) strain with the endogenous thymidylate synthase gene inactivated by transposon-mediated insertion, was a gift from Dr W. S. Dallas (Glaxo Wellcome, UK).

### Nematode growth and synchronization

The wild-type *C. elegans* strain N2, variety Bristol, was used, with stocks maintained at 25 °C on agar plates streaked with *E. coli* strain OP50 (Brenner, 1974). Worms to be used for determination of specific activity or purification of thymidylate synthase were grown with addition of *E. coli* strain TX61<sup>-</sup> (Cieřla *et al.* 2002) as the bacterial food. In other cultures the OP50 strain (a gift from the Caenorhabditis Genetic Center, University of Minnesota-St Paul) was used. In order to obtain a large number of worms, 1 litre of liquid culture in the complete S medium (Lewis & Fleming, 1995) at 25 °C with forced aeration was performed. *C. elegans* cultures were synchronized and worms at specific developmental stages obtained according to earlier described procedures (Lewis & Fleming, 1995). Briefly, dauer larvae were obtained by adding the stock culture to 1 litre of the S medium containing 50 g of bacteria and growing, unless otherwise indicated, for 2 weeks at 25 °C. Dauer larvae, constituting 67–100% of the resulting worm population, were cleaned by sedimentation at 1 g and either stored frozen at –30 °C or used (after culturing in the S medium containing bacteria) as a source of worms growing to adulthood. The adult worms were cleaned by sedimentation and flotation on 30% (w/v) sucrose in water and either stored at –30 °C or treated with alkaline hypochlorite solution to obtain embryos. Embryos were cleaned in the M9 buffer (Lewis & Fleming, 1995) by centrifugation at 150 g

for 5 min and either stored frozen at –30 °C or suspended in buffer M9 and allowed to develop to starvation-arrested L1 larvae (17–24 h at 25 °C). The L1 larvae were cleaned in M9 buffer by centrifugation at 150 g for 5 min and either stored frozen at –30 °C or allowed to resume synchronous growth in S medium at 25 °C (after reintroduction to bacterial food) to develop to L3 larvae. These were cleaned by centrifugation at 100 g for 5 min and stored frozen at –30 °C.

### Preparation of crude extracts

The worms were homogenized by grinding with quartz sand in a mortar (placed on ice) with 3–5 volumes of homogenizing buffer and centrifuged (20 000 g for 20 min), and the supernatant (crude extract) was saved. For assays of thymidylate synthase and dUTPase activities the worms were homogenized in ice-cold 0.05 M phosphate buffer, pH 7.5, containing 0.1 M KCl and 0.01 M 2-mercaptoethanol. For assays of thymidine kinase activity the worms were homogenized in 0.05 M Tris-HCl buffer, pH 7.5, containing 0.1 M KCl, 2 mM EDTA, protease inhibitor cocktail (Complete, Mini tablets from Roche; concentration corresponding to 1 tablet dissolved in 10 ml) and 0.01 M 2-mercaptoethanol. For assays of dihydrofolate reductase the worms were homogenized in 0.05 M Tris-HCl buffer, pH 7.0, containing 0.1 M KCl and 0.01 M 2-mercaptoethanol.

### Enzymatic studies

*C. elegans* crude extract activities of thymidylate synthase (Cieřla *et al.* 1995a), dihydrofolate reductase (Mathews, Scrimgeour & Huennekens, 1963), dUTPase (Gołos & Rode, 1999) and thymidine kinase (Nakata *et al.* 1985) were assayed according to previously published procedures. The recombinant thymidylate synthase activity was assayed spectrophotometrically (Wahba & Friedkin, 1961). The activity unit was defined as the enzyme amount required to convert 1  $\mu$ mol of substrate per 1 min at 37 °C. Kinetic studies were done as previously described (Dąbrowska *et al.* 1996; Rode *et al.* 2000).

### Protein content

Protein concentrations were determined by the procedure of Spector (1978), with bovine serum albumin used as a standard.

### RNA purification

Total RNA was isolated from *C. elegans* adult worms, and L1, L3 and dauer larvae, using Trizol ultra-pure reagent from GibcoBRL.

### RT-PCR, cDNA cloning and C. elegans thymidylate synthase expression system

General methods used for DNA manipulations were as described (Sambrook, Fritsch & Maniatis, 1989). Reverse transcription was performed on total RNA with Moloney Murine Leukemia Virus Reverse Transcriptase (Promega) and oligo(dT) primer, with an additional round of reverse transcription reaction. After the first round the sample was denatured for 3 min at 95 °C, cooled on ice and, after addition of reverse transcriptase (50 units), incubated for 1 h at 42 °C. In the PCR procedure the following primers, designed based on the NCBI database sequence (Accession no. NM\_059131), were used: forward 5'-**GGAATTCATATGGAAGTCATGAACAAA GAA**-3', identical to the initial 21 nucleotide region of the coding sequence (bold), and reverse 5'-**AAAGCAAGCTTTCAAACAGCCATATCCCAT**-3', complementing the last 19 bases of the coding region (bold). *EcoRI* and *NdeI* (in the forward primer) or *HindIII* (in the reverse primer) restriction sites (underlined) were included to permit cloning of the amplified sequence (a direct cloning into *NdeI/HindIII* sites was unsuccessful). This sequence, being the thymidylate synthase coding region, was ligated into the *EcoRI/Hind III* sites of the pBluescript SK<sup>-</sup> vector, the ligation mixture was used to transform *E. coli* DH5αF' cells and the sequence of 1000 bp inserts in the isolated recombinant plasmids was confirmed. The coding region was restricted from pBluescript as an *NdeI/Hind III* fragment and ligated into the pPIGDM4+stop expression vector (Mikiewicz *et al.* 1997) as previously described (Cieśła *et al.* 2002). A pPIGDM4+stop/thymidylate synthase plasmid pool was used to transform the thymidylate synthase-deficient *E. coli* strain TX61<sup>-</sup>, in order to obtain a *C. elegans* thymidylate synthase overexpressing system.

### Northern blot analysis

Total RNA was size-fractionated on 1% agarose-formaldehyde gel then subjected to Northern transfer onto Hybond-N<sup>+</sup> nylon membranes (Amersham) and hybridized with one of the following specific probes: (i) a 647 bp long cDNA fragment, corresponding to a part of *C. elegans* thymidylate synthase coding region or (ii) a reference probe – 612 bp long *T. spiralis* rDNA fragment (Kuratli *et al.* 1999; cf Dąbrowska *et al.* 2004). All procedures were performed as previously described (Dąbrowska *et al.* 2004).

### C. elegans thymidylate synthase overexpression

This was performed as previously described (Cieśła *et al.* 2002; Dąbrowska *et al.* 2004).

### Thymidylate synthase purification and electrophoretic analysis

A portion (25 ml) of a pelleted mixed population of different developmental *C. elegans* forms, stored at –20 °C, was used to prepare crude extract that underwent ultracentrifugation (100 000 g for 60 min) and the resulting supernatant served as a starting material to purify thymidylate synthase by the previously described (Cieśła *et al.* 1995a) affinity chromatography procedure, with Triton X-100 present only in the buffer eluting the enzyme from the concentrating DEAE-cellulose column. The recombinant enzyme was purified (Cieśła *et al.* 1995b), with protein contents determined and electrophoretic analyses performed (Cieśła *et al.* 1995a) as earlier reported. Thymidylate synthase capacity to form a ternary complex with [<sup>3</sup>H] FdUMP and CH<sub>2</sub>H<sub>4</sub>PteGlu allowed identification on the gel (Dąbrowska *et al.* 1996).

### Thymidylate synthase protein level immunoblot analysis

After PAGE-SDS (1 μg of purified recombinant thymidylate synthase or 50 μg of crude extract protein per lane), proteins were transferred into PVDF membrane. Thymidylate synthase was detected by treatment with polyclonal antibodies raised in rabbits against purified recombinant thymidylate synthase (antibodies were purified on the same recombinant thymidylate synthase preparation, immobilized on CNBr-Sepharose in the reaction using 2 mg of the recombinant thymidylate synthase protein per 1 ml of CNBr-Sepharose). Anti-rabbit IgGs coupled with HRP (Sigma) were used as secondary antibodies and colour was developed with the DAB/metal enhancer reagent (Bio-Rad). Density of bands was measured using the Scion Image program.

### Statistically evaluated results

Unless otherwise indicated, these are presented as means ± S.E.M. or means ± % difference between the mean and each of the two results, followed by the number of experiments (*N*) in parentheses.

## RESULTS

### Enzyme activities involved in dTMP biosynthesis

The specific activities of 4 enzymes involved in thymidylate biosynthesis, thymidylate synthase, dUTPase, dihydrofolate reductase and thymidine kinase, were high in adult forms, L1, L3 and dauer larvae (Table 1). Thymidylate synthase activity was not lowered when dauer larvae remained up to 6 weeks in the culture (Table 1). When measured in an extract from dauer larvae that were obtained from a starved stock culture based on their climbing behaviour (Riddle & Albert, 1997), thymidylate

Table 1. Specific activities of selected enzymes involved in thymidylate biosynthesis in extracts of *Caenorhabditis elegans* adult forms (A), and L1, L3 and dauer larvae

Developmental stage	Enzyme specific activity [nmol/min/mg protein]			
	Thymidylate synthase	Dihydrofolate reductase	dUTPase	Thymidine kinase
Adult forms	0.12 ± 17% (2)*	7.4 ± 12% (2)	3.9 ± 7.7% (2)	0.032 ± 0.006 (3)
L1 larvae	0.11 ± 45% (2)	13.2 ± 27% (2)	2.7 (1)	0.019 ± 0.009 (3)
L3 larvae	0.40 ± 0.15 (3)	7.8 ± 6.4% (2)	4.2 ± 1.35 (3)	0.016 ± 25% (2)
Dauer larvae	0.10 ± 0.03 (3)	14.0 ± 4.3% (2)	0.83 ± 0.15 (3)	0.013 ± 0.005 (3)
Dauer larvae (4 weeks at 25 °C)†	0.10 ± 0.00 (3)	NT	NT	NT
Dauer larvae (6 weeks at 25 °C)†	0.09 (1)	NT	NT	NT

\* For statistical evaluation of results see Materials and Methods section.

† The culture prepared to obtain dauer larvae was continued for the time indicated.

NT, not tested.

synthase activity was found to be similarly high (0.18 nmol/min/mg protein;  $N=1$ ).

#### Expression of thymidylate synthase mRNA and 18S rRNA in adult worms, embryos and L1, L3 and dauer larvae of *C. elegans*

The results of Northern blot analyses of *C. elegans* total RNA are presented in Fig. 1(A, B). It should be noted that previously obtained 612 bp long *T. spiralis* rDNA fragment (Dąbrowska *et al.* 2004; cf GeneBank Accession no. [AY497012](#)) was used as a reference probe, as the corresponding *C. elegans* rDNA sequence (GeneBank Accession no. [AY268117](#)) is over 80% identical. Thymidylate synthase mRNA, relative to 18S rRNA, level was found to be constant in *C. elegans* adult worms, embryos and L1 and L3 larvae, only several-fold higher than that in dauer larvae. On the other hand, thymidylate synthase mRNA level in dauer larvae was not lowered when the larvae remained up to 6 weeks in the culture (Fig. 1A, B).

#### Purification and properties of native and recombinant *C. elegans* thymidylate synthases

Purification of thymidylate synthase from *C. elegans* worms (asynchronous liquid culture), involving affinity chromatography based on dUMP-dependent enzyme binding on immobilized N<sup>10</sup>-formyl-5, 8-dideazafolate (Rode *et al.* 1979), resulted in highly enriched (2079-fold, 35% yield, specific activity of 0.4 unit/mg protein), albeit not homogeneous, preparation of the enzyme (Fig. 2). Of note is that successful application of the affinity chromatography system indicates an ordered addition of dUMP, before CH<sub>2</sub>H<sub>4</sub>PteGlu, to the enzyme. Thymidylate synthase monomer (identified based on bound [<sup>3</sup>H] FdUMP) molecular weight, determined by SDS polyacrylamide gel electrophoresis (Fig. 2), was 34 800 ± 13% (2).

Expression of the recombinant thymidylate synthase in *E. coli* cells confirmed the sequence identity. Its purification resulted in 38.5 mg of electrophoretically homogeneous preparation (not shown) with specific activity of 1.82 unit/mg and monomer molecular weight of 35 100 (identified based on bound [<sup>3</sup>H] FdUMP).

Considerable differences in affinities for substrates were found with highly purified recombinant and endogenous enzyme preparations. The  $K_m^{app}$  values for dUMP and (6RS,αS) N<sup>5,10</sup>-methylene tetrahydrofolate (CH<sub>2</sub>H<sub>4</sub>PteGlu) of 1 ± 20% μM (2) and 18 ± 14% μM (2) with the endogenous thymidylate synthase, and 2.7 ± 47% μM (2) and 107 ± 6% μM (2) with the recombinant thymidylate synthase, respectively, pointing to possible influence of post-translational modification(s) on enzyme properties. FdUMP showed linear competitive inhibition of the endogenous *C. elegans* thymidylate synthase, described by the  $K_i^{app}$  value of 0.002 ± 9% μM (2). When pre-incubated with the enzyme in the presence of CH<sub>2</sub>H<sub>4</sub>PteGlu, the analogue caused time-dependent inactivation of the enzyme, consistent with the behaviour of a slow-binding inhibitor (Morrison, 1982). The inactivation rate decreased after about 2 min pre-incubation (for similar pattern cf Dąbrowska *et al.* 1996), reflected by a biphasic plot of log (remaining activity) versus time, suggesting differing interactions of FdUMP with the two known binding sites on the thymidylate synthase molecule. Inhibition constants and inactivation rate constants, calculated with the use of apparent inactivation rate constants during the initial (0.0–1.5 min) and later (4–10 min) periods of pre-incubation with various FdUMP concentrations, were for the endogenous enzyme  $K_i' = 1.0 ± 0.11$  nM (3) and  $k_2' = 0.15 ± 0.03$  min<sup>-1</sup> (3), and  $K_i'' = 0.57 ± 0.01$  nM (3) and  $k_2'' = 0.03 ± 0.02$  min<sup>-1</sup> (3), respectively. The corresponding values for the recombinant enzyme were  $K_i' = 2.1 ± 1.2$  nM (3) and  $k_2' = 0.38 ± 0.12$  min<sup>-1</sup> (3),

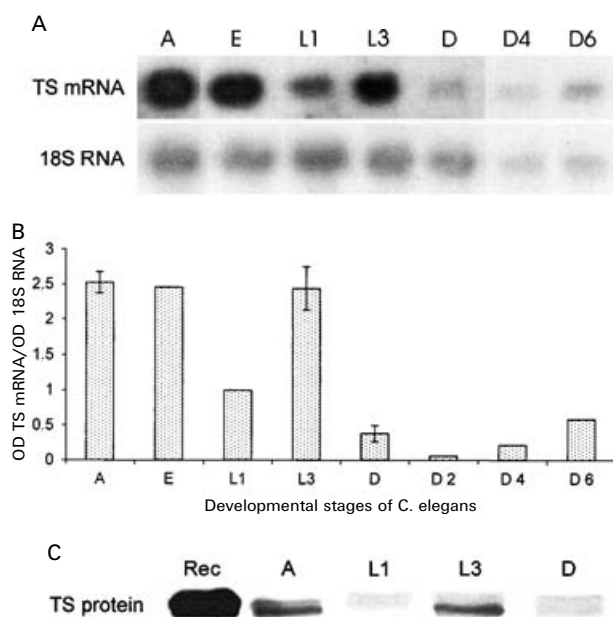


Fig. 1. (A) Thymidylate synthase (TS) mRNA and 18S rRNA bands in *Caenorhabditis elegans* adult forms (A), embryos (E), L1, L3 and dauer larvae (L1, L3 and D, respectively), and dauer larvae isolated from cultures prolonged up to 4 and 6 weeks at 25 °C (D4 and D6, respectively). (B) Thymidylate synthase mRNA level in *C. elegans* adult forms (A), embryos (E), L1, L3 and dauer larvae (L1, L3 and D, respectively), and dauer larvae isolated from cultures prolonged up to 4 and 6 weeks at 25 °C (D4 and D6, respectively), related to 18S rRNA level. Densitometric results are expressed as arbitrary units. Results for A, L3 and D are presented as mean result of  $N$  separate experiments  $\pm$  s.d. ( $N=3$  for A and L3, and  $N=4$  for D) and those for E and L1 as mean result of 2 separate experiments, differing from each of the two results by no more than 2% (E) or 13% (L1).  $N=1$  for D4 and D6. (C) Thymidylate synthase protein level in *C. elegans* adult forms (A), and L1, L3 and dauer larvae (L1, L3 and D, respectively). Immunoblot analysis with purified *C. elegans* recombinant thymidylate synthase preparation (Rec) used as a positive control. After PAGE-SDS (1  $\mu$ g of RecTS or 50  $\mu$ g of crude extract protein per lane), proteins were transferred into PVDF membrane, thymidylate synthase was detected by treatment with polyclonal antibodies raised against the recombinant enzyme. Anti-rabbit IgG coupled with HRP (Sigma) were used as secondary antibodies and colour was developed with the DAB/metal enhancer reagent (Bio-Rad). Density of bands was measured using the Scion Image program. The study was repeated twice with virtually identical results.

and  $K_i''=0.61 \pm 0.08$  nM (3) and  $k_2''=0.05 \pm 0.01$  min<sup>-1</sup> (3).

#### Expression of thymidylate synthase protein in adult worms, and L1, L3 and dauer larvae of *C. elegans*

Thymidylate synthase protein level was assessed by immunoblot analysis in crude extracts of *C. elegans* adult forms (A), and L1, L3 and dauer larvae by

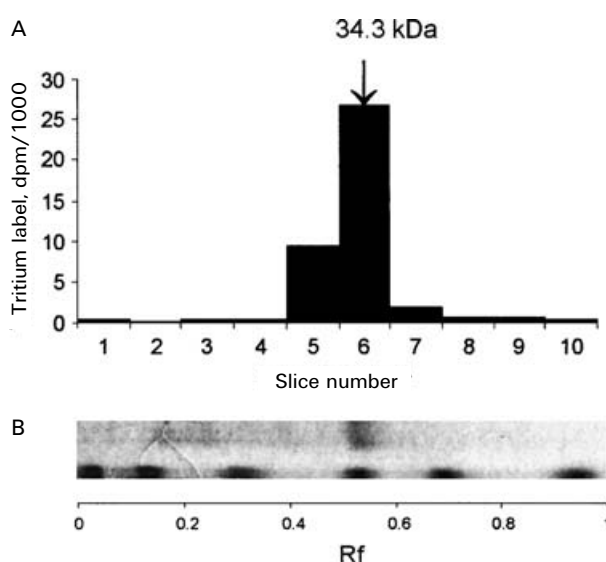


Fig. 2. PAGE-SDS of *Caenorhabditis elegans* purified endogenous thymidylate synthase preparation (5  $\mu$ g). (A) Distribution of radioactivity following separation of thymidylate synthase preparation pre-incubated with [<sup>3</sup>H] FdUMP in the presence of CH<sub>2</sub>H<sub>4</sub>PteGlu (Dąbrowska *et al.* 1996). (B) Separated thymidylate synthase preparation (upper lane) and molecular weight markers (lower lane; molecular weights 113 000, 91 000, 49 900, 35 100, 28 400 and 20 800 kDa).

comparing with the level detected in the extract prepared from adult forms, run in each experiment on the same gel and considered 100%. Following electrophoretic analysis of each crude extract, the enzyme protein level was detected on blots with a polyclonal antibody raised against purified recombinant *C. elegans* thymidylate synthase (tested to react also with endogenous *C. elegans*, as well as recombinant *T. spiralis*, rat, mouse and human thymidylate synthases). Thymidylate synthase levels found in L1, L3 and dauer larvae, expressed as a percentage of the level detected in the extract from adult forms (Fig. 1C), were  $25 \pm 7\%$  (2),  $114 \pm 20$  (6) and  $26 \pm 7$  (6), respectively. It should be noted that the enzyme shows some heterogeneity, observed previously for thymidylate synthases from *T. spiralis* and other sources (Gołos *et al.* 2002), pointing to a possibility of a post-translational modification.

#### DISCUSSION

All enzyme activities studied were present at high levels throughout the life-cycle of *C. elegans* (Table 1). This includes thymidine kinase, which could not be detected in muscle larvae of *Trichinella spiralis* and *T. pseudospiralis* (Rode *et al.* 2000), as well as in certain other parasitic nematodes (Farland & MacInnis, 1978; So, Wong & Ko, 1992), but was detected in *Brugia pahangi* and *Dirofilaria immitis* (Jaffe, Comley & Chrin, 1982). Expression of high activity of this enzyme in dauer larvae, in relation to

other developmental stages, provides further support for the cell cycle arrest hypothesis, as thymidine kinase activity is known to be dramatically lower in G<sub>0</sub> than in cycling cells (Pardee, 1989). Moreover, the three remaining enzyme activities studied were also high in developmentally arrested dauer larvae, in spite of the fact that the three enzymes, similar to thymidine kinase, are known to be associated with cell proliferation or cell cycling (Kit, 1976; Johnson, Fuhrman & Wiedemann, 1978; Vilpo, 1983; Blakley, 1984; Santi & Danenberg, 1984; Hokari *et al.* 1987; Pestalozzi *et al.* 1995; Békési *et al.* 2004) and no cell divisions occur in metabolically depressed dauer larvae (Riddle, 1988; Wadsworth & Riddle, 1988; Euling & Ambros, 1996). It is noteworthy that dauer larva specific activities of thymidylate synthase and dUTPase are comparable to those (0.1 nmol/min/mg protein and 0.7 nmol/min/mg protein, respectively) found in regenerating rat liver extracts (Cieśła *et al.* 1995a; Gołos & Rode, 1999).

The steady-state level of thymidylate synthase mRNA is known to be very low in quiescent mammalian cells, increasing 10 to 20-fold as such cells progress to the S phase and decreasing again with differentiation (discussed in Dąbrowska *et al.* 2004). When compared in a variety of cells and tissues, it reflects differences in cell proliferation rates (Lee, Shen & Johnson, 1999). The pattern of thymidylate synthase mRNA levels in *C. elegans* development is rather poorly correlated with proliferation, as dauer larvae, lacking cell divisions (Riddle, 1988; Wadsworth & Riddle, 1988; Euling & Ambros, 1996), show considerable levels of this mRNA. In accordance with thymidylate synthase specific activity, the enzyme mRNA level persists in dauer larvae for at least 6 weeks. Additionally, comparison of the enzyme activity, enzyme protein and mRNA levels in adult worms, and L1, L3 and dauer larvae, shows only the enzyme protein and mRNA patterns to be correlated. Interestingly, the enzyme activity pattern parallels the two former patterns only with L1, L3 and dauer larvae, with the enzyme activity level in adult forms being low, relative to both mRNA and enzyme protein levels. The latter points to some regulation of enzyme protein expression and activity during the development. Of note is that *T. spiralis* thymidylate synthase mRNA levels showed no differences between adults, newborns and muscle larvae, with the enzyme specific activities in adults and muscle larvae also being comparable (Rode *et al.* 2000; Dąbrowska *et al.* 2004).

Expression patterns of the three enzymes, thymidylate synthase, dihydrofolate reductase and dUTPase, indicate the unusual cell cycle regulation pattern to be shared by developmentally arrested larvae of *C. elegans* and the two *Trichinella* species (Rode *et al.* 2000; Dąbrowska *et al.* 2004). Thus, the cell cycle arrest hypothesis concerning *Trichinella* muscle larvae (Rode *et al.* 2000; Dąbrowska *et al.*

2004) is supported, as with *C. elegans* dauer larvae, a life-cycle stage corresponding to *T. spiralis* muscle larvae (Bürglin *et al.* 1998), a state of global cell cycle arrest has been hypothesized also based on other observations (Euling & Ambros, 1996; Hong *et al.* 1998). However, the apparent global cell cycle arrest in muscle larvae, reflected by high expression of the enzymes involved in thymidylate biosynthesis, does not seem to be specific for *Trichinella* parasites, as in view of the present results it may be characteristic also for developmentally arrested forms of other species of nematodes.

Notably, the possibility of global cell cycle arrest in non-developing *T. spiralis* muscle larvae poses a question of the relation of this unusual cell cycle regulation to changes induced by the larvae in muscle cells. Those changes, involving cell cycle re-entry and induction of DNA synthesis, followed by the apparent G<sub>2</sub>/M arrest in the cell cycle (Jasmer, 1995), point to muscle larvae as a source of cell cycle-specific signals.

High expression of thymidylate synthase in both *C. elegans* dauer and *T. spiralis* muscle larvae, where the enzyme's catalytic activity seems to be irrelevant, is interesting also in view of results suggesting possible non-catalytic functions of the enzyme protein in translational regulation (Liu *et al.* 2002) and neoplastic transformation (Rahman *et al.* 2004) of mammalian cells.

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