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Sequence Analysis and Functional Study of Thymidylate Synthase from Zebrafish, *Danio rerio*

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The thymidylate synthase (TS), an important target for many anticancer drugs, has been cloned from different species. But the cDNA property and function of TS in zebrafish are not well documented. In order to use zebrafish as an animal model for screening novel anticancer agents, we isolated TS cDNA from zebrafish and compared its sequence with those from other species. The open reading frame (ORF) of zebrafish TS cDNA sequence was 954 nucleotides, encoding a 318-amino acid protein with a calculated molecular mass of 36.15 kDa. The deduced amino acid sequence of zebrafish TS was similar to those from other organisms, including rat, mouse and humans. The zebrafish TS protein was expressed in *Escherichia coli* and purified to homogeneity. The purified zebrafish TS showed maximal activity at 28°C with similar K_m value to human TS. Western immunoblot assay confirmed that TS was expressed in all the developmental stages of zebrafish with a high level of expression at the 1–4 cell stages. To study the function of TS in zebrafish embryo development, a short hairpin RNA (shRNA) expression vector, pSilencer 4.1-CMV/TS, was constructed which targeted the protein-coding region of zebrafish TS mRNA. Significant change in the development of tail and epiboly was found in zebrafish embryos microinjected pSilencer4.1-CMV/TS siRNA expression vector.

Key words: cloning and expression, development of zebrafish embryos, enzymatic activity, thymidylate synthase, zebrafish.

Thymidylate synthase (TS) is a folate-dependent enzyme that catalyzes the reductive methylation of 2′-deoxyuridine-5′-monophosphate(dUMP) by the reduced folate 5,10 methylenetetrahydrofolate (CH_2THF) to thymidylate (dTMP) and dihydrofolate (*1*). Once synthesized, dTMP is further metabolized intracellularly by two successive enzymatic steps to dTTP, an essential precursor for DNA synthesis. Although dTMP can be formed through the salvage pathway catalyzed by thymidine kinase, the TS-catalyzed reaction provides for the sole intracellular de novo source of dTMP (*2*, *3*). Given its central role in DNA biosynthesis, and given that inhibition of this reaction results in immediate cessation of cellular proliferation and growth, TS represents an important target for cancer chemotherapy (*4*, *5*).

The zebrafish is an excellent system that is well suited for studies in genetics, embryology, and developmental and cell biology (*6–8*). The externally developing embryos are transparent, allowing visualization of organ systems. Zebrafish embryos exhibit unique characteristics, including ease of maintenance and drug administration, short reproductive cycle, and transparency that permits visual assessment of developing cells and organs (*9*, *10*). The zebrafish embryo has become an important vertebrate model for assessing drug effects in recent years.

TSs from more than 20 organisms have been cloned and sequenced, and the X-ray structure of several TSs from different species have been resolved. Sequence analysis of at least 17 TS proteins over a broad range of phylogenetic sources reveals that this enzyme, which is a dimer of identical subunits of 30–35 kDa, is one of the most conserved proteins identified to date (*11*, *12*). It is now well established that the regulation of expression of human and *E. coli* TSs is controlled at multiple levels including transcription, translation and post-translation. However, little is known about the regulation of TS expression in fish species. Also, the role of TS in development is not well established. In order to use zebrafish as an animal model for screening new anticancer drugs and developing a novel TS inhibitor (*13*, *14*), we cloned zebrafish cDNA and compared the cDNA sequence with *E. coli*, mouse and human TSs. We also expressed the open reading frame (ORF) of zebrafish TS in *E. coli* and purified it to homogeneity. Western immunoblot analysis confirmed that the zebrafish TS was expressed in all of the developmental stages with the highest expression levels at 1-cell to 4-cell stages.

Gene silencing *via* double-stranded RNAs has had a significant impact on the ability to suppress gene expression in a wide range of species including mice, human cells, plants, *Drosophila melanogaster*, *Caenorhabditis elegans* and yeast. Short interfering RNA-mediated gene targeting has been proved a powerful technique to study gene function in zebrafish development (*15*, *16*). However, all of the studies to date in zebrafish have used synthesized siRNA molecules. In our present report, we construct a shRNA expression vector, pSilencer 4.1-CMV/TS, to

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express short hairpin RNA (shRNA). After microinjection of the shRNA expression vector into zebrafish embryos, the TS gene was successfully silenced, and the development of the embryos was significantly affected.

MATERIALS AND METHODS

1. Total RNA Extraction—Zebrafish were raised and maintained according to standard laboratory conditions. The embryos were reared in 0.003% PTU to prevent pigmentation and staged at 28°C according to Kimmel *et al.* (*17*). Total RNA extraction was performed using Trizol RNA extraction kit (Invitrogen, CA). In brief, 10×10^2 zebrafish eggs were homogenized in 1 ml of TRIZOL buffer, and the sample was centrifuged. The aqueous phase was precipitated using isopropyl alcohol. The RNA pellet was washed 3 times with 75% ethanol, resolved in 1% agarose gel, and stained with ethidium bromide.

2. RT-PCR Analysis and cDNA Synthesis—Total RNA (5 μg) was isolated from zebrafish embryos as described above. Synthesis of the first strand cDNA was performed using Invitrogen Reverse Transcription kit (Invitrogen, CA) following the manufacturer's protocol. Briefly, 5 μg of total RNA was mixed with dNTPs in cDNA synthesis buffer. Ten units of reverse transcriptase were added to the mixture. After denaturing at 65°C for 5 min, the mixture was incubated at 45°C for 1 h. The resulting single-strand cDNA was then used as template, and 1.5 mM $MgCl_2$ was included in the PCR reaction.

3. Cloning of Zebrafish TS cDNA—Two sets of degenerated primers were designed according to the conserved regions of human and *E. coli* TSs. The sequences of the primers were as follows:

Sense: 5′-GTT TAT GGY TTY CAR TGG-3′;
Antisense: 5′-GGC RAT GTT GAA NGG RAC-3′

(where R is a purine, N is any nucleotide, and Y is pyrimidine). The PCR program parameters were initial denaturation at 94°C for 5 min followed by 31 cycles of 94°C for 60 s, 57°C for 60 s, 72°C for 90 s, and a final extension at 72°C for 10 min.

To obtain the full-length zebrafish TS cDNA sequence, 5′race and 3′race cloning systems (Clontech, CA) were used following the manufacturer's instructions. The sequences of the gene-specific PCR primers were as follows:

Sense: 5′-GGCATTTTGGAGCCGAATAC-3′
Antisense: 5′-GAGACCGATGTCTCCCGATC-3′

The PCR conditions were according to the Perkin Elmer protocol, 94°C for 60 s, 57°C for 60 s, 72°C for 90 s, 31 cycles, followed by extension at 72°C for 10 min.

4. Synthesis of TS Expression Plasmid—The complete ORF encoding the zebrafish TS gene was amplified by PCR and was inserted in pMD-18T vector at *Hin*dIII and *Eco*RI sites to synthesize a clone plasmid, pMD-18T/Z-TS. The sequences of the primers were as follows:

Sense: 5′-AAATGCCCGACACGGCAGTG-3′
Antisense: 5′-CTAAACCAGCCATTTGCATTTTG-3′

The pMD-18T/Z-TS plasmid was isolated and the nucleotide sequence of the insert was confirmed. After digesting with *Eco*RI and *Sal*I, the TS-coding region was inserted into the corresponding sites in pET-28a to construct an expression vector, pET-28/Z-TS (Fig. 1).

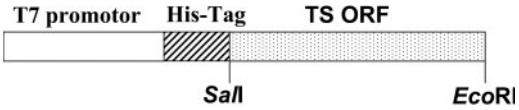

Fig. 1. **Synthesis of zebrafish TS expression vector pET-28/Z-TS.** Construction of the zebrafish TS expression vector pET-28/Z-TS is described in "MATERIALS AND METHODS." The T7 promoter is indicated by the blank box, the His-tag by the hatched box, and the cDNA ORF by the dotted box.

5. Purification of Recombinant Zebrafish TS Protein—Recombinant zebrafish TS was purified using a Qiagen Ni-NTA spin kit (Qiagen, CA) according to previously described methods (*18*). In brief, *E. coli* BL21 (DE3) cells were transformed with the zebrafish TS expression vector pET-28/Z-TS. The transformed cells were grown overnight at 37°C in liquid LB medium containing 50 μg/ml kanamycin. For protein expression, cells were diluted with fresh LB medium and grown at 37°C to an A_{600} of 0.8, and the isopropyl-β-D-thiogalactoside was added to a final concentration of 1 mM. After 3 h, cells were harvested by centrifugation. Cells were sonicated six times for 10 s each time with 5 s pauses in 1 ml of lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0). The lysate was centrifuged at $10{,}000 \times g$ for 10 min, and the supernatant was added onto the Ni-NTA spin column (Qiagen, CA). After washing the column 3 times with 600 μl of washing buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8.0), the zebrafish His-tag TS was eluted with 200 μl of elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 300 mM imidazole, pH 8.0).

6. Western Immunoblot Analysis—Western immunoblot analysis was performed as previously described (*18*). Briefly, equivalent amounts of protein (10 ng of purified TS protein or 10 μg of crude protein from zebrafish embryos) were resolved on SDS-PAGE (15% acrylamide) using the method of Laemmli (*19*). Gels were electroblotted onto nitrocellulose membranes (Bio-Rad), and the filter membranes were then incubated in blocking solution (1× PBS, 0.2% Tween-20, 5% non-fat dry milk powder) for 2 h at room temperature. Membranes were incubated overnight at 4°C with a 1:2,000 dilution of the primary antibody, anti-TS106 monoclonal antibody. After four 15-min washes in PBST (1× PBS, 0.2% Tween-20), membranes were incubated with a dilution of 1:2,000 of horseradish peroxidase–conjugated secondary antibodies (IgG goat anti-mouse, Bio-Rad) for 1 h at room temperature. After an additional four 15-min PBST washes, membranes were processed by the enhanced chemiluminescence method (SuperSignal Substrate, Pierce), and protein bands were visualized by autoradiography.

7. Determination of TS Enzyme Activity—The catalytic activity of zebrafish TS proteins was determined as previously described (*18*). This assay was performed in a total volume of 200 μl containing 10^{-5} M [5-^{3}H]dUMP (specific activity, 20 Ci/mM), 100 mM 2-mercaptoethanol, 50 mM KH_2PO_4, pH 7.2, 150 μM CH_2THF and 0.5 μg of TS protein. Each reaction mixture was incubated at 4°C, 20°C, 28°C, 37°C and 40°C for 30 min respectively. The reaction was terminated by addition of 100 ml of ice-cold 20%

trichloroacetic acid. Residual [5-^{3}H]dUMP was removed by adding 200 ml of an albumin-coated activated charcoal solution. Samples were vortexed and allowed to stand at room temperature for 10 min. The charcoal was removed by centrifugation at 10,000 × *g* for 30 min. A 250-ml sample of the supernatant was then measured for ^3H_2O radioactivity by liquid scintillation counting.

8. shRNA Design and Microinjection of the Zebrafish Embryos—The potential shRNA target site in the zebrafish was determined using the Qiagen siRNA design program (Qiagen), and the sequence was BLAST-confirmed for specificity. The targeted zebrafish TS mRNA sequence was contained within nucleotides 134–152 . The forward and reverse synthetic oligonucleotides were annealed and inserted into the *Hin*dIII and *Bam*HI sites of the pSilencer 4.1-CMV neoVector (Ambion, TX) to construct a shRNA expression vector, pSilencer 4.1-CMV/TS. pSilencer 4.1-CMV neo vector (Ambion, CA) is a circular plasmid encoding a hairpin siRNA whose sequence is not found in the mouse, human, or rat genome database, and could be used as an ideal negative control plasmid. The sequence of the siRNA is as follows: AGC TTA ACT ACC GTT GTT ATA GGT GTC TCT TGA ACA CCT ATA ACA ACG GTA AGT G. Aliquots of 0.3 ng of parent pSilencer 4.1-CMV neo Vector and 0.3 ng of pSilencer 4.1-CMV/TS were microinjected into 300 zebrafish embryos separately at the 1–2 cell stage. After 24 h, the embryos were collected and fixed with 4% paraformaldehyde in PBS (pH 7.4) for 30 min at room temperature. The development of zebrafish embryos was evaluated under light microscopy (*20*).

RESULTS

1. Isolation of the cDNA—Degenerated oligonucleotide primers were used for PCR amplification of zebrafish TS. A single 270-bp product was amplified from zebrafish cDNAs, and their nucleotide sequence was determined. By comparison with other TSs and examination of the multiple sequence alignments, the sequence exhibited a high degree similarity to other species. The isolation of partial zebrafish TS cDNA and determination of its nucleotide sequence allowed the use of the 5′race and 3′race techniques to obtain the 5′ and 3′ ends of zebrafish TS cDNA, using gene-special PCR primers. Both the 5′ and 3′ ends of the zebrafish TS gene were obtained, which allowed us to assemble the complete TS nucleotide sequence. The sequence obtained is 1,181 bp in length and contained an ORF that would encode a protein of 318 amino acids with a predicted molecular mass of 36,152 Da. There is a polyadenylation signal in the end of 3-untranslated sequence to form a poly A tail. (Fig. 2). A GC-rich region was found around nucleotide 34 to 41.

2. Comparison of Amino Acid Sequence—The deduced amino acid sequence of the TS was compared with those from human (*21*), mouse (*22*, *23*), rat (*24*), *Escherichia coli* (*25*), *Bacteriophage* T_4 (*26*) and *Leishmania major* (*27*) (Fig. 3). The amino acid identity was expressed as percentage of the aligned amino acids shared by all enzymes (Table 1). The results revealed that zebrafish TS is most closely related to mouse TS with 79% sequence identity, and related to the human TS with 76% sequence identity. As indicated in previous studies, the TS amino acid sequence is highly conserved during evolution, with 53% sequence identity between human and *E. coli*, and 60% identity between *E. coli* and *L. casei*. Thus, our study reveals that the zebrafish TS is also conservative. The folate-binding site from position 79 to position 93 is highly similar to that of the human TS. Of the 15 residues involved in the folate binding, 14 are identical between human TS and zebrafish TS. The FdUMP binding from position 195 to 205 is identical to the human TS (*28*).

Phylogenetic analysis of known TS genes using the DNAMAN program showed that the zebrafish TS is on the same branch as mammals. Zebrafish TS is much more closely related to human TS than to *E. coli*, *bacteriophage* T4 and *Bacillus* (Fig. 4).

3. Expression and Purification of the TS Enzyme—To study the enzymatic activity of zebrafish TS, we cloned the coding region of zebrafish TS cDNA into a pET-28a vector to construct an expression plasmid, pET-28/Z-TS, as shown in Fig. 1. The expression plasmid pET-28/Z-TS was transformed into *E. coli* strain BL21 (DE3) cells. Because a His-tag was introduced into the 5′ end of zebrafish TS, the Ni-NTA spin column (Promega, WI) was used to purify the enzyme from the total cellular proteins. As shown in Fig. 5, the enzyme was purified to near homogeneity on SDS-PAGE gel (Fig. 5A). The molecular mass of the His-tag zebrafish TS, including the linking sequence, was about 40.2 kDa, as compared with the 38.5 kDa of human His-tag TS. Since there was a high degree of homology between the protein sequence of TS isolated from human and zebrafish, we used human monoclonal antibody to check the immunoactivity of purified zebrafish TS. As seen in Fig. 5B, zebrafish TS protein interacts with human TS-106 monoclonal antibody with a similar affinity to human TS.

4. Zebrafish TS Activity and Kinetic Study—The activity of zebrafish TS was analyzed using a well-characterized radioactive [5-^{3}H]dUMP assay. Five temperature conditions were used to determine the effect of temperature on TS activity. As shown in Table 2, zebrafish TS manifested the highest activity at 28°C, different from the 37°C for human TS. Zebrafish TS also displayed high thermostability over a range of 10–37°C, maintaining >80% of its maximal activity over the range. Another interesting result was that with the temperature decrease, the activity of both zebrafish and human TS went down. However, the zebrafish TS showed a higher activity at low temperature than human TS. At 4°C, zebrafish TS manifested >70% of its maximal activity, while human TS showed only about 40% of its maximal activity.

We determined the K_m values of the purified zebrafish His-tag TS. At 28°C, the K_m values for the recombinant enzyme interaction with dUMP and (6*RS*,*aS*)-$N^{5,10}$-menylenetetrahydrofolate were 2.7 ± 0.4 μM and 35.6 ± 2.8 μM respectively, similar to the TSs from other source species.

5. Developmental Stage Expression of Zebrafish TS—To study the TS expression profile in zebrafish development, Western immunoblot analysis was used to determine the expression level of TS in zebrafish embryos. Human monoclonal antibody anti–TS-106 was used as primary antibody. As shown in Fig. 6, zebrafish TS was significantly expressed during the period from 20 min to 72 h (1-cell

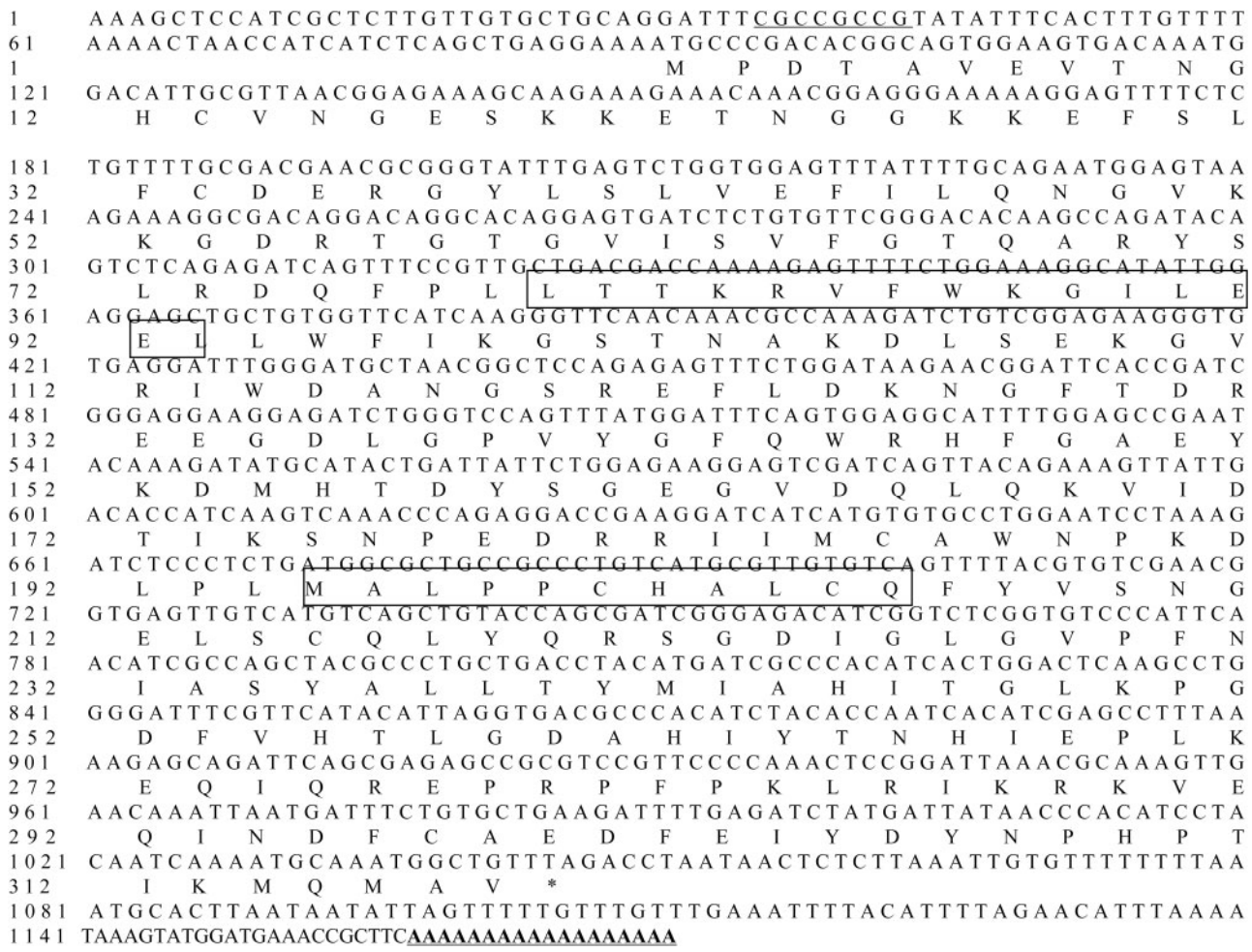

Fig. 2. **Nucleotide and deduced amino acid sequence of zebrafish TS.** The first and second boxed regions represent the folate-binding and fdUTP-binding sites, respectively. The underlined bases indicate the GC-rich region. The bold and underlined bases represent the polyadenylation signal.

stage to adult). Highest expression of zebrafish TS was found during 1-cell to 4-cell stages (Fig. 6, lane 1, 2).

6. Effect of TS mRNA Interference on Embryonic Development—To study the function of the TS gene in zebrafish development, we generate a shRNA-based vector, and microinjected into zebrafish embryos at the 1–2 cell stages. Our results suggest that the development of embryos was significantly affected after microinjecting pSilencer 4.1-CMV/TS vector, with delay in the tail and head development (Fig. 7A, 3) (of the total 300 embryos injected, abnormality was found in almost every embryo), while the development of the embryos in the control or parent vector injected showed normal tail and head development (Fig. 7A, 1 and 2). Of note, the parent vector, pSilencer 4.1-CMV neo, transcribing an shRNA which is not related within zebrafish genome, could be used as an ideal control plasmid. To confirm if the salvage pathway occurred in zebrafish, we added 10 nM thymidine in the incubation buffer of zebrafish embryos, and then injected shRNA expression vector. We found that the development of zebrafish embryos was normal with no change in the tail and head development (Fig. 7A, 4). To confirm that the development of zebrafish was inhibited by TS mRNA, Western immunoblot analysis was performed in treated or untreated embryos. Our results confirmed that the expression of TS protein in zebrafish embryos was significantly decreased in shRNA expression vector-injected embryos (Fig. 7B, lane 3, and lane 4), compared with untreated embryos (Fig. 7B, lane 1), and embryos treated with control vector, pSilencer 4.1-CMV neo (Fig. 7B, lane 2).

DISCUSSION

In this report, we cloned the full-length TS cDNA from zebrafish embryos. Sequence comparison analysis confirmed a very high homology among the TS sequences of different species, including human, mouse, rat and zebrafish TS. Western immunoblot analysis revealed that zebrafish TS was expressed in all the developmental stages, with a high level expression in the 1-cell to 4-cell stages. TS gene silencing experiment confirmed that TS plays an important role in the development of zebrafish embryos.

It is interesting that the same binding site for folate is found in human and zebrafish TS, and that the binding site

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zfin        MPDTAVEVTNGHCVNGESKKETNGGKKEFSLFCDERGYLSLVEFILQNGV      50
rat         ..........MLVEGSELQSGAQQPRTEAPQ.HGELQYLRQVEHIMRCGF      39
mouse       ..........MLVVGSELQSDAQQLSAEAPR.HGELQYLRQVEHILRCGF      39
human       .....MPVAGSELPRRPLPPAAQERDAEPRPPHGELQYLGQIQHILRCGV      45
L casei     ..............................NHEERQYLELIDRIMKTGI      19
E coli      ...............................XKQYLELMQKVLDEGT      16
ph T4       ..............................MKQYQDLIKDIFENGY      16
Consensus                                 e    h e gyl l   il  g
zfin        KKGDRTGTGVISVFGTQARYSLRDQF.PLLTTKRVFWKGILEELLWFIKG      99
rat         KKEDRTGTGTLSVFGMQARYSLRDEF.PLLTTKRVFWKGVLEELLWF.KG      87
mouse       KKEDRTGTGTLSVFGMQARYSLRDEF.PLLTTKRVFWKGVLEELLWFIKG      88
human       RKDDRTGTGTLSVFGMQARYSLRDEF.PLLTTKRVFWKGVLEELLWFIKG      94
L casei     VKEDRTGVGTISLFGAQMRFSLRDNRLPLLTTKRVFWRGVCEELLWFLRG      69
E coli      QKNDRTGTGTLSIFGHQMRFNLQDGFPLVTTKRCHLRSIIHELLWFLQGD      66
ph T4       ETDDRTGTGTIALFGSKLRWDLTKGFPAVTTKKLAWKACIAELIWFLSGS      66
Consensus     k drtgtgtlsvfg qaryslrd f pllttkrvfwkgvleellwf kg
zfin        STNAKDLSEKGVRIWDANGSREFLDKNGFTDREEGD..LGPVYGFQWRHF     147
rat         STNAKELSSKGVRIWDANGSRDFLDSLGFSARQEGD..LGPVYGFQWRHF     135
mouse       STNAKELSSKGVRIWDANGSRDFLDSLGFSARQEGD..LGPVYGFQWRHF     136
human       STNAKELSSKGVKIWDANGSRDFLDSLGFSTREEGD..LGPVYGFQWRHF     142
L casei     ETSAQLLADKDIHIWDGNGSREFLDSRGLTENKEMD..LGPVYGFQWRHF     117
E coli      TNIAYLHENNVTIWDEWADEN.............GD..LGPVYGKQWRAW     101
ph T4       TNVNDLRLIQHDSLIQGKTVWDENYENQAKDLGYHSGELGPIYGKQWRDF     116
Consensus   stnak ls kgv iwdangsrdflds gf  r egd  lgpvygfqwrhf
zfin        GAEYKDMHTDYSGEGVDQLQKVIDTIKSNPEDRRIIMCAWNPKDLPLMAL     197
rat         GADYKDMDSDYSGQGVDQLQKVIDTIKTNPDDRRIIMCAWNPKDLPLMAL     185
mouse       GAEYKDMDSDYSGQGVDQLQKVIDTIKTNPDDRRIIMCAWNPKDLPLMAL     186
human       GAEYRDMESDYSGQGVDQLQRVIDTIKTNPDDRRIIMCAWNPRDLPLMAL     192
L casei     GADYKGFEANYDGEGVDQIKLIVETIKTNPNDRRLLVTAWNPCALQKMAL     167
E coli      PTPDGRHI.........DQITTVLNQLKNDPDSRRIIVSAWNVGELDKMAL     143
ph T4       G..............GVDQIIEVIDRIKKLPNDRRQIVSAWNPAELKYMAL     153
Consensus   ga ykdm  dysg gvdqlq vidtiktnpddrriimcawnp dlplmal
zfin        PPCHALCQFYVSNG..ELSCQLYQRSGDIGLGVPFNIASYALLTYMIAHI     245
rat         PPCHALCQFYVVNG..ELSCQLYQRSGDMGLGVPFNIASYALLTYMIAHI     233
mouse       PPCHALCQFYVVNG..ELSCQLYQRSGDMGLGVPFNIASYALLTYMIAHI     234
human       PPCHALCQFYVVNS..ELSCQLYQRSGDMGLGVPFNIASYALLTYMIAHI     240
L casei     PPCHLLAQFYVNTDTSELSCMLYQRSCDMGLGVPFNIASYALLTILIAKA     217
E coli      APCHAFFQFYVADGKLSCQLYQRSCCVFLGLPFNIASYALLVHMMAQQCD     193
ph T4       PPCHMFYQFNVRNGYLDLQWYQRSVDVFLGLPFNIASYATLVHIVAKMCN     203
Consensus   ppchalcqfyv ng  elscqlyqrsgdmglgvpfniasyalltymiahi
zfin        TGLKPGDFVHTLGDAHIYTNHIEPLKEQIQREPRPFPKLRIKRKVEQIND     295
rat         TGLQPGDFVHTLGDAHIYLNHIEPLKIQLQREPRPFPKLRILRKVETIDD     283
mouse       TGLQPGDFVHTLGDAHIYLNHIEPLKIQLQREPRPFPKLRILRKVETIDD     284
human       TGLKPGDFIHTLGDAHIYLNHIEPLKIQLQREPRPFPKLRILRKVEKIDD     290
L casei     TGLRPGELVHTLGDAHVYRNHVDALKAQLERVPHAFPTLIFKEERQYLED     267
E coli      LEVGDFVWTGGDTHLYSNHMDQTHLQLSREPRPLPKLIIKRKPESIFDYR     243
ph T4       LIPGDLIFSGGNTHIYMNHVEQCKEILRREPKELCELVISGLPYKFRYLS     253
Consensus   tgl pgdfvhtlgdahiy nhieplk qlqreprpfpkl ilrkve i d
zfin        FCAE.........DFEIYDYNPHPTIKMQMA                       317
rat         FKVE.........DFQIEGYNPHPTIKMEMA                       305
mouse       FKVE.........DFQIEGYNPHPTIKMEMA                       306
human       FKAE.........DFQIEGYNPHPTIKMEMA                       312
L casei     YELT.........DMEVIDYVPHPAIKMEMA                       289
E coli      FEDF.........EIEGYDPHPGIKAPVAI                        264
ph T4       TKEQLKYVLKLRPKDFVLNNYVSHPPIKGKMA                      285
Consensus   fk e           df i  ynphptikmema
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Fig. 3. **Comparison of zebrafish TS with those from human, mouse, rat, *Escherichia coli*, *Leishmania major*, and *Bacteriophage* T4.** Amino acid sequences of zebrafish TS and other species are aligned using the NCI Blast-amino acids program. Residues that are identical in four or more sequences are indicated by shaded amino acids and expressed in lower case letters.

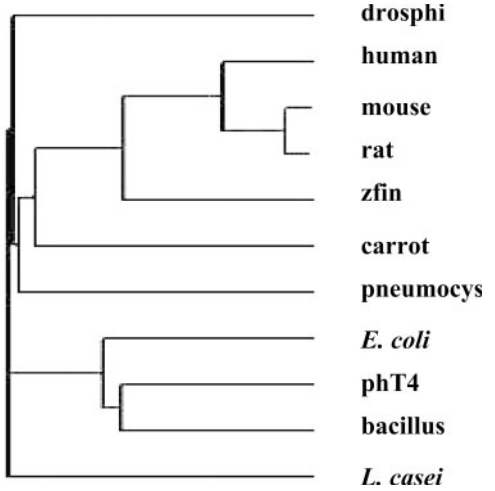

Fig. 4. **Phylogenetic tree showing the relationship of zebrafish TS with other species.** The rooted tree was drawn using the DNAMAN program. All the TS sequences were obtained from published papers or GeneBank databases: *Drosophila melanogaster* (AAC27622); *Daucus carota* (S35272); *Pneumocystis carinii* (ICI7_A); *Bacilus subtilis* (CAB13652). All the other references are presented in Fig. 3.

Table 1. **Amino acid similarity of zebrafish TS with those from other species.**

Species	Amino acid sequence identity (%)
Mouse	79
Rat	78
Human	76
L. casei	60
E. coli	53
Phage T4	44

shows a very high homology between the two species (93%). Only one amino acid residue difference was found in the folate-binding region between human and zebrafish TS in the total of 15 amino acid residues. The binding site for dUMP from position 195 to 205 was identical with human TS. Our results suggest that similar regulation mechanisms of TS may exist in zebrafish and human.

In human TS, a tandem repeat sequence is present in the 5′-UTR, consisting of either 2 or 3 repeats (*29*, *30*). Previous studies have shown that the variable number of tandem repeats of the TS gene is one of the genetic variations that can potentially predict the effectiveness

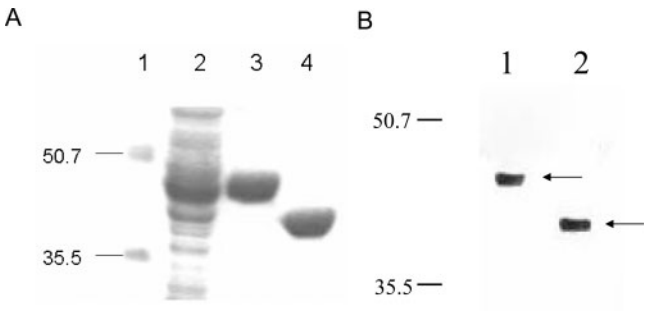

Fig. 5. **SDS-PAGE and Western immunoblot analysis of purified zebrafish His-tag TS protein.** A: SDS-PAGE of purified zebrafish TS. Crude extract of homogenized BL21 cells with a protein content of 30 μg (lane 2), or 1 μg of purified zebrafish TS (lane 3) and 1 μg of purified human TS (lane 4) were resolved on SDS-Page gel, and stained with coommassie blue. B: Western immunoblot analysis of purified zebrafish TS. Purified zebrafish TS protein and human TS protein were detected with a human TS-106 monoclonal antibody as described in "MATERIALS AND METHODS." Lane 1, purified zebrafish TS protein; lane 2, purified human TS protein.

Table 2. **Enzymatic activity of zebrafish TS at different temperatures.** The catalytic activity of zebrafish TS was determined using radioenzymatic assay as outlined in "MATERIAL AND METHODS." One unit of activity is defined as 1 mM of thymidylate formed per min at specific temperature under the conditions of the assay.

Temperature (°C)	Specific activity (U/mg)	
	Zebrafish TS	Human TS
4	0.40 ± 0.05	0.23 ± 0.03
10	0.48 ± 0.06	0.31 ± 0.04
20	0.56 ± 0.07	0.40 ± 0.05
25	0.57 ± 0.08	0.45 ± 0.06
28	0.59 ± 0.08	0.48 ± 0.07
37	0.38 ± 0.04	0.50 ± 0.08
40	0.30 ± 0.03	0.48 ± 0.08

of 5-fluorouracil–based chemotherapy (*31*). However, there was no similar sequence in the cDNA of zebrafish TS, although A GC-rich sequence was found in zebrafish TS from nucleotides 34–41 (CGCCGCCG) (Fig. 2). Further studies are needed to address if the sequence plays a role in zebrafish TS regulation. In our previous research, we found that TS could bind with its cognate mRNA and repress the expression of TS mRNA. Two binding sites have been identified on human the TS mRNA molecule. One binding site is located in the 5′ region, encompassing a 30-nts sequence including the translation start site in a stable stem-loop structure. The second binding site is a 70-nts sequence in the protein-coding region corresponding to nucleotides 480–550 (*32*). A careful analysis failed to reveal any similar sequences in the corresponding regions of the zebrafish TS mRNA. However, a stable stem-loop structure is found around nucleotides 18–38 in the 5′ untraslation region of zebrafish TS mRNA. Further studies are in progress in our lab to address whether zebrafish TS could bind with its own mRNA and repress the TS mRNA expression.

Zebrafish are normally maintained in aquaria heated to 28°C (*33*). In their natural habitat, however, they are

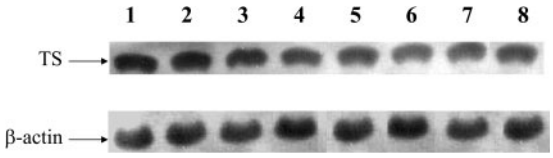

Fig. 6. **TS expression in different developmental stages of zebrafish embryos.** Samples of 30 μg of total proteins from different embryonic stages were used in the Western immunoblot analysis. Human TS-106 monoclonal antibody was used to interact with zebrafish TS as described in "MATERIALS AND METHODS." Lane 1, 1-cell stage (0.2 hpf); lane 2, 4-cell stage (1 hpf); lane 3, 1k-cell stage (3 hpf); lane 4, 6-somite stage (12 hpf); lane 5, prim-6 stage (25 hpf); lane 6, high-pec stage (42 hpf); lane 7, long-pec stage (48 hpf); lane 8, adult.

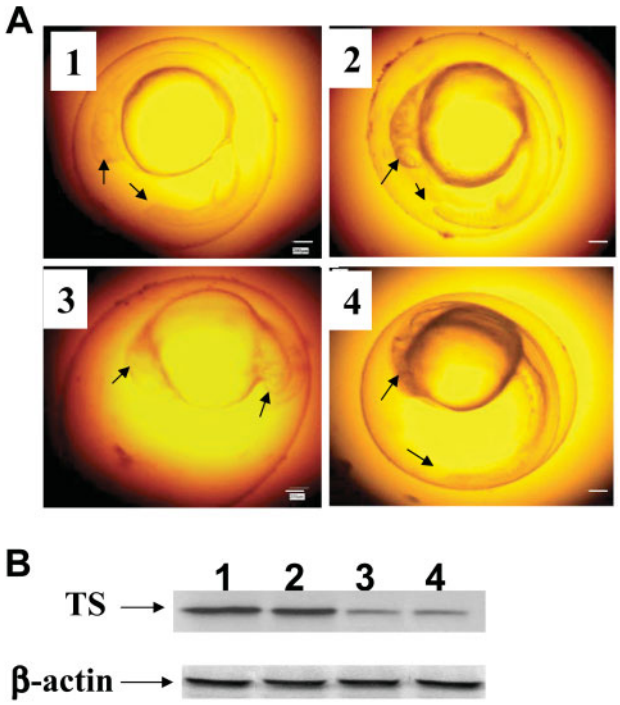

Fig. 7. **Effect of TS RNA interference on the development of embryos.** A: Light micrographs of shRNA-injected embryos. Three hundred embryos at the 1 to 2-cell stage were untreated (1), treated with 0.3 ng of control pSilencer 4.1-CMV neo plasmid (2) , 0.3 ng of pSilencer 4.1-CMV/TS plasmid (3), or incubated with 10 nM thymidine first and then microinjected with 0.3 ng of TS pSilencer 4.1-CMV/TS plasmid (4) . Embryos were harvested at 24 hpf, and analyzed under a light microscope. Scale bars: 200 μm. The arrows indicate the head and tail regions. B: TS expression in zebrafish embryos. Western immunoblot analysis was performed on embryos that were untreated (lane 1), treated with 0.3 ng of parent pSilencer 4.1-CMV neo Vector (lane 2), 0.3 ng of pSilencer 4.1-CMV/TS (lane 3), or incubated with 10 nM thymidine first and then microinjected with 0.3 ng of TS pSilencer 4.1-CMV/TS plasmid. Each of the above experiments was repeated four times.

subjected to fluctuation in body temperature. An intriguing issue, therefore, is the stability of the enzyme at different temperatures. We studied the enzymatic property of zebrafish TS in 5 different conditions with dUMP as a substrate, and confirmed that zebrafish TS had similar enzymatic activity as human TS. However, TS from zebrafish displayed its maximal activity at 28°C, different from

human TS at 37°C. Also, zebrafish TS showed higher activity than human TS at low temperature, as shown in Table 2. Activity data obtained also indicated that the zebrafish TS was stable over a range of temperature from 10 to 37°C, maintaining more than 80% of its maximal activity. The results are consistent with previously studied enzymes from zebrafish, including SULT1 cytosolic sulfotransferase (*34*) and SULT2 cytosolic sulphotransferase (*35*), which display their maximal activity at around 30°C.

The importance of TS in the development of vertebrates has not been well documented. In our study, we first checked the expression level of TS in different developmental stages, and then made use of the siRNA technique to interfere with the TS gene expression in the zebrafish embryos to see if knock-down of the TS gene could affect the development of zebrafish embryos. Our results confirmed that the TS was expressed in all of the stages, with high level expression in the 1-cell to 4-cell stages in the development of zebrafish. The results suggested that TS was needed to catalyze the synthesis of dTMP in the zebrafish development. Previous study has also shown that TS activity remains at a constant level in the crude extracts from muscle larvae, as well as from adult worms of *Trichinella spiralis* (*37*). TS is also expressed in different stages of maize development, and displays a high level of expression in the development of maize kernels from 8 to 20 DAP (days after pollination) (*38*). RNA interference experiment revealed that the development of the tail and epiboly was seriously affected after silencing the TS gene. This is the first demonstration that TS plays an important role in the development of a vertebrate. Further studies are needed to address if knock-down of the TS gene affects the expression of related genes, as is found in the human cancer cells (*39*). We also found a higher death rate in the siRNA injected embryos compared with untreated embryos or embryos treated with the control plamid (data not shown). Further study is needed to address if the TS gene interference could result in apoptosis in zebrafish embryos. Previous study has confirmed that transfection of TS siRNA into human colon cancer cells resulted in a dose-dependent inhibition of TS expression and restored the chemosensitivity of TS-resistant cancer cells to various TS inhibitors (*40*). Our study has shown that TS siRNA targeted to the coding region of TS mRNA could significantly inhibit the expression of TS, suggesting that a similar mechanism may occur in the zebrafish to that in human cancer cells.

dTMP can be synthesized by TS-catalyzed reaction as well as by the thymidine kinase-catalyzed salvage pathway using thymidine as a substrate in most vertebrates and invertebrates. To confirm if thymidine kinase–catalyzed reaction is present in zebrafish, we add thymidine in the incubation buffer, and then interfered with the TS expression using TS shRNA. Our experiments revealed that 10 nM thymidine could reverse the abnormality of zebrafish embryo development caused by TS gene interference. The results suggested that a salvage pathway occurred in the zebrafish embryos. Thymidine kinase is also an important target in cancer therapy. However, little is known about the thymine kinase in zebrafish. Our lab is trying to clone the thymidine kinase from zebrafish and study its function in the development of zebrafish embryos.

The zebrafish has become an important vertebrate model for assessing drug effects in recent years. Use of zebrafish bioassays for assessing toxicity, angiogenesis, and apoptosis has been reported (*41*). Although purified human TS has been used as a target for screening TS inhibitor *in vitro* for many years, the *in vitro* screening model does not always reflect the drug effects *in vivo*. For example, some TS inhibitors, including 5-FU, exert their effects by converting intracellularly to various nucleotide forms (*42*). Whole-animal-throughput using zebrafish allows screening of multiple targets simultaneously, and could get more information than cell-based or cell-free high-throughput screening (HTS) models. High homology between human and zebrafish TSs suggest that zebrafish could be developed as a potential tool for searching for novel TS-inhibitors. However, more efforts are needed to develop suitable indicators for TS-inhibitor screening in the whole-animal model.

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