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The identification of thymidylate synthase peptide domains located in the interface region that bind thymidylate synthase mRNA☆

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Abstract

Thymidylate synthase (TS) is a critical chemotherapeutic target and intracellular levels of TS are an important determinant of sensitivity to TS inhibitors. Translational autoregulation represents one cellular mechanism for controlling the level of expression of TS. This mechanism involves the binding of TS protein to its own messenger RNA (mRNA), thus, repressing translational efficiency. The presence of excess substrate or inhibitors of TS leads to derepression of protein binding to mRNA, resulting in increased translational efficiency and ultimately increased levels of TS protein. TS protein has been shown to bind to two distinct areas on its mRNA. The goal of the present work is to define the TS domains responsible for this interaction. Using a separate series of overlapping 17-mer peptides spanning the length of both the human and *Escherichia coli* TS sequences, we have identified six potential domains located in the interface region of the TS protein that bind TS mRNA. The identified domains that bind TS mRNA include three concordant regions in both the human and *E. coli* peptide series. Five of the six binding peptides contain at least one invariant arginine residue, which has been shown to be critical in other well-defined protein–RNA interactions. These data suggest that the identified highly conserved protein domains, which occur at the homodimeric interface of TS, represent potential participating sites for binding of TS protein to its mRNA.

Keywords: Thymidylate synthase; Peptides; Protein–RNA interaction

Thymidylate synthase (TS, EC 2.1.1.45) represents a central enzyme in the metabolic pathway for the de novo synthesis of thymidylate. This enzyme has been the focus of chemotherapeutics development for well over 4 decades [1]. The nucleotide prodrug, 5-fluorouracil, and more recently developed antifolates including Raltitrexed (ZD1694) [2,3] and Pemetrexed (Alimta, LY231514, MTA,) [4] specifically target TS and have been shown to have broad clinical utility in the treatment of cancer [1,5]. Cellular resistance to TS inhibitors has been shown to occur most commonly by increases in the intracellular levels of the target enzyme TS, a mechanism that commonly occurs with antimetabolites [1]. Further support for the role of TS as a critical determinant of fluoropyrimidine sensitivity comes from work demonstrating that lowering TS levels through the use of antisense molecules results in enhanced sensitivity to fluoropyrimidines [6]; and that the intracellular level of TS in the malignant tissue of patients may be used to predict response to fluoropyrimidine-based therapies [7,8].

Reports from several laboratories using murine and cell culture models and the malignant tissues of patients with breast cancer have demonstrated relatively rapid 3–5-fold increases in TS enzyme levels within 24 h following exposure to fluoropyrimidines in the absence of changes in TS mRNA [9–11]. TS protein has been shown to regulate its own translational efficiency through an interaction with its mRNA [12–14]. This protein–mRNA interaction has been demonstrated in both cell-free and intact cell models [10,12,14]. Binding of the TS protein to its message requires that the enzyme be unbound by substrates/inhibitors and in a non-

☆ *Abbreviations:* TS, thymidylate synthase; *E. coli*, *Escherichia coli*; EMSA, electrophoretic gel mobility shift assay; SPA, scintillation proximity assay.

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oxidized state [12,14]. Intracellular TS enzyme levels may be rapidly and precisely regulated via translational autoregulation wherein high levels of intracellular substrate (or inhibitors) lead to increased translational efficiency by relieving protein bound to RNA with resultant elevation of intracellular levels of TS [15,16]. It has also been shown that the TS protein of *Escherichia coli* (*E. coli*) binds to its own mRNA, thus, suggesting evolutionary conservation of this autoregulatory phenomenon [17]. Interestingly, the *E. coli* TS protein also binds to the human TS mRNA [17]. Other levels of control of intracellular TS exist and include regulation at the transcriptional level and possible stabilization of TS protein through an interaction of the enzyme with its substrates or inhibitors [18–20]. The autoregulation of TS translational efficiency has been shown to occur through the interaction of TS protein with either or both of the two regions on TS mRNA. The first of these two regions includes the translational start sequence and occurs at nucleotides (nt) 81–110 and the second lies between nucleotides 450 and 520 in the protein-coding region [12,21]. The first binding region has been predicted to form a stem-loop structure containing at least one bulge-C in the stem [10,12,22]. TS protein has also been shown to interact with several other mRNAs including c-myc and p53, thus, suggesting that TS protein may have broad regulatory functions in addition to its well-described catalytic function [16,23,24].

Given that the intracellular level of TS is a critical determinant of sensitivity to nucleotide and antifolate inhibitors of this enzyme, we hypothesized that the rapid induction in the level of TS following exposure to these inhibitors constituted a potentially important mechanism of clinical resistance. We reasoned that modulating the induction of TS might be a means to sensitize malignant tissues to the cytotoxic effects of TS inhibitors. The goal of the present work is to identify TS protein domain(s) that participate in the interaction with the first TS mRNA binding site (nt 81–110). Using separate series of overlapping 17-mer peptides made from the sequences of both the human and the *E. coli* TS proteins, we have identified the interface region located between the monomeric subunits of TS protein as a possible site of interaction with the TS mRNA binding site.

Materials and methods

[5-^{3}H]dUMP (21.1 Ci/mmol) was obtained from Moravek Biochemicals (Brea, CA). [^{32}P]CTP (spec. act. 200 Ci/mmol) was purchased from Perkin–Elmer Life Sciences (Boston, MA), [^{32}P]ATP (3000 Ci/mmol) was purchased from Amersham Pharmacia Biotech (Piscataway, NJ), and Tween 20, activated charcoal, BSA, and dextran were purchased from Sigma Chemicals (St. Louis, MO). Rnase inhibitor was purchased from Eppendorf (Long Island, NY). T4 polynucleiotide kinase was purchased from Promega (Madison, WI). Goat anti-mouse immunoglobin was purchased from BIO-RAD (Hercules, CA). TCA was purchased from Mallinkrodt (Paris, KY). Biosafe II scintillation cocktail was purchased from RPI (Mount Prospect, IL). Protein A – agarose beads were purchased from Invitrogen (Carlsbad, CA).

In vitro RNA synthesis. The full-length TS mRNA was synthesized with SP6 RNA polymerase following linearization of the pcEHTS plasmid with *Hin*dIII [25]. In vitro transcription reactions were performed according to the Promega (Madison, WI) supplied protocol. Radioactively labeled RNA transcripts were made by inclusion of [^{32}P]CTP (spec. act. 200 Ci/mmol) in the reaction. A 30-nt RNA sequence corresponding to nt 81–110 that includes the translational start site was also used as RNA probe. The smaller RNA probe was prepared by end-labeling the RNA with [^{32}P]ATP (3000 Ci/mmol) using T4 polynucleotide kinase. A 150 bp fragment of Actin RNA was synthesized with T7 RNA polymerase following linearization of the PGEM4Z plasmid with AVAI. In vitro transcription reactions were performed as described above.

Peptide synthesis. Two different sets of TS peptides were synthesized by Chiron Mimotopes (Raleigh, NC). The first set derived from the Human TS protein sequence consisted of 61 17-mer peptides, which spanned the entire length of the protein with an overlap of 12 residues per peptide. The peptides were biotinylated at the amino terminus through a serine–glycine–serine–glycine spacer. The second peptide set synthesized in the same manner as the human consisted of 52 17-mer peptides, which spanned the entire *E. coli* TS protein. The peptides were provided as a lyophilized powder and reconstituted in 40% acetonitrile/water (final concentration = 1 μg/μl).

RNA–protein binding assay. RNA protein binding experiments were performed using an electrophoretic gel mobility shift assay (EMSA) [12,25]. Radiolabeled TS RNA (1–2.2 fmol, 100,000 cpm) was incubated with TS peptides (1–2 μg) in a reaction mixture containing 10 mM Hepes, pH 7.4, 40 mM KCl, 3 mM $MgCl_2$, 250 mM 2-mercaptoethanol, 0.1 U/μl Rnase Inhibitor (Eppendorf), and 5% (v/v) glycerol for 15 min at room temperature. With TS full-length RNA, Rnase T1 (9 U) was added for 10 min, followed by heparin sulfate (5 mg/ml, Sigma, St. Louis, MO) for an additional 10 min. The reaction samples were resolved on a 4% non-denaturing polyacrylamide gel (acrylamide/methylenebisacrylamide ratio, 60:1), dried, and visualized by autoradiography. The EMSA was also performed with the TS 30-mer RNA using the same method as the full-length probe but without treatment with RnaseT1.

Scintillation proximity assay. A modified version of the scintillation proximity assay (SPA) developed by Amersham (Piscataway, NJ) to measure the activity of the MAP kinase enzyme was used to complement the electrophoretic gel mobility shift assay [26]. TS peptide (1 ug) was incubated with TS 30-mer RNA probe (100,000 cpm) for 15 min at RT in the same reaction mix used in the gel shift assay. A 200 μl STOP solution containing 500 μg streptavidin-coated beads and 50 μM ATP, 5 mM EDTA, and 0.1% Triton 100 in PBS was added to the assay reaction and the mixture was incubated at RT for 10 min. The samples were then centrifuged at 10,000*g* for 5 min to pellet the RNA/peptide/bead complex. The supernatant containing the free radiolabel was removed and the pellet was counted in a Beckman Scintillation Counter. Background counts were determined using a reaction mix that contained radiolabeled RNA, but no peptide. Competition experiments were performed by incubating the TS peptides and radiolabeled TS 30-mer RNA in the presence of competitor RNAs at concentrations in 500-fold excess of the labeled TS RNA. Both unlabeled TS-30 and actin RNA were used as competitors in the assay.

Preparation of TS protein. The human TS plasmid was provided by Dr. Dan Santi (University of California, San Francisco). A bacterial culture containing the TS plasmid was grown by the Bacterial Cell Production Lab, at the Frederick Cancer Research and Development Center. After pelleting the disrupted bacteria, the protein was purified using a 30–65% $(NH_4)_2SO_4$ fraction and the final pellet was frozen for further purification. The Chemical Synthesis and Analysis Laboratory at FCRDC further isolated and purified the TS protein by suspending

the pellet in 50 mM Tris, pH 7.5, 1 mM EDTA, and 0.2% 2-mercaptoethanol (Buffer A). The protein solution was loaded onto a Blue Sepharose column equilibrated with Buffer A and the column was washed with the same buffer, followed by elution of the protein with Buffer B (50 mM Tris, 1 M KCl, 1 MM EDTA, and 0.2% 2-me, ph 7.5). Fractions were assayed for TS activity and pooled. The pooled mix was dialyzed overnight at 4° against Buffer C (50 mM Tris, pH. 0, 1 mM EDTA, 12 mM 2-me, 0.1% Triton X, and 10% glycerol). The sample was then loaded onto a Q-Sepharose fast flow column equilibrated with Buffer C. Three volumes of 0–50 mM KCl gradient, followed by 10 volumes of a 50–150 mM KCl gradient in Buffer C, was passed through the column as fractions were collected. The fractions were assayed for TS activity and the peak fractions were pooled and concentrated. The purified TS protein product had a concentration of 0.97 mg/ml.

Equilibrium dialysis. Protein samples were dialyzed in a 1 ml dialysis cell chamber (Bel-Art Products, Pequannock, NJ) with a 50,000 MW cut-off cellulose ester membrane (Spectrum Laboratories, Rancho Domingas, CA). The protein samples were placed in cell A in a total volume of 500 μl of 10 mM Tris buffer. Cell B contained 500 μl of 10 mM Tris buffer alone. The sample was allowed to equilibrate at 4 °C for approximately 115 h. At the completion of dialysis, the samples were removed from both cell A and cell B and placed in centrifuge tubes for further analysis. Approximately 90% of the samples was recovered from each side of the dialysis cell.

Western blot analysis. All protein samples were resolved on a 12.5% SDS–polyacrylamide gel and electroblotted onto a nitrocellulose membrane as previously described [12]. The membrane was incubated with Blotto blocking solution (5% non-fat milk, 10 mM Tris, and 0.01% thimerosal) for 1 h and then incubated with the TS monoclonal antibody, TS106 (1–200 dilution in Blotto), for 1 h at room temperature [27]. The blots were washed with 1× PBS/0.02% Tween and incubated with goat anti-mouse immunoglobin (1–600-fold dilution) for 1 h at room temperature. After the washing was complete, the blots were subjected to a chemiluminescent substrate (Pierce Chemicals, Rockford, IL) for protein signal detection.

TS catalytic assay. The previously described catalytic assay [28] was performed in a final volume of 200 μl containing 10^{-5} M [5-^{3}H]dUMP, 2000 pmol dUMP, 200 pmol of 5–10 methylene tetrahydrofolate, 20 μM β-mercaptoethanol, and TS protein source. Samples were incubated at 37 °C for 25 min. The reaction was terminated by the addition of 100 μl ice-cold 20% TCA. Unmetabolized [5-^{3}H]dUMP was removed by addition of 200 μl albumin-coated activated charcoal solution. The samples were vortexed and allowed to stand at room temperature for 10 min. The charcoal was then settled by centrifugation at 13,000*g* for 30 min. A 200 μl aliquot of the supernatant was counted in a Beckman liquid scintillation counter to determine the amount of radioactivity representing the $[^3H]H_2O$.

Immunoprecipitation of TS protein/analysis by mass spectrometry. TS protein was incubated with a TS polyclonal antibody, TS547, at a 1–10 dilution in 0.1 M, Tris pH 7.0, 0.15 M NaCl, and 5 mM EDTA for 1 h at 4 °C on a rotating shaker. Protein A – agarose beads (75 μl) were then added to the protein/antibody mix and incubated for an additional hour at 4 °C with rotating. The sample was centrifuged and the resulting pellet was washed with buffer (120 mM NaCl) 4 times. The TS protein was then eluted with a mass spectrometric solution (α-cyano-4-hdyroxy-cinnamic acid) and analyzed by Matrix Assisted Laser Desorption/Ionization (MALDI) mass spectrometry on a Voyager DE-Pro mass spectrometer (Perspective Biosystems, Framingham, MA). The peaks detected on the spectra indicate the MW of the various proteins present in the immunoprecipitated sample.

Results

Using the RNA EMSA, we first determined which of the 61 human peptides were able to bind the radiolabeled full-length human TS mRNA. Five of the 61 human peptides screened were found to be positive for binding to the TS mRNA. The sequence and location of these peptides (9, 14, 29, 38, and 43) within the full-length TS protein are illustrated in Fig. 1B. Each binding peptide is highlighted on the ribbon structure of one of the monomeric units of the human TS homodimeric protein pictured in Fig. 1A. When these five peptides were incubated with the full-length human TS RNA probe, an RNA–protein complex was formed and detected by autoradiography (Fig. 2A, lanes 3–7). The other peptides screened did not bind TS as illustrated by the absence of a complex with an exemplary non-binding peptide (Fig. 2A, lane 2). A control reaction containing TS mRNA but no peptide also did not result in complex formation (Fig. 2A, lane 1). The relative complex quantitation was determined by densitometry and is indicated in arbitrary units under the respective lane (Fig. 2A). A binding titration of peptides 9 and 14 using 0.5, 1, and 2.5 μg peptide in the reaction mix with the human TS mRNA probe was performed and the EMSA is illustrated in Fig. 2B. The signal intensity increases in proportion to the amount of the peptide used in the reaction until it reaches a saturation level. The peptides that bound full-length TS mRNA were also tested for their ability to bind the shorter TS 30-mer RNA probe (nt 81–110) in the EMSA. All of the peptides that bound the full-length TS RNA also bound the TS 30-mer RNA (data not shown).

Because *E. coli* TS protein was previously shown to bind to human TS mRNA [17], a second set of peptides derived from the *E. coli* TS protein sequence were synthesized and tested for their ability to bind the human TS RNA. Given the evolutionary conservation of the TS protein/mRNA interaction, we reasoned that any *E. coli* peptides that bound to the human mRNA and matched conserved binding regions of the human protein might represent important TS protein/RNA contacts. Using the human TS full-length probe in the EMSA, four *E. coli* peptides were found to bind the human TS mRNA (peptides 8, 25, 28, and 33). The amino acid sequences of both the human and *E. coli* peptides that bind RNA are illustrated in Table 1 for comparison. Of the four binding *E. coli* peptides, three of them (peptides 8, 28, and 33) corresponded to the identical region of a human TS peptide (14, 38, and 43, respectively) that bound to the human TS mRNA, thus, strongly suggesting that these particular peptides may represent important contact points between the TS protein and its mRNA. *E. coli* TS peptide 25 did bind TS mRNA, but did not have a corresponding human peptide that bound TS. The two *E. coli* peptides whose sequences corresponded to the human binding peptides 9 and 29 did not bind the human TS mRNA. Although the charge of the peptide may play a non-specific role in the binding of peptides to the TS mRNA, our data show that the binding peptides had various isoelectric values (Pi's) ranging from 6.1 to

Table 1
Conserved regions of the human and *E. coli* TS peptides that bind human TS mRNA

Peptide	Sequence
Human 9	L Q Y L G Q I Q H I L R C G V R K
E. coli 3[a]	M K Q Y L E L M Q K V L D E G T Q K
Human 14	L S V F G M Q A R Y S L R D E F P
E. coli 8	T L S I F G H Q M R F N L Q D G F
Human 29	L G P V Y G F Q W R H F G A E Y R
E. coli 21[a]	G P V Y G K Q W R A Q P T P T D G R
Human 35[a]	L Q R V I D T I K T N P D D R R I
E. coli 25	Q I T T V L N Q L K N D P D S R R
Human 38	R I I M C A W N P R D L P L M A L
E. coli 28	R R I I V S A W N V G E L D K M A
Human 43	F Y V V N S E L S C Q L Y Q R S G
E. coli 33	Q F Y V A D G K L S C Q L Y Q R S

Human and *E. coli* TS peptides are listed in pairs that represent identical regions of the full-length protein; where either both or a least one of the peptides binds the TS mRNA as determined by a electrophoretic mobility shift assay.

[a] Peptides that do not bind the human TS mRNA, but do have a corresponding TS sequence in the other species which is able to bind TS mRNA.

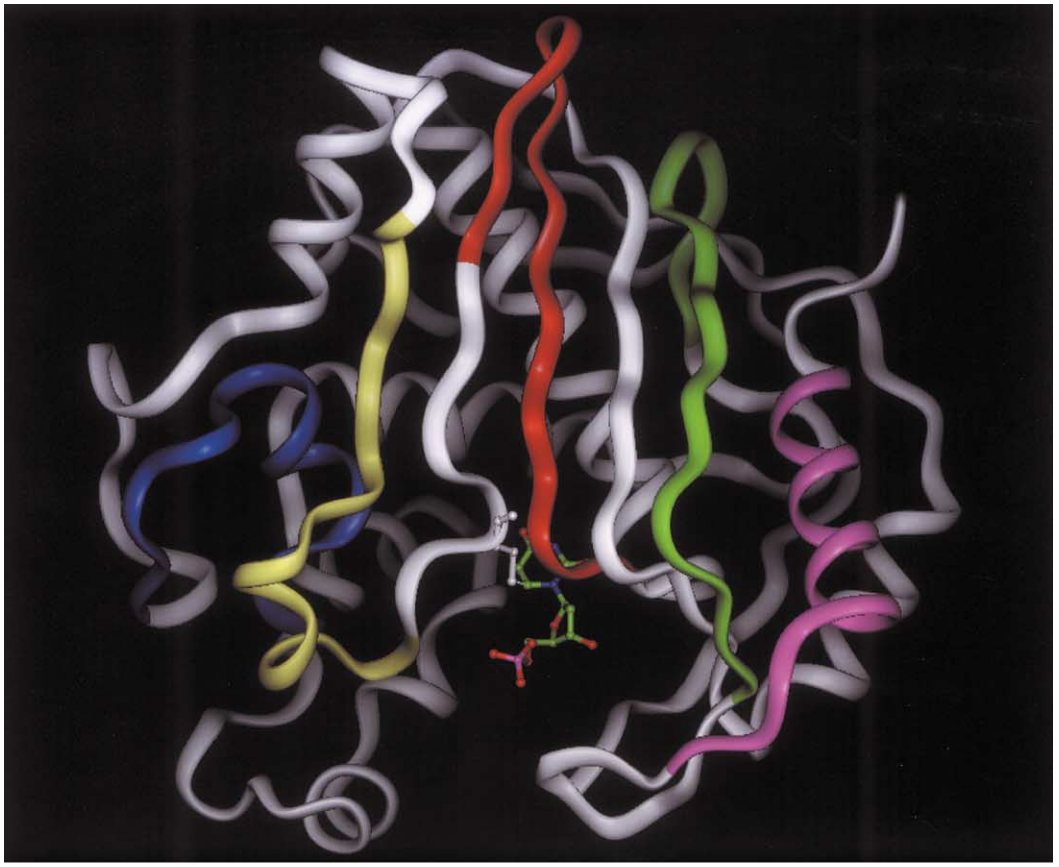
11.2, thus, suggesting that non-specific charge interactions are not responsible for the protein–RNA binding.

To further support this interaction, the SPA was used to test the binding of the EMSA positive peptides to TS mRNA. With this assay, the smaller TS 30-mer mRNA probe was used, since this RNA was found to bind the peptides with an affinity equivalent to the full-length TS mRNA. Peptides that were able to bind TS RNA in the gel shift were also found to bind the human TS 30-mer mRNA in the SPA assay. Competition experiments were performed in which both unlabeled TS mRNA and β-actin mRNA as a control were used to compete for binding to the selected peptides with the radiolabeled TS 30-mer RNA probe. The TS 30-mer RNA, but not the control actin RNA, was able to compete for binding to each of the peptides, as indicated by the decrease in radiolabeled complex formation (data not shown). The relative binding affinities of the TS peptides for the TS 30-mer RNA in the SPA assay were determined by Scatchard analysis. These affinities ranged from 1.35 to 5.65 μM. The relative binding affinities were determined for a select number of peptides based on results of the EMSA and found to be similar for their binding to either the TS full-length or the 30-mer RNA

Given that the peptide regions likely to be involved in the protein/RNA interaction were in the interface region between two monomeric subunits where access by a macromolecule like RNA would be limited, we reasoned that some fraction of the TS protein might exist in solution in a monomeric form. While catalytic function requires the enzyme to be in its dimeric form, it is conceivable that binding to RNA occurs with the enzyme in a monomeric state.

To study the possibility of TS existing in a monomeric form as well as the dimeric form, we employed an equilibrium dialysis chamber to determine if a lower MW form of TS could be identified by its passage through a membrane with a specific exclusion size of 50 kDa. Since the MW of a monomeric form of TS is approximately 35 kDa, a 50 kDa MW cut-off cellulose membrane would be expected to exclude the dimeric form of the enzyme while allowing passage of the monomeric form. As a control, both BSA and carbonic anhydrase were tested in the dialysis chamber to determine the sensitivity and selectivity of the 50 kDa MW cut-off membrane. After 72 h of dialysis at 4 °C, the BSA with a MW of 66 kDa did not pass through the membrane, but the carbonic anhydrase with a MW of 32 kDa equilibrated on both sides of the membrane. Both BSA and carbonic anhydrase were present in the aliquot removed from cell A. (Fig. 3A, lane 2). The aliquot removed from cell B after dialysis illustrates the absence of BSA protein and the presence of carbonic anhydrase as seen on the Coomassie gel (Fig. 3A, lane 3). Dialysis of the TS protein sample in the chamber for approximately 115 h at 4 °C resulted in the passage of TS through the 50 kDa membrane. An aliquot of the dialyzed samples removed from both cell A and cell B (Fig. 3B, lanes 3 and 4), as well as control protein standards, carbonic anhydrase (Fig. 3B, lane 1) and human TS (Fig. 3B, lane 2), were resolved on a 12.5% SDS–PAGE and analyzed by Western immunoblot using the TS 106 monoclonal antibody. As seen in the aliquot removed from cell B (Fig. 3B, lane 4), there is a protein band present at 35 kDa representing TS protein. As expected, a 35 kDa band is also present in the aliquot removed from cell A after dialysis, indicating the presence of the equilibrated TS, which remained in the original cell (Fig. 3B, lane 3). The TS monoclonal antibody does not cross-react with the carbonic anhydrase standard (Fig. 3B, lane 1), which is very similar in MW to TS. With dialysis times ranging from 72 to 115 h, the TS protein was able to pass through the 50 kDa MW cut-off membrane in three separate experiments. The Western blot analysis of these equilibrium dialysis experiments indicates that approximately $54 \pm 9.9\%$ of the TS protein existed in monomeric form and equilibrated on both sides of the dialysis membrane.

To further confirm that the TS protein which passed through the membrane into cell B and detected by Western marker was not degraded and fully functional, we used an in vitro TS catalytic assay to detect TS enzyme activity in the sample. Activity was determined to be present in both samples obtained from cell A and cell B. A 100-μl aliquot of the sample in cell A resulted in 172.4 pmol/min/ml, while the aliquot tested from cell B resulted in 81.6 pmol/min/ml of TS activity. These data



Peptide 9
10 50
MPVAGSELPR RPLPPAAQER DAEPRPPHGE **LQYLGQIQHI LRCGVRK**DDR
Peptide 14
60 100
TGTGT**LSVFG MQARYSLRDE FP**LLTTKRVF WKGVLEELLW FIKGSTNAKE
Peptide 29
110 150
LSSKGVKIWD ANGSRDFLDS LGFSTREEGD **LGPVYGFQWR HFGAEYR**DME
Peptide 38
160 200
SDYSGQGVDQ LQRVIDTIKT NPDDR**RIIMC AWNPRDLPLM AL**PPCHALCQ
Peptide 43
210 250
FYVVNSELSC QLYQRSGDMG LGVPFNIASY ALLTYMIAHI TGLKPGDFIH
260
TLGDAHIYLN HIEPLKIQLQ REPRPFPKLR ILRK

Fig. 1. Ribbon drawing and sequence of the human TS protein. The top panel represents a ribbon structure of the monomeric subunit of the human thymidylate synthase protein with each of the human binding peptides superimposed and highlighted as follows: peptide 9 (magenta); peptide 14 (green); peptide 29 (blue); peptide 38 (yellow); and peptide 43 (red). For orientation, the nucleotide substrate dUMP is illustrated in the active site of the enzyme. The bottom panel illustrates the sequence of the full-length human thymidylate synthase protein with the sequences of the binding peptides indicated in bold and bracketed. The highly conserved arginines within these peptide regions are underlined.

indicate that approximately 47% of the TS in the starting material was able to pass through the 50 kDa cut-off membrane in a non-degraded and fully functional state.

An additional assay employed to show that in solution TS, in part, exists in monomeric form was MALDI mass spectroscopy. The TS protein sample was incubated with the TS 547 antibody or with either an anti-tubulin antibody or buffer alone as controls. The spectra from the sample containing TS protein incubated with TS 547 demonstrated a significant peak representing a protein with a MW of 36.911 Da. Neither spectrum generated using the anti-tubulin antibody or buffer alone contained a peak corresponding to the peak at

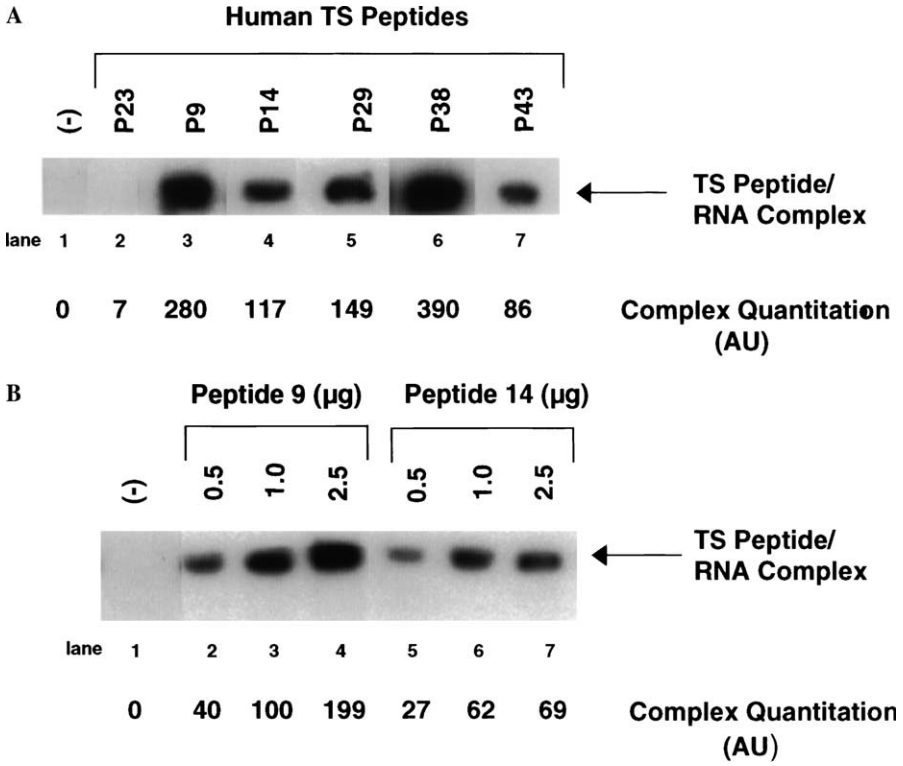


Fig. 2. Binding of human TS peptides to full-length TS mRNA. EMSA assays were performed using the full-length TS mRNA as probe. Samples were resolved on a 4% non-denaturing acrylamide gel. The peptide/RNA complex is indicated. (A) TS mRNA probe was incubated in the absence (lane 1) or presence of 2 µg peptide (lanes 2–7). The relative quantitation of the peptide/RNA complex in arbitrary units (AU) was determined by densitometry and is indicated under the respective complex. (B) TS mRNA was incubated in the absence (lane 1) of peptide or in the presence of increasing concentrations (0.5, 1.0, and 2.5 µg) peptide 9 (lanes 2–4) or increasing concentrations (0.5, 1.0, and 2.5 µg) peptide 14 (lanes 5–7). The relative quantity of the peptide/RNA complex in AU was determined by densitometry and is indicated below each respective complex.

36 kDa seen with the TS antibody. This result lends additional support for the existence of the monomeric form of TS.

Discussion

In this study, we have identified five domains on the human TS protein and four domains on the *E. coli* TS protein that bind the TS mRNA. The region of the protein represented by three of the five human peptides (14, 38, 43) corresponded to the exact protein region of one of the four *E. coli* peptides (8, 28, 33) also found to bind human TS mRNA.Several lines of evidence suggest that these particular binding regions may represent critical RNA contact points that participate in the interaction of TS protein with its mRNA including: (1) the known strong evolutionary conservation of the TS RNA/protein interaction; (2) that three domains of the human protein exactly match those of the *E. coli* protein that bind human mRNA; and (3) that all binding peptides fall in the interface region between the monomeric subunits with the present data supporting the existence of the monomeric form of TS in solution. The relative binding affinities determined for the peptides based on both the EMSA and SPA assay systems differed by approximately 7-fold. This difference is most likely due to the differences in the techniques used to generate the data; however, both methods consistently demonstrated binding affinities in the micromolar range.The micromolar affinities for the binding peptides compared with the nanomolar of the complete TS protein [25] suggest that either the conformation of the peptides is sub-optimal compared with the full length protein and/or each peptide represents only part of the complete mRNA binding site present in the full length protein. While it is unlikely that all six of the binding peptide regions identified from either the human and/or *E. coli* peptides are part of the protein binding site, it is likely that the binding peptides that represent identical regions in both the human and *E. coli* proteins represent important binding domains with the RNA. Furthermore, despite the apparent specificity of binding of the peptides to TS mRNA but not actin RNA, it is unlikely that the peptides which likely represent only portions of the complete binding site present in the full-length TS protein would preserve the level of specificity associated with the full-length protein.

It is interesting that each of the six regions defined by either the binding human and/or *E. coli* peptides lies at

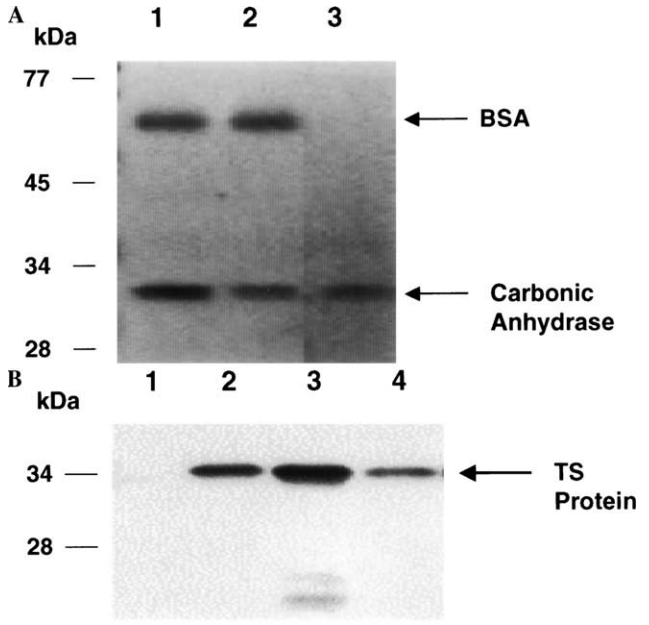


Fig. 3. Equilibrium dialysis of human TS and control proteins. A dialysis cell chamber was used to equilibrate proteins with a 50 kDa cellulose membrane. (A) BSA and carbonic anhydrase were subjected to dialysis for 72 h and an aliquot from cell A prior to dialysis (lane 1), an aliquot from cell A after dialysis (lane 2), and an aliquot from cell B after dialysis (lane 3) were resolved on a 12.5% SDS–polyacrylamide gel and analyzed by Coomassie blue staining. The proteins are indicated. (B) Human TS full-length protein was subjected to 115 h of dialysis at 4 °C. Control protein standards carbonic anhydrase (lane 1) and human TS (lane 2), an aliquot from cell A after dialysis (lane 3) and an aliquot from cell B after dialysis (lane 4) were resolved on a 12.5% SDS–PAGE and analyzed by Western immunoblot using TS 106 antibody as described.

the interface of the two monomeric subunits of the dimeric TS protein. Given that the interface region would be expected to have limited access to macromolecules such as mRNA, we felt it critical to demonstrate the presence of monomeric TS in solution. Two separate experimental systems were employed to demonstrate that a monomeric form of TS protein exists in solution, despite the well-established fact the enzyme is an obligate dimer for its catalytic function. The equilibrium dialysis experiment shows specific passage of a 35 kDa polypeptide that retains full catalytic activity and, thus, does not represent a degraded portion of dimeric TS enzyme. The inability of the 66 kDa albumin protein to pass through the membrane supports the ability of the membrane to exclude dimeric TS. The identification by mass spectroscopy of a protein precipitated by anti-TS antibody with a MW of 36.91 kDa further supports the presence of the monomeric form of TS.

We scanned the GenBank database for sequence similarities with the identified peptide regions that bind TS RNA and found that these particular regions of TS are very highly conserved amongst 11 different species whose TS protein has been sequenced [29]. These species include Human, *E. coli*, Mouse, Human herpesvirus 8, Bacteriophage T4, *Lactobacillus casei*, *Saccharomyces cerevisiae*, *Candida albicans*, *Cryptococcus neoformans*, *Plasmodium berghei*, *Leishmania major*, and Soy plant. In particular, each of the human binding peptide regions, except human peptide 9 which has the weakest affinity and whose *E. coli* counterpart did not bind, contains at least one arginine that is conserved amongst the TS of all 11 species. The conserved arginine in the different peptide regions is underlined in the sequence illustrated in Fig. 1B. Other human peptides including 25 and 34 that contain arginine were not able to bind TS mRNA, suggesting that binding is not the result of simply the presence of arginine, but depends as well on its context. Since arginine has been implicated in well-studied protein–mRNA interactions [30], the conservation of these arginines within regions of the protein that bind to the TS mRNA further supports the notion that these peptide regions may be important in the TS protein–mRNA interaction.

A preliminary investigation was performed to begin to understand the role of the arginines in the binding interaction. Single amino acid mutations were made to the conserved arginines in two of the binding peptides, peptides 14 and 43. When the arginines in each of these peptides were mutated to alanine, the peptide lost its ability to bind the human TS mRNA, thus, supporting the importance of the arginine in these interactions. No secondary structure changes were predicted to occur with the substitution of alanine for the arginines.

These data demonstrate that TS protein contains several regions that bind to both the full-length human TS mRNA and a 30-mer RNA that represents the first of the two RNA binding sites identified on the TS mRNA. Five of the identified peptides contain at least one arginine that is evolutionarily highly conserved across the TS of at least 11 different species from bacteriophage to human. These conserved aginines are all situated on the interface region of the protein. In addition, three of the highly conserved peptides of 61 human and 52 *E. coli* peptides tested represent identical regions of the protein and all contain at least one invariant arginine. These data, taken together, support the importance of these particular peptide areas as potential mRNA contact points. This information along with mutational studies of both the putative peptide binding areas and the 30-mer mRNA binding region are currently being used to develop a three-dimensional model of the TS protein–mRNA interaction. This model will form the basis for the identification and development of clinical reagents designed to limit the intracellular level of TS protein which is associated with the clinical resistance to TS inhibitors, thus, sensitizing malignant cells to the effects of TS-directed cytotoxins. This molecular model will also be used to explore chemical

libraries with the goal of identifying specific small molecule inhibitors of TS mRNA that may be used as malignant cell sensitizers in conjunction with commercially available TS inhibitors.

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