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Characterization of a specific interaction between *Escherichia coli* thymidylate synthase and *Escherichia coli* thymidylate synthase mRNA

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ABSTRACT

Previous studies have shown that human TS mRNA translation is controlled by a negative autoregulatory mechanism. In this study, an RNA electrophoretic gel mobility shift assay confirmed a direct interaction between *Escherichia coli* (*E.coli*) TS protein and its own *E.coli* TS mRNA. Two *cis*-acting sequences in the *E.coli* TS mRNA protein-coding region were identified, with one site corresponding to nucleotides 207–460 and the second site corresponding to nucleotides 461–807. Each of these mRNA sequences bind TS with a relative affinity similar to that of the full-length *E.coli* TS mRNA sequence (IC_{50} = 1 nM). A third binding site was identified, corresponding to nucleotides 808–1015, although its relative affinity for TS (IC_{50} = 5.1 nM) was lower than that of the other two *cis*-acting elements. *E.coli* TS proteins with mutations in amino acids located within the nucleotide-binding region retained the ability to bind RNA while proteins with mutations at either the nucleotide active site cysteine (C146S) or at amino acids located within the folate-binding region were unable to bind TS mRNA. These studies suggest that the regions on *E.coli* TS defined by the folate-binding site and/or critical cysteine sulfhydryl groups may represent important RNA binding domains. Further evidence is presented which demonstrates that the direct interaction with TS results in *in vitro* repression of *E.coli* TS mRNA translation.

INTRODUCTION

Thymidylate synthase (TS) catalyzes the reductive methylation of 2′-deoxyuridine-5′-monophosphate (dUMP) by 5,10-methylenetetrahydofolate (5,10-methylene-H_4PteGlu to generate thymidylate (dTMP) and dihydrofolate (H_2Pte Glu) (1). Because this enzymatic reaction provides for the sole intracellular *de novo* source of dTMP, an essential precursor for DNA biosynthesis, TS represents an important target enzyme in cancer chemotherapy (2,3).

Previous studies demonstrated that the translation of human TS mRNA is controlled by its own protein product through an autoregulatory feedback mechanism (4). Using an RNA electrophoretic gel mobility shift assay (EMSA), this translational autoregulatory process was found to be mediated by an interaction between TS protein and two *cis*-acting elements on its corresponding TS mRNA (5). The first site corresponds to a 30 nt sequence that includes the translational start site contained within a putative stem–loop structure while the second site corresponds to a 100 nt sequence located within the protein-coding region. Recent studies using an immunoprecipitation–RT-PCR technique identified a TS ribonucleoprotein (RNP) complex in cultured human colon cancer cells composed of TS protein and TS mRNA, providing direct evidence for the existence of a TS mRNA–protein complex in intact cells (6).

Over the past few years, significant efforts have been placed on elucidating the role of translational control mechanisms in the regulation of gene expression (7,8). While TS is the first eukaryotic gene whose expression was determined, in part, by translational autoregulation, this mechanism appears to play an important role in the regulated synthesis of various bacteriophage T4 (9–13) and *E.coli* proteins (14–17). Since TS represents one of the most highly conserved protein species identified to date (3), we determined whether the mechanism of translational autoregulation observed for human thymidylate synthase is conserved in evolution. Studies by Kisliuk *et al.* (18,19) found that TS isolated from MTX-resistant *Streptococcus faecium* was bound to a poly G tetraribonucleotide sequence. While the nature of this particular RNA–protein interaction was not further characterized, the authors postulated that this short RNA sequence might, itself, affect TS enzyme activity or that this tetranucleotide piece might be part of a longer RNA sequence with a specific regulatory function. Given these findings, the fact that the synthesis of a number of *E.coli* TS proteins is controlled via translational autoregulatory events, and the availability of both wild-type and mutant *E.coli* TS recombinant proteins, the bacterial *E.coli* TS system was chosen for

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investigation. In this study, we demonstrate the existence of a specific interaction between *E.coli* TS and its corresponding *E.coli* TS mRNA which results in the inhibition of *E.coli* TS mRNA translation. Further evidence is presented which identifies a potential RNA binding region on *E.coli* TS protein.

MATERIALS AND METHODS

Synthesis of plasmid constructs and *in vitro* mRNA transcripts

The full-length *E.coli* TS cDNA was excised from the Stratagene Bluescript vector at the *Xho*I and *Bam*HI restriction sites and cloned into pGEM-7Z plasmid vector (Promega). The sequence was confirmed by the dideoxynucleotide sequencing method of Sanger *et al.* (20). Full-length *E.coli* TS mRNA was synthesized with T7 RNA polymerase after linearization of the pGEM-7Z/*E.coli* WT-TS with *Bam*HI. Truncated *E.coli* TS cDNA constructs corresponding to the 5′-untranslated region (UTR), 3′-UTR and nucleotides 207–460, 461–807 and 808–1015, respectively, were prepared by PCR amplification from pGEM-7Z/*E.coli* WT-TS using specific 5′ and 3′ primers. All primers were synthesized on an Applied Biosystems model 391 DNA synthesizer. Their sequences are as follows (the underlined bases are not part of the target gene sequence and represent the unique *Xba*I and *Hin*dIII restriction enzyme digestion sites);

E.coli TS-1, ACCTCTAGAATGAAACAGTATTTAGAA (sense);
E.coli TS-2, ACCAAGCTTTTCGTCCCAGATGGTGAC (antisense);
E.coli TS-3, ACCTCTAGATGGGCCGATGAAAACGGC (sense);
E.coli TS-4, ACCAAGCTTAACTCAGATC (antisense);
E.coli TS-5, ACCTCTAGATTTGTCTGGACCGGTGGC (sense);
E.coli TS-6, ACCAAGCTTTCGTATTAGATAGCCACC (antisense);
E.coli TS-7, ACCTCTAGAAAGCTTGGCTGTCTCAGGT (sense);
E.coli TS-8, ACCAAGCTT GGTTCCTCAGGAAACGTGT (antisense);
E.coli TS-9, ACCTCTAGAATGAAACAGT ATTTAGAAC (sense);
E.coli TS-10, ACCAAGCTTTTAGATAGCCACCCGGCGCT (antisense);
E.coli TS-11, ACCTCTAGATTACGAAACATCCTGCCAG (sense);
E.coli TS-12, ACCAAGCTTAAGCTTGGCCAGTTTCTAT (antisense).

PCR amplifications were carried out under standard conditions (21) using 100 pmol of each primer and 0.5 pmol of DNA template. Amplified DNA products were digested with *Hin*dIII and *Xba*I and then cloned into the *Hin*dIII and *Xba*I restriction sites of pGEM-7Z (Promega). The corresponding RNA transcripts were synthesized with T7 polymerase after linearization with *Hin*dIII according to the Promega protocol.

TS protein purification

Homogeneous recombinant wild-type and mutant *E.coli* TS proteins were purified as previously described but without the affinity column step (22). Mutants were prepared using a high expression system (L. Changchien and F. Maley, manuscript in preparation) by means of a slight modification of the procedure of Taylor *et al.* (23) as outlined in the Amersham Mutagenesis kit. Following purification, TS was stored in a buffer containing 25 mM Tris–HCl (pH 7.4), 25 mM 2-ME and 10% ethylene glycol and kept at –20°C. Human recombinant TS was purified as previously described (4,5).

RNA–protein binding assay

RNA electrophoretic gel mobility shift assays (EMSA) were performed using previously published methods (4,5,24). In brief, radiolabeled RNA (1–2.2 fmol; 100 000 c.p.m.) was incubated with recombinant *E.coli* TS (3 pmol) in a reaction mixture containing 10 mM HEPES, pH 7.4, 3 mM $MgCl_2$, 40 mM KCl, 5% (vol/vol) glycerol and 250 mM 2-mercaptoethanol (total volume, 20 µl) for 15 min at room temperature. RNase T1 (9 U; Boehringer Mannheim) was then added for 10 min, followed by incubation with heparin (5 mg/ml; Sigma) for an additional 10 min at room temperature. Samples were electrophoresed on a 4% non-denaturing polyacrylamide gel (acrylamide/methylenebisacrylamide weight ratio, 60/1), transferred to Whatman filter paper, dried and subjected to autoradiography.

Competition experiments were performed by incubating *E.coli* TS (3 pmol) and ^{32}P-radiolabeled TS mRNA (2.2 fmol; 100 000 c.p.m.) in the presence of various unlabeled competitor RNAs, at concentrations in 0–1000-fold molar excess over that of radiolabeled TS mRNA. The relative binding affinity (IC_{50}) of each competitor RNA was measured in terms of the concentration at which specific binding of radiolabeled full-length TS mRNA to TS protein was inhibited by 50%. Each competition experiment was performed three to five times. Quantitation was performed by densitometry on a ScanJet Plus scanner (Hewlett-Packard) using NIH IMAGE 1.36 software (Wayne Rasband, National Institutes of Mental Health, Bethesda, MD).

RNase T1 mapping of TS protein binding sites

For elution of the RNA–protein complex, RNA binding reactions were performed with 220 fmol of ^{32}P-radiolabeled *E.coli* TS mRNA and 30 pmol *E.coli* TS according to the conditions as stated above. Following treatment with RNase T1 and heparin, the RNA–protein complex was then resolved on a 4% polyacrylamide gel, visualized by wet autoradiography following a 5–6 h exposure, and eluted from the gel in a buffer containing 0.5 M ammonium acetate, 10 mM magnesium acetate and 0.1% SDS overnight at 37°C. Eluted RNAs were then subjected to phenol–chloroform extraction and ethanol precipitation, resolved on a denaturing 8 M urea–15% acrylamide gel and visualized by autoradiography.

UV crosslinking of RNA–protein complexes

RNA–protein binding reaction mixtures containing ^{32}P-radiolabeled full-length *E.coli* TS RNA sequence with either wild-type or mutant *E.coli* TS proteins (3 pmol) were incubated at room temperature for 15 min and subsequently treated with RNase T1 and heparin as described above. The reaction mix was then subjected to UV cross-linking for 10 min at 254 nm (Stratalinker 1800 UV crosslinker, Stratagene), and the RNA–protein complexes were resolved on an SDS–12.5% polyacrylamide gel. The gel was dried and subjected to autoradiography.

In vitro translation

Translation reactions (final volume, 30 µl) were performed using a rabbit reticulocyte protein-processing *in vitro* translation system (New England Nuclear, Boston, MA) as previously described (4). Translation reactions were also performed using either the wheat germ extract system (Promega, Madison, WI) or the *E.coli* S30 prokaryotic *in vitro* translation system (Promega, Madison, WI)

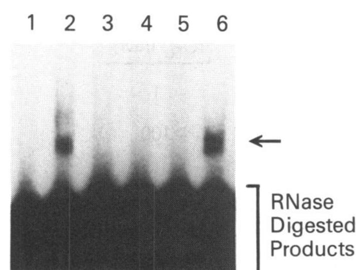

Figure 1. Specific binding of *E.coli* TS protein to *E.coli* TS mRNA. Labeled *E.coli* TS mRNA (2.2 fmol, 100 000 c.p.m.) was incubated in the absence (lane 1) or presence (lane 2) of recombinant *E.coli* TS (3 pmol). TS (3 pmol) was first denatured at 95°C for 15 min (lane 3) and then incubated in a reaction mix containing radiolabeled *E.coli* TS mRNA. Radiolabeled *E.coli* TS mRNA (2.2 fmol, 100 000 c.p.m.) was incubated with bovine serum albumin (3 pmol; lane 4), glutathione-*S*-transferase–max fusion protein (3 pmol; lane 5), or with human recombinant TS (3 pmol; lane 6). Samples were electrophoresed in a non-denaturing 4% acrylamide gel. The specific complex is indicated by the arrow.

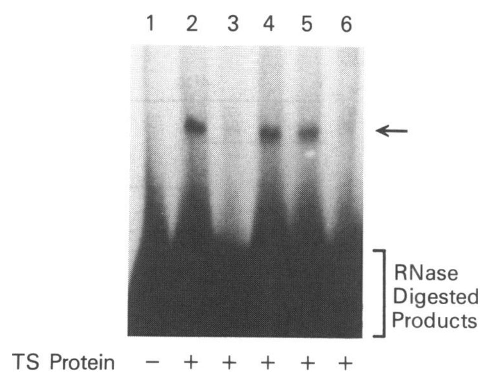

Figure 2. Competition experiment using various *E.coli* TS mRNA sequences with *E.coli* TS. ^{32}P-radiolabeled *E.coli* TS mRNA (2.2 fmol, 100 000 c.p.m.) was incubated in the absence (lane 1) or presence, where indicated, (lanes 2–6) of TS (3 pmol). A 100-fold molar excess of an unlabeled *E.coli* TS mRNA sequence represented by either the 1165 nt full-length (lane 3), 5′-UTR (lane 4), 3′-UTR (lane 5), or the 808 nt protein-coding region (lane 6) was also included in the reaction mix. Samples were electrophoresed in a non-denaturing 4% acrylamide gel and visualized by autoradiography. The specific complex is indicated by the arrow.

according to the Promega protocol. Translation protein products were analyzed by SDS–PAGE (12.5% acrylamide) according to the method of Laemmli (25), and the gels were processed as previously described (4). After drying for 2 h, the translation products were visualized by autoradiography.

RESULTS

Specific RNA–protein binding

Using the RNA electrophoretic gel shift assay, we first determined whether there was a specific interaction between *E.coli* TS and *E.coli* TS mRNA. When full-length radiolabeled *E.coli* TS RNA probe was incubated with wild-type recombinant *E.coli* TS, an RNA–protein complex was formed (Fig. 1, lane 2). However, when TS was first denatured either by heat (Fig. 1, lane 3) or by treatment with proteinase K (data not shown), no complex was formed. The fact that denatured *E.coli* TS was unable to bind to its target RNA provides evidence that the native state of TS is a critical requirement for RNA binding. No complex was formed when radiolabeled *E.coli* TS mRNA was incubated with unrelated proteins including either bovine serum albumin (Fig. 1, lane 4) or a glutathione-*S*-transferase-max fusion protein (Fig. 1, lane 5). The inability of denatured *E.coli* TS as well as unrelated proteins to complex with *E.coli* TS RNA lends support for the specific nature of this RNA–protein interaction. The incubation of radiolabeled *E.coli* TS RNA with human recombinant TS also yielded an RNA–protein complex (Fig. 1, lane 6). In addition, an RNA–protein complex was formed when radiolabeled human TS mRNA probe was incubated with wild-type *E.coli* TS (data not shown). These findings suggest that the specific region(s) on TS protein that mediates RNA binding is conserved.

Identification of *cis*-acting RNA sequences

To begin to identify the RNA binding site(s) on *E.coli* TS RNA, the ability of critical regions on *E.coli* TS mRNA to interact with *E.coli* TS was examined. RNA sequences corresponding to either the 5′- or 3′-UTR or the entire protein-coding region were synthesized *in vitro* and then used as unlabeled competitors in EMSAs to determine their relative affinity to TS protein (Fig. 2). Full-length *E.coli* TS mRNA effectively blocked RNA–protein complex formation (Fig. 2, lane 3). In contrast, TS RNA sequences corresponding to either the 5′-UTR (Fig. 2, lane 4) or the 3′-UTR (Fig. 2, lane 5) did not inhibit complex formation. The TS RNA sequence corresponding to the entire protein-coding region (nucleotides 207–1015) effectively inhibited formation of complex to the same extent as that observed with full-length *E.coli* TS RNA (Fig. 2, lane 6). This initial set of competition experiments suggested that the key *cis*-acting element(s) on *E.coli* TS RNA was located within the protein-coding region.

To more precisely define the RNA binding site(s) within the protein-coding region of *E.coli* TS mRNA, three different RNA sequences were synthesized *in vitro* and subsequently employed in competition experiments. As shown in Figure 3, a 253 nt *E.coli* TS RNA sequence corresponding to nucleotides 207–460 and a 346 nt sequence corresponding to nucleotides 461–807 demonstrated similar relative binding affinities (IC_{50} = 1.8 and 1.5 nM, respectively) when compared to either full-length *E.coli* TS mRNA (IC_{50} = 1 nM) or to the entire protein-coding region *E.coli* TS RNA sequence (IC_{50} = 1 nM). While the *E.coli* TS RNA sequence defined by nucleotide positions 808 to 1015 also competed with radiolabeled RNA probe for protein binding, its relative binding affinity for TS was significantly lower than that of the *E.coli* TS RNAs represented by either the full-length or the protein-coding region (IC_{50} = 5.1 nM; $P < 0.001$).

To determine the size of the RNA species involved in the formation of the RNA–protein complex, the RNA was isolated from the complex and subsequently resolved on a denaturing gel. Using either the full-length wild-type *E.coli* TS mRNA or the TS RNA sequence corresponding to the protein-coding region, we observed a single RNA fragment that was ~30 nt in length (data not shown). Given that the competition experiments demonstrate the presence of at least three different binding sites on the *E.coli* TS mRNA, this finding suggests that the predominant size of the

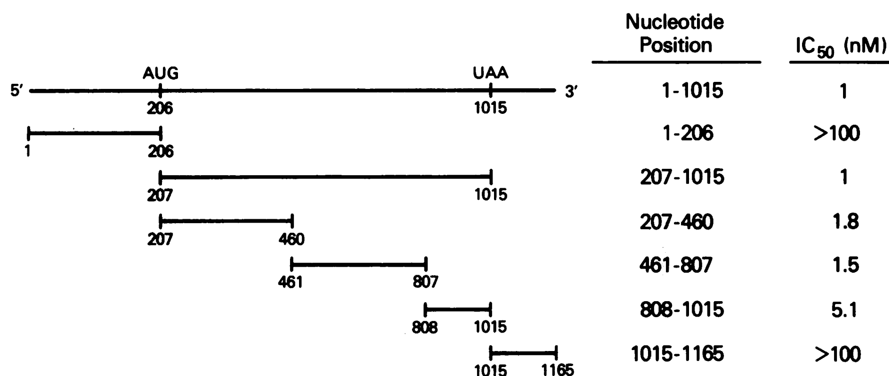

Figure 3. Competition experiment to determine relative binding of various *E.coli* TS RNA sequences. Binding of *E.coli* TS to different *E.coli* TS RNAs relative to full-length *E.coli* TS mRNA was determined by comparing the concentration of unlabeled RNA at which specific binding of ^{32}P-radiolabeled full-length *E.coli* TS mRNA was inhibited by 50%. Each value represents the mean of three to five experiments. Details for synthesis of the recombinant *E.coli* TS RNAs are presented in Materials and Methods.

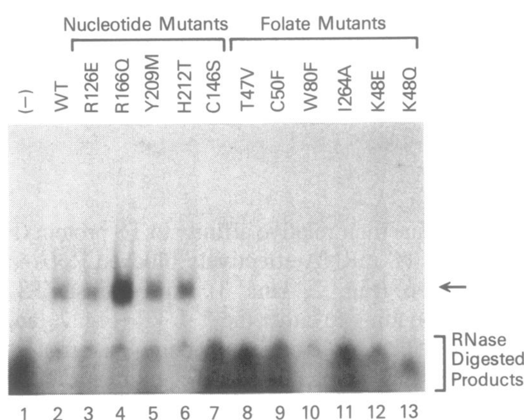

Figure 4. Binding of mutant *E.coli* TS proteins with *E.coli* TS mRNA. Radiolabeled *E.coli* TS mRNA (2.2 fmol, 100 000 c.p.m.) was incubated in the absence (lane 1) or presence of wild-type *E.coli* TS (3 pmol, lane 2). Labeled *E.coli* TS mRNA was also incubated with 3 pmol of mutant *E.coli* TS proteins R126E (lane 3), R166Q (lane 4), Y209M (lane 5), H212T (lane 6), C146S (lane 7), T47V (lane 8), C50F (lane 9), W80F (lane 10), I264Am (lane 11), K48E (lane 12) and K48Q (lane 13). The specific complex is indicated by the arrow.

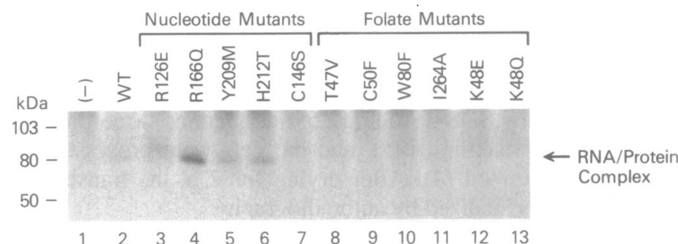

Figure 5. UV cross-linking analysis of radiolabeled *E.coli* TS mRNA with wild-type and mutant *E.coli* TS proteins. ^{32}P-radiolabeled full-length *E.coli* TS mRNA was incubated in the absence (lane 1) or presence of either wild-type *E.coli* TS (3 pmol; lane 2) or mutant *E.coli* TS proteins (3 pmol), where indicated. UV cross-linking was performed as described in Materials and Methods. The RNA–protein complex is indicated by the arrow.

RNase T1-protected RNA species for each of these *cis*-acting elements is approximately the same size.

RNA binding of mutant *E.coli* TS proteins

In order to identify the potential RNA binding domain(s) on *E.coli* TS, we examined the ability of various mutant recombinant *E.coli* TS proteins to form a complex with *E.coli* TS mRNA. The amino acids altered in these mutant proteins were largely in the enzyme active site and as determined by X-ray crystallographic studies, they were all involved in ligand binding (26–29). Those amino acids in direct contact with or near residues in contact with the nucleotide substrate were designated as belonging to the nucleotide-binding region, while those amino acids in contact with or near those in contact with the folylpolyglutamate substrate were designated as being part of the folate-binding region. Of note, all but two of the mutated amino acid residues, C50 and I264, are invariant among all TS sequences characterized to date (30). As shown in Figure 4, all *E.coli* TS proteins with point mutations involving the nucleotide-binding region (Fig. 4, lanes 3–6) retained their RNA binding function with the exception of the C146S mutant (Fig. 4, lane 7). In contrast, TS proteins with point mutations in the folate-binding region were unable to interact with *E.coli* TS RNA (Fig. 4, lanes 8–13). Identical results were obtained when these mutant proteins were employed in binding experiments using radiolabeled human TS RNA as probe (data not shown). This finding provides additional evidence that the critical RNA binding domain(s) on TS protein appears to be conserved in evolution.

A UV cross-linking analysis was performed to determine the approximate molecular weight of the RNA–protein complex as well as to identify the size of the *E.coli* TS RNA fragment bound to TS protein. A complex resolving at ~70 kDa was observed when either wild-type *E.coli* TS (Fig. 5, lane 2) or the nucleotide-binding region mutant proteins R126E (Fig. 5, lane 3), R166Q (Fig. 5, lane 4), Y209M (Fig. 5, lane 5), H212T (Fig. 5, lane 6) were UV cross-linked with radiolabeled *E.coli* TS RNA probe. However, no complex was observed when either the nucleotide mutant protein C146S (Fig. 5, lane 7) or any of the mutant proteins of the folate-binding domain were employed (Fig. 5, lanes 8–13).

To determine the size of the cross-linked RNA fragment, we compared the molecular weight of the RNA–protein complex when either full-length *E.coli* TS mRNA or a 35 nt TS RNA

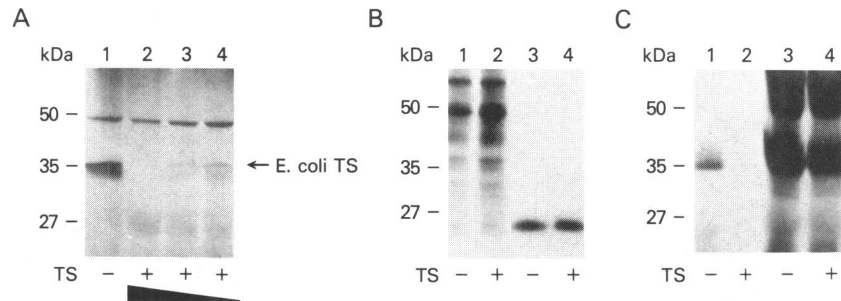

Figure 6. Inhibition of *E.coli* TS mRNA translation by *E.coli* TS protein *in vitro*. (**A**) Translation reactions containing rabbit lysate were incubated with *E.coli* TS mRNA (0.7 pmol) in the absence (lane 1) or presence of 6 pmol (lane 2), 3 pmol (lane 3) and 1.5 pmol (lane 4) of wild-type *E.coli* TS. (**B**) Translation reactions containing rabbit lysate were incubated with either 0.7 pmol human chromogranin mRNA (lanes 1,2) or 0.7 pmol human folate receptor mRNA (lanes 3 and 4). *E.coli* TS (6 pmol), where indicated, was included in the reaction mixture. Translation reactions were incubated at 37°C for 60 min, and the protein products were analyzed by SDS–PAGE and autoradiography as described in Materials and Methods. The *in vitro*-translated TS protein is indicated by the arrow. (**C**) Translation reactions containing *E.coli* S30 extract were incubated with either linearized full-length *E.coli* TS cDNA (7 pmol) (lanes 1 and 2) or a linearized reporter plasmid containing luciferase (luc) cDNA, pBESTluc (7 pmol) (lanes 3 and 4). Wild-type *E.coli* TS (70 pmol), where indicated, was included in the reaction mixture. Translation reactions were incubated at 37°C for 2 h according to the Promega protocol (Promega), and the protein products were analyzed by SDS–PAGE and autoradiography as described in Materials and Methods.

sequence representing the upstream *cis*-acting element of the human TS RNA were used in UV cross-linking experiments. Both the 35 nt TS RNA and the full-length *E.coli* TS mRNA sequences yielded complexes with identical molecular weights (~70 kDa) (data not shown). This finding suggests that the size of the *E.coli* TS RNA sequence bound to TS protein and protected from RNase T1 digestion is ~30–35 nt in length.

Effect of *E.coli* TS on *E.coli* TS mRNA translation *in vitro*

To determine whether binding of *E.coli* TS to its own TS mRNA was associated with translational repression, we employed a rabbit reticulocyte lysate *in vitro* translation system. Translation of rabbit lysate in the absence of exogenous RNA yielded a protein product with a molecular weight of ~47 kDa. This protein is present in all of the reaction samples (Fig. 6A, lanes 1–5). When *E.coli* TS mRNA was included in the lysate reaction, the corresponding *E.coli* TS protein resolving at 30 kDa was observed (Fig. 6A, lane 1). The effect of exogenous *E.coli* TS on the translation of *E.coli* TS mRNA was next determined. With the addition of decreasing concentrations of TS (Fig. 6A, lanes 2–4), there was a dose-dependent inhibition of *E.coli* TS mRNA translation. This inhibitory effect of *E.coli* TS appeared to be specific in that the translation of unrelated mRNAs including human chromogranin (Fig. 6B, lanes 1 and 2) and human folate receptor (Fig. 6B, lanes 3 and 4) were unaffected. Similar studies were performed using the wheat germ *in vitro* translation system which confirmed that the inhibition of *E.coli* TS mRNA translation by *E.coli* TS was specific (data not shown). We also employed an S30 prokaryotic *in vitro* coupled transcription–translation system to test more directly the specificity of *E.coli* TS regulation. When linearized full-length *E.coli* TS cDNA was included in the S30 extract, the corresponding *E.coli* TS protein was synthesized resolving at ~30 kDa (Fig. 6C, lane 1). In the presence of exogenous *E.coli* TS, however, the synthesis of [^{35}S]methionine-labeled *E.coli* TS was completely repressed (Fig. 6C, lane 2). In contrast, the expression of a reporter construct encoding for luciferase remained the same either in the absence (Fig. 6C, lane 3) or presence (Fig. 6C, lane 4) of exogenous *E.coli* TS.

DISCUSSION

In this report, we present evidence that demonstrates a specific interaction between *E.coli* TS and its corresponding *E.coli* TS RNA. A series of RNA gel shift competition experiments revealed that the binding site on *E.coli* TS mRNA was localized to at least three different sites within the protein-coding region. One site is a 253 nt sequence that includes nucleotides 207–460 while the second site is a 346 nt sequence that corresponds to nucleotides 461–807. The affinity of *E.coli* TS for either of these sequences is approximately the same as that observed for full-length *E.coli* TS mRNA (IC_{50} = 1 nM)). While *E.coli* TS was also able to interact with a third TS RNA sequence located within the C-terminal part of the protein-coding region corresponding to nucleotides 808–1015, the affinity of this interaction was significantly lower than that observed for the other *cis*-acting sites (IC_{50} = 5.1 nM). Preliminary analysis of these three *cis*-acting *E.coli* TS RNA sequences reveals no consensus with regard to sequence and/or secondary structure. However, more precise RNA sequencing studies are required to completely characterize each of these binding sites.

Using three different *in vitro* translation methods, a prokaryotic *E.coli* S30 extract, a wheat germ extract and a rabbit reticulocyte lysate system, our studies show that formation of this specific RNA–protein complex is associated with translational inhibition of *E.coli* TS mRNA. This finding suggests that the synthesis of *E.coli* TS protein may be controlled by a translational autoregulatory mechanism in a manner identical to that previously reported for human TS (4,5). Translational regulation of human TS was first proposed by Takeishi *et al.* (31) who noted that three tandem repeat sequences were located within the 5′-UTR of the TS cDNA with the potential to form stable secondary stem–loop structures. In a follow-up study using a TS-negative murine FM3A cell line and DNA-mediated gene transfer techniques, Kaneda *et al.* (32) observed that deletion of these three repeat elements significantly increased the translational efficiency of transcribed TS mRNA suggesting that these sequences might act either *in cis* by virtue of

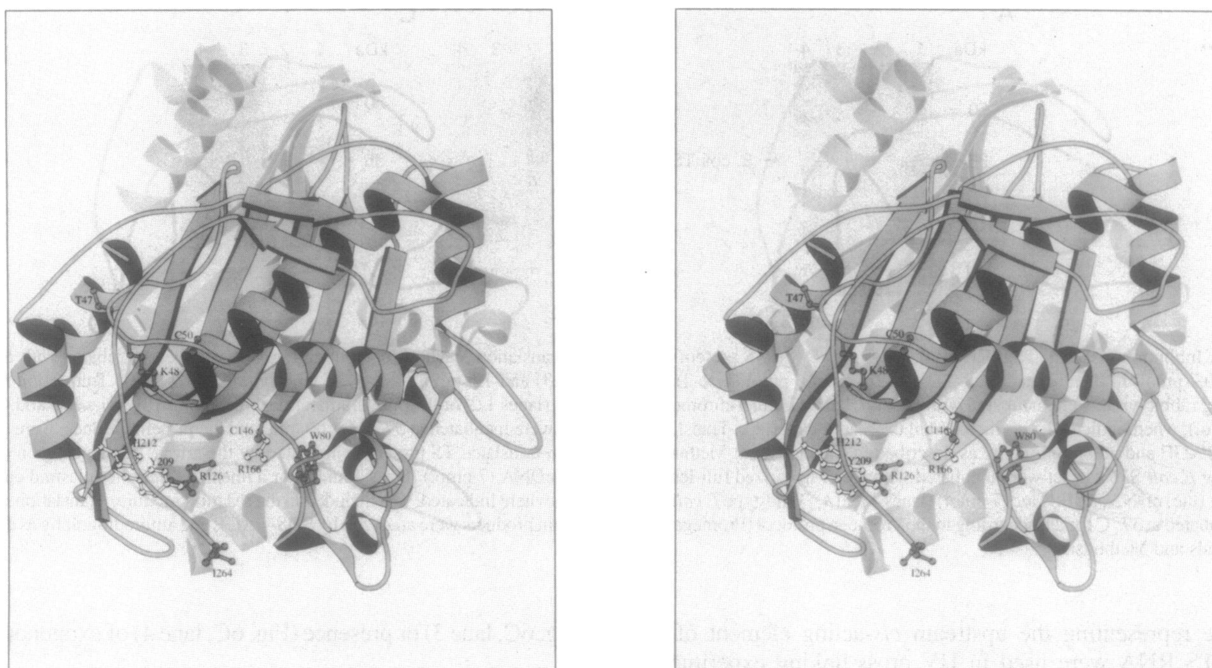

Figure 7. Stereo ribbon drawing of *E.coli* TS. Amino acids whose mutation results in *E.coli* TS proteins with RNA binding are displayed with open bonds while those amino acids whose mutation results in loss of RNA binding are indicated by shaded bonds. Arginine 126 is contributed by the 'second' monomer of TS and is labeled R126'. Sequence numbers are for the *E.coli*-derived TS. This figure was produced using the program MOLSCRIPT (45).

secondary structure or *in trans* with a cellular factor to inhibit the translation of TS mRNA. Recent *in vitro* studies have shown that short-term exposure of human H630 colon cancer cells to 5-fluorouracil is associated with an increased expression of TS protein due to an enhanced translational efficiency of TS mRNA (33). In addition, a similar induction of TS protein expression with no corresponding change in the levels of TS-specific mRNA was observed when human MCF-7 breast cancer cells were exposed to the folate analog ZD1694 (34). These studies, taken together, demonstrate that TS expression in response to cytotoxic stress is controlled by a translational event and provide further support for the biological relevance of TS translational autoregulation. Moreover, the fact that TS translational autoregulation is evolutionarily conserved indicates that this mechanism represents a fundamental regulatory event underlying the expression of TS. Although translational autoregulatory events have only recently been described for eukaryotic genes including TS (4) and dihydrofolate reductase (35), this process appears to play a critical role in the regulated synthesis of a number of bacteriophage proteins (9–13) and *E.coli* ribosomal proteins (14–17). Thus, the interaction between *E.coli* TS and its corresponding *E.coli* TS mRNA, as described in the present study, provides another example of a gene product which may directly regulate its own expression.

Using a series of mutant recombinant *E.coli* TS proteins in RNA binding experiments, we have shown that *E.coli* TS proteins with mutations in the nucleotide-binding region with the exception of the nucleotide active site cysteine mutant (C146S) maintained their ability to bind to either human or *E.coli* TS RNA. Of note, these nucleotide site mutant proteins have significantly reduced TS enzymatic activity. In contrast, TS proteins with mutations in the folate-binding region including the C50F mutant protein and the nucleotide active site C146S mutant completely lose their RNA binding function. These studies suggest that the active site cysteine sulfhydryl and the folate-binding site may represent important domains on *E.coli* TS for RNA binding. Moreover, the fact that catalytically inactive mutant proteins retain their ability to bind *E.coli* TS mRNA indicates that the TS functions of RNA binding and enzyme catalysis are not simultaneously controlled by a single common site on TS protein. This observation is supported by recent studies which determined that the reduced form of TS engaged in RNA binding and enzyme catalysis is not mediated by a single common redox site but may involve multiple and/or coupled redox sites (36). Although the specific mechanism(s) by which the cysteine sulfhydryls and the folate-binding region mediate RNA binding remains unclear, it is conceivable that these regions either directly interact with their target RNA or that they maintain TS in a certain conformational state that allows for optimal RNA binding.

The location for the amino acid residues altered in the *E.coli* mutant TS proteins are shown in Figure 7. The amino acids represented by the filled bonds appear to be important for RNA binding. Based on the X-ray crystal structure of wild-type *E.coli* TS, each of these amino acids are in direct contact with or are near residues which make contact with the reduced folate substrate 5,10-methylenetetrahydrofolate (26–29). Mutation of amino acid residues K48, C50 and T47 completely abolished RNA binding suggesting that the RNA binding domain may overlap with the binding site for the folylpolyglutamate tail (37). Although T47 is largely internal and points away from the active site, mutation of this residue may alter the position of the loop in which it is contained. Amino acid residues C146 and W80 are located deep within the active site pocket. Preliminary molecular modeling studies suggest that a stem–loop RNA structure may be able to fit into the active site and make direct contact with C146. W80

appears to be part of a conformationally mobile domain in TS which may facilitate its interaction with RNA (27). Finally, the inability of the C-terminal deletional mutant protein, I264Am, raises the possibility that positioning of the C-terminal loop may be important for RNA binding. X-ray crystallographic studies are currently in progress for each of the mutant proteins used in this study. While no gross structural rearrangements have been detected thus far, detailed characterization of the RNA binding domain(s) on TS protein awaits identification of the precise amino acid contact points or X-ray crystallographic and/or NMR resolution of the specific *E.coli* TS RNA–protein complex.

Various studies have identified the 5′-UTR as being critical in regulating mRNA translation (8,38). A well-studied system for the regulation of translational initiation is the iron-dependent regulation of ferritin mRNA (24,38,39). In this case, binding of the iron-regulatory factor (IRF) to a stem–loop structure termed the iron-responsive element located within the 5′-UTR of ferritin mRNA results in translational repression. Recently, sequences within the 3′-UTR have also been shown to control the translation of various mRNAs (40–44). Although it has now been shown that RNA–protein interactions occurring in the 3′-UTR can affect translational initiation, the underlying mechanism(s) by which this takes place remain ill-defined. One issue raised by the present study relates to how binding of *E.coli* TS to RNA sequences in the protein-coding region leads to translational inhibition. Presumably this RNA–protein interaction interferes with binding of the translational initiation machinery including the ribosomes and/or the translation initiation factors to the mRNA. Such a mechanism would suggest some type of interaction between the 5′-end, the translational start site and the RNA sequences within the protein-coding region. Further studies are required, however, to elucidate the precise molecular events for this process.

The studies presented herein show that direct binding of *E.coli* TS to its corresponding *E.coli* TS mRNA may represent an important mechanism by which TS protein negatively regulates translation. Work is currently in progress to characterize, in more detail, the molecular basis for this RNA–protein interaction. However, this study provides new insights into the elements that control TS protein synthesis and offers further evidence for the important role of translational autoregulation in determining TS expression.

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