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*Articles*

# Human Cytoplasmic Serine Hydroxymethyltransferase Is an mRNA Binding Protein†

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ABSTRACT: The 5′ untranslated region (UTR) of the human cytoplasmic serine hydroxymethyltransferase (cSHMT) message is alternatively spliced, creating a full-length 5′ UTR (LUTR) encoded within exons 1−3 and a shorter UTR (SUTR) that results from excision of exon 2. The role of the 5′ UTRs in cSHMT expression was investigated by fusing the cSHMT 5′ UTRs to the 5′ end of the luciferase gene. Human cSHMT protein at 10 $\mu$M inhibits in vitro translation of cSHMT 5′ UTR-luciferase fusion mRNA templates by more than 90%, but does not inhibit translation of the luciferase message lacking the UTR. Translation inhibition is independent of amino acid and folate substrate binding to the cSHMT enzyme. The cSHMT SUTR-luciferase mRNA binds to the cSHMT·glycine·5-formyltetrahydrofolate ternary complex with an apparent $K_d$ of 10 $\mu$M. Gel mobility shift assays demonstrate that the human cSHMT protein binds to the cSHMT LUTR-luciferase fusion mRNA in the presence and absence of glycine and 5-formyltetrahydrofolate pentaglutamate. The fusion cSHMT SUTR-luciferase message at 65 $\mu$M inhibits the cSHMT-catalyzed cleavage of allothreonine as a partial mixed type inhibitor, reducing both $k_{cat}$ and $K_m$ by 40 and 75%, respectively, while tRNA has no effect on cSHMT catalysis. These studies indicate that the cSHMT protein can bind mRNA, and displays increased affinity for the 5′ untranslated region of its mRNA.

Translational regulation of mRNA is a common mechanism associated with the control of gene expression (*1*). Generally, the rate of protein synthesis can be regulated either by modulation of the amount of ribosomes or initiation factors or by a change in their activity due to phosphorylation or dephosphorylation. However, specific translational control, which affects only one mRNA or a class of mRNA molecules, usually involves regulation at the level of initiation. Stimulation of translational initiation can be mediated by sequences in the coding region, by polyadenylation at the 3′ end, or by the length and sequence of the 5′ untranslated region (UTR). Proteins whose expression is tightly regulated often have 5′ UTRs[1] that are longer than the average of 100−140 nucleotides and form stable secondary structures (*2*). This mode of regulation appears to be particularly adapted to regulating the expression of enzymes

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[1] Abbreviations: THF, (6*S*)-tetrahydrofolate; 5-formylTHF, 5-formyltetrahydrofolate; DHF, dihydrofolate; PLP, pyridoxal phosphate; SAM, *S*-adenosylmethionine; SAH, *S*-adenosylhomocysteine; cSHMT, cytoplasmic serine hydroxymethyltransferase; mSHMT, mitochondrial serine hydroxymethyltransferase; dUMP, deoxyuridine monophosphate; 5′ UTR, 5′ untranslated region; ORF, open reading frame; TS, thymidylate synthase; DHFR, dihydrofolate reductase; LUTR, full-length cSHMT 5′ UTR; SUTR, alternatively spliced cSHMT UTR lacking exon 2; Lluc, fusion mRNA template containing the cSHMT 5′ LUTR fused to the luciferase gene; Sluc, fusion mRNA template containing the cSHMT 5′ SUTR fused to the luciferase gene; IRP, iron regulatory protein; IRE, iron response element.

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and proteins involved in various aspects of cellular metabolism and nutrient storage. Translational regulation has been demonstrated to mediate gene expression under conditions of changing nutrient status (*3*) and redox state of the cell, as well as metabolic perturbations resulting from pharmacological agents (*4*). In many instances, translational regulation is critical to maintaining homeostasis, because it permits rapid induction of protein synthesis (*5*).

One of the best-characterized mechanisms of specific translational control has been elucidated for the iron-storage protein ferritin. Ferritin expression is regulated by a reversible mRNA−protein interaction in the 5′ UTR of ferritin mRNA. The iron regulatory protein (IRP) binds iron-responsive elements (IREs) located in the 5′ UTR of ferritin mRNA that form moderately stable hairpin loops. The binding site is within approximately 40 nucleotides of the cap structure at the 5′ end of the mRNA. Stable association of the 43S preinitiation complex cannot occur in the presence of IRP, and therefore, the small ribosomal subunit does not bind (*3*). IRP contains an iron-binding site that allows this mechanism to be iron-responsive. At low iron concentrations, IRP will bind the IRE and prevent translation. Translation of ferritin and aminolevulinate synthase, an enzyme involved in heme synthesis, is regulated in this manner (*1*, *5*).

Translational regulation can also occur by an autoregulatory mechanism, by which translation products bind to and repress the translation of their corresponding mRNA. This type of autoregulatory control was first characterized in a prokaryotic system. The coat protein of bacteriophage R17 binds a hairpin structure containing the initiation codon of the R17 replicase mRNA, repressing its translation (*6*, *7*). Similar mechanisms have been described in eukaryotic systems, including the cytoplasmic folate-dependent enzymes thymidylate synthase (TS) and dihydrofolate reductase (DHFR) as well as p53. These proteins bind specifically to their own mRNAs and inhibit translation (*8*−*10*). The mechanism of their binding involves a nucleophilic attack by the sulfhydryl group of an active site cysteine upon a uracil ring of the mRNA. A similar mechanism has been proposed for the binding of IRP and the R17 coat protein to mRNA (*11*).

It has been suggested that TS and DHFR, along with cSHMT, form a metabolic cycle that methylates deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP) (Figure 1, scheme A). In this cycle, TS utilizes methyleneTHF as a cofactor that serves as a reductant and a one-carbon donor. DHFR and cSHMT then regenerate methyleneTHF from DHF (*4*). Folate substrates repress the mRNA binding properties of TS and DHFR, suggesting a mechanism for folate-dependent metabolic regulation of both TS and DHFR (*9*).

The SHMT enzyme catalyzes the THF-dependent reversible conversion of serine to glycine (reaction 1):

$$\text{serine} + \text{THF} \leftrightarrow \text{glycine} + \text{5,10-methyleneTHF} \quad (1)$$

This reaction, when catalyzing serine cleavage, generates one-carbon units for use in folate-dependent anabolic reactions and is the major source of one-carbon units in mammals (Figure 1, scheme A) (*12*). SHMT exists in both the mitochondria and cytoplasm, and the two proteins are encoded by distinct genes (*13*). While the reaction is fully

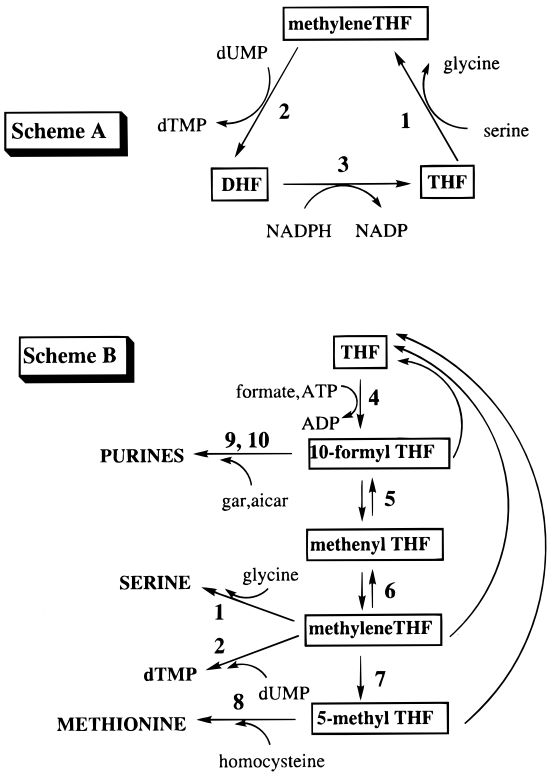


FIGURE 1: Two alternative schemes describing the role of cytoplasmic serine hydroxymethyltransferase in folate metabolism. In scheme A, cSHMT supplies one-carbon units for one-carbon metabolic cycles. In scheme B, cSHMT catalyzes serine synthesis and competes with other metabolic cycles for one-carbon units in the form of methyleneTHF: (1) serine hydroxymethyltransferase, (2) thymidylate synthase, (3) dihydrofolate reductase, (4) 10-formylTHF synthetase, (5) methenylTHF cyclohydrolase, (6) methyleneTHF dehydrogenase, (7) methyleneTHF reductase, (8) methionine synthase, (9) 10-formyltetrahydrofolate:5′-phosphoribosylglycinamide formyltransferase, and (10) 10-formyltetrahydrofolate:5′-phosphoribosyl-5-amino-4-imidazolecarboxamide formyltransferase.

reversible in vitro, the two isozymes of SHMT behave differently in vivo (*14*−*17*). Chinese hamster ovary cells lacking mitochondrial SHMT (mSHMT) become auxotrophic for glycine (*18*), suggesting that the mSHMT enzyme is responsible for glycine synthesis and the generation of one-carbon units (Figure 1, scheme B) (*19*). There is evidence that cSHMT catalyzes serine synthesis in the cytoplasm and that this reaction serves to control the flux of one-carbon units used in homocysteine remethylation. It also serves as the initial step in the conversion of glycine to glucose (*20*, *21*). When cSHMT is catalyzing serine synthesis, cSHMT competes with MTHFR and TS for methyleneTHF and depletes the supply of one-carbon units (Figure 1, scheme B). The cSHMT enzyme also catalyzes a second reaction (reaction 2):

$$\text{5,10-methenylTHF} \rightarrow \text{5-formylTHF} \quad (2)$$

The product of this reaction, 5-formylTHF, is a slow tight binding inhibitor of cSHMT (*22*), which indicates that the enzyme autoregulates itself, producing in a secondary reaction an inhibitor of its principal reaction. Depletion of intracellular 5-formylTHF in neuroblastoma activates cSHMT activity, suggesting that cSHMT is inhibited by 5-formylTHF in vivo (*20*).

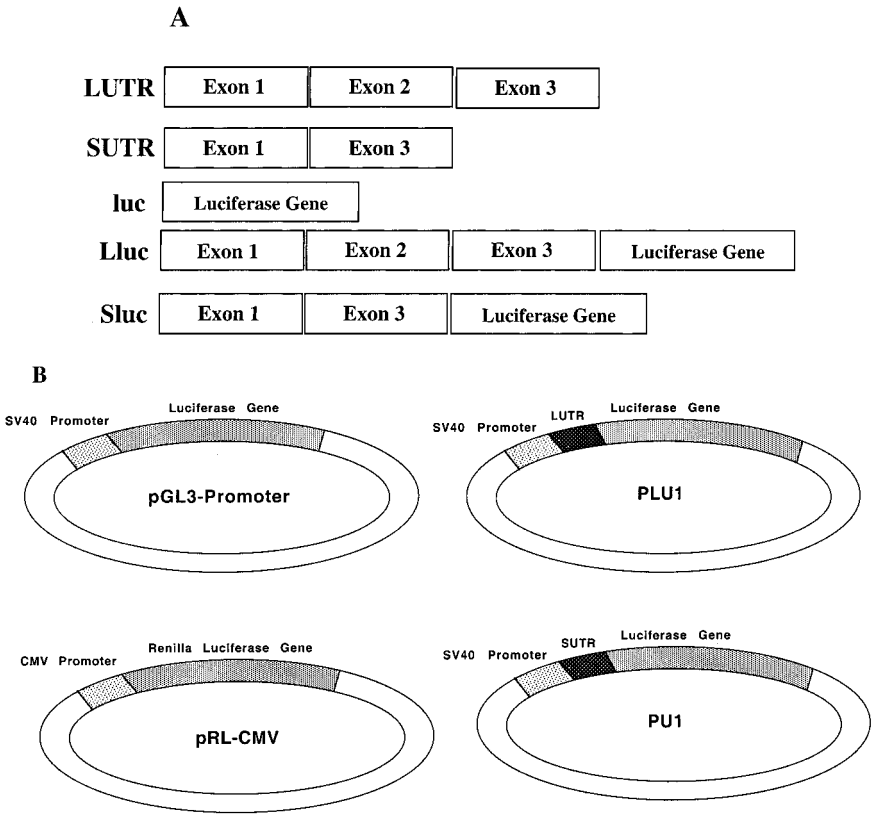


FIGURE 2: Vectors and mRNA transcripts. (A) The full-length cSHMT 5′ UTR (LUTR) encoded by exons 1−3 was fused to the 5′ end of the luciferase gene (luc) to create the Lluc mRNA transcript. The shortened alternatively spliced cSHMT 5′ UTR (SUTR) encoded by exons 1 and 3 was fused to the 5′ end of the luciferase gene to create the Sluc mRNA transcript. (B) The Lluc and Sluc cDNAs were cloned into the pGL3 vector to create the PLU1 and PU1 vectors, respectively. The pRL-CMV vector was used in all transfection experiments to normalize experimental readings.

In this study, we examined whether cSHMT was capable of binding to its the 5′ UTR of its mRNA as demonstrated for TS and DHFR. The cSHMT message contains a longer than average, multiply spliced 5′ UTR (*23*), giving further relevance to this question. The cSHMT cDNA has a 329 bp 5′ UTR encoded within the first three exons of the gene. Alternative splicing of exon 2 results in a 191 bp 5′ UTR. Both splice forms have been predicted to form stable stem−loop structures (*23*). These long UTRs, rich in secondary structure, are likely candidates for translational regulation via binding of the translation product. In this study, we demonstrate that the cSHMT enzyme can bind to the 5′ UTR of its mRNA and inhibit in vitro translation of an UTR-luciferase reporter gene.

## MATERIALS AND METHODS

*Materials*. Tetrahydrofolic acid, 5-formyltetrahydrofolic acid, allothreonine, alcohol dehydrogenase, and NADH were obtained from Sigma Chemical. [α-$^{32}$P]ATP was obtained from NEN. Other chemicals were reagent grade. Fetal bovine serum and α minimal essential media (αMEM) were obtained from Hyclone Laboratories, Inc. All other enzymes, including restriction endonucleases, were obtained from Gibco BRL, Promega, or Stratagene.

*Cell Lines and Media*. The human MCF-7 mammary adenocarcinoma cells (HTB22) were obtained from the American Type Culture Collection (ATCC). Cells were cultured in αMEM with 11% fetal calf serum and maintained at 37 °C in a 5% $CO_2$ atmosphere.

*SHMT Activity Assays*. Michaelis−Menten constants were determined for the human cSHMT-catalyzed cleavage of allothreonine in the presence and absence of RNA. Initial velocity measurements for the reaction were taken using a coupled assay system with alcohol dehydrogenase as described previously (*24*). The rate of decrease in absorbance at 340 nm was recorded after the addition of 100 pmol of cSHMT to a 1 mL cuvette containing 0.15 mM NADH, 0.05 mg of alcohol dehydrogenase, 65 $\mu$M RNA, and variable concentrations of allothreonine using a Shimadzu UV-2401 PC recording spectrophotometer.

*General Molecular Biology*. Total RNA was isolated from cultured cells ($1 \times 10^7$) that were harvested with 10% trypsin and washed twice with phosphate-buffered saline (PBS). RNA was isolated using the Purescript RNA Isolation Kit (Gentra Systems) as directed by the manufacturer. The yield was quantified and the purity determined by spectroscopy. The mRNA was converted to cDNA using Tth polymerase (Promega) at 70 °C. The First Strand cDNA Synthesis Kit (Clontech) was also occasionally used to make cDNA by following the manufacturer's instructions. Plasmid preparations were performed by the method of alkaline lysis, using Macherey-Nagel's Nucleospin Kit, or using Qiagen's QIAprep or Plasmid Mini/Maxi Kits.

*Generation of the cSHMT 5′ UTR-Luciferase Constructs*. The cSHMT 5′ UTRs were fused to the 5′ end of the luciferase gene to create reporter gene constructs that are sensitive to and reflect translational regulation that is mediated through the cSHMT 5′ UTRs (Figure 2). The two

spliced forms of the cSHMT UTR, the full-length one (LUTR) and the one lacking exon 2 (SUTR), were generated by PCR (primers, 5′-TTCACAAGCTTGCCTGGCGCGCA-GAGTGCCC-3′ and 5′-GTAGTCCATGGCACTGGTTC-GAAGCTGCC-3′) following reverse transcription of mRNA isolated from MCF-7 cells (Figure 2A). The PCR products were cloned into pGL3 luciferase reporter vectors (Promega). The region around the cSHMT start codon was mutated from CAATGG to CCATGG, the *Nco*I recognition site, so that the UTRs could be inserted in front of a reporter gene, thereby maintaining their natural relationship to the start codon. The forward primer for both spliced and full-length UTRs was engineered to contain a *Hin*dIII restriction site at their 5′ ends. The UTRs were cloned directionally into the *Hin*dIII and *Nco*I sites of the pGL3 Promoter vector, fusing them to the luciferase ORF just as they are natively fused to the cSHMT ORF. This procedure generated the plasmids PLU1, which has the full-length UTR, and PU1, which has the spliced UTR (Figure 2B). The pGL3 Promoter vector contains a SV40 promoter immediately upstream of the *Hin*dIII site, so vector sequences should add at most 20 ribonucleotides to the 5′ end of each 5′ UTR when transcribed.

All cSHMT nucleotide sequences used to create these constructs were generated by PCR using the high-fidelity *Pfu* polymerase. All PCR products were originally cloned into the pCR2.1 vector by TA cloning (Stratagene) as directed by the manufacturer. All pCR2.1 clones were verified by sequencing both strands. The orientation of inserts in the pGL3 constructs was determined by PCR.

*Synthesis of mRNA.* For in vitro translation experiments, mRNA encoding luciferase with and without the cSHMT UTR fusion was generated from pGL3 and pGL3-cSHMT UTR vectors (PLU1 and PU1). The plasmids PLU1 and PU1 were linearized with *Xba*I and purified. RNA corresponding to luciferase mRNA (luc-mRNA), luciferase mRNA with the cSHMT 5′ UTR fusion (Lluc-mRNA), and luciferase mRNA with the shortened alternatively spliced cSHMT 5′ UTR fusion (Sluc-mRNA) were generated using the Ribomax Large Scale RNA Production System-T7 kit, according to the Promega protocol. The RNA was extracted with a phenol−chloroform mixture and then precipitated overnight in 2 M LiCl at 4 °C. The RNA generated by this method contained 12 nucleotides of vector sequence added to its 5′ end. All RNA procedures were conducted under RNase-free conditions, and all mRNA was stored with placental RNase inhibitor. The purity was verified by electrophoresis.

*Purification of the cSHMT Protein*. Recombinant cSHMT protein was isolated from an *Escherichia coli* strain expressing human or mouse cSHMT protein using a previously described procedure (*25*). The enzyme concentration was determined by measuring the absorbance at 425 nm, using an extinction coefficient of 6500 $mol^{-1}$ $cm^{-1}$ L, for a pyridoxal phosphate (PLP) enzyme. The protein was estimated to be more than 95% pure by spectroscopy and SDS−PAGE. Rabbit cSHMT was purified from liver as previously described (*26*). All protein preparations were treated and stored with placental RNase inhibitor.

*Spectroscopic Assessment of RNA−Protein Binding.* Binding of RNA to the cSHMT·glycine·5-formylTHF ternary quinonoid complex was assessed using a spectrophotometer. A solution containing 50 mM HEPES (pH 7.2), 5 mM glycine, 200 $\mu$M (6*R*,*S*)-5-formylTHF, and 4.0 $\mu$M human cSHMT was titrated with increasing concentrations of Sluc mRNA or luc mRNA (1−25 $\mu$M). The absorbance spectrum of the enzyme was recorded from 550 to 450 nm after each addition of RNA, and the loss of absorbance at 502 nm was recorded. The amount of RNA bound was quantified by determining the loss of absorbance at 502 nm ($\lambda_{max}$ = 502 nm, $\epsilon_{502}$ = 40 000 $mol^{-1}$ $cm^{-1}$ L).

*Gel Mobility Shift Assays*. [α-$^{32}$P]ATP-labeled RNA ($10^5$ cpm) was incubated with 10 $\mu$M recombinant human cSHMT in binding buffer [20 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, and 1 mM EDTA] containing 8 mM DTT, 3 $\mu$g of BSA, 1.25 $\mu$g of yeast tRNA, 40 units of RNaseOut RNase inhibitor, and 15% (v/v) glycerol (final volume of 10 $\mu$L) for 15 min at 37 °C. The reaction mixtures were electrophoresed on a nondenatured 3.5% polyacrylamide gel. For substrate competition assays, the incubation buffer contained 1 mM glycine and 50 $\mu$M (6*S*)-5-formylTHFpentaglutamate for occupying cSHMT substrate binding sites. Unlabeled luc or Lluc mRNA was added as a competitor to test the specificity of the RNA−protein interaction.

*In Vitro Translation.* Translation reaction mixtures (final volume of 25 $\mu$L) containing rabbit reticulocyte lysate (16.5 $\mu$L), 1 mM MgOAc, 2 mM DTT, 35 mM KCl, supplied in the Flexi Rabbit Reticulocyte Lysate System kit (Promega), and 2.5 $\mu$g of yeast tRNA were incubated at 30 °C for 35 min with Sluc or Lluc mRNA template. All experiments were performed with a final mRNA concentration of 5 $\mu$g/mL (or about 2 nM RNA) to ensure that the catalytic system was well below saturation. Inhibition assays were performed by adding varying concentrations of human, murine or rabbit cSHMT enzyme, 2 mM glycine, or 200 $\mu$M 5-formylTHF. All enzyme−mRNA incubations contained 20 units of RNaseOut RNase inhibitor. Prior to all experiments, mRNA was heated to 70 °C for 10 min and then cooled to room temperature before adding it to the reaction mixtures to allow a more uniform secondary structure to form. Following a 35 min incubation at 30 °C, the translation reactions were quenched in an ice−water slurry. Luciferase activity was determined by the addition of 100 $\mu$L of LAR II reagent (Promega) and luminescence quantified with a Berthold Lumar luminometer and Promega's Dual-Luciferase Reporter Assay System, using a 10.0 s reading time. Results are reported as a percentage of the luminosity of the control, which was a reaction mixture containing 5 ng/$\mu$L Sluc RNA without cSHMT protein.

*Transient Transfection and Luciferase Reporter Gene Assays*. The pGL3-promoter, PLU1, and PU1 vectors were isolated for transfection using Qiagen's Plasmid Maxi Kit. Transfection of human MCF-7 cells was carried out by $CaPO_4$ coprecipitation. Cultured cells were exposed to transfecting $CaPO_4$ precipitate for 4−5 h (*27*). Culture media were replaced and the cells cultured for 2−3 days and assayed for luciferase activity using a Berthold Lumat luminometer. To account for possible variations in transfection efficiency, every experimental vector was cotransfected with pGL-CMV, a coreporter vector that expresses *Renilla* luciferase from a CMV promoter (Promega). All results are described as a luciferase luminosity ratio mean (LLRM), which is the mean ratio of firefly luciferase activity levels, generated from the experimental pGL3-cSHMT constructs, to *Renilla* luciferase levels, generated from the coreporter

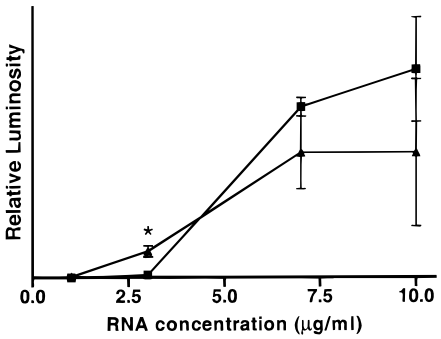


FIGURE 3: In vitro translational efficiency of the Lluc and Sluc mRNA transcripts. Luciferase activity was measured following a 35 min in vitro translation reaction using a Sluc mRNA template (■) or a Lluc mRNA template (▲) as described in Materials and Methods. Data are the mean of five measurements. Variance is expressed as the standard error of the mean (SEM). Translation of Lluc and Sluc templates is significantly different at 3 $\mu$g of mRNA/mL (the asterisk denotes $p < 0.06$, *t*-test assuming unequal variances).

vector. Transfections had experimental vector:coreporter vector ratios of 1:80 to 1:120, but the ratios were uniform within each experiment.

*Primer Synthesis, DNA Sequencing, and Computer Analyses*. All primers were synthesized by Cornell University's Oligonucleotide Synthesis Facility. Sequencing was done either by Cornell University's DNA Sequencing Facility (Fluorescent Dye-Terminator) or as directed by Epicentre Technology's Cycle Sequencing Kit using radiolabeled primers. DNA sequence analysis was carried out using the DNAstar suite of programs from DNAstar Inc. Data analyses were performed using the programs Prism and Microsoft Excel.

## RESULTS

*Role of 5′ UTR Alternative Splicing in cSHMT Translation.* There is accumulating evidence that the cSHMT enzyme displays complex regulation. The enzyme shows a wide range of tissue specific expression (*23*), and metabolically autoregulates its activity by synthesizing its own inhibitor, 5-formylTHF (*20*). In this study, the role of the cSHMT 5′ UTR in cSHMT expression was investigated by fusing the cSHMT UTRs to the 5′ end of the luciferase reporter gene. Initially, the influence of 5′ UTR alternatively splicing on cSHMT translation was investigated. An mRNA template was constructed that contained the luciferase open reading frame (luc) with the cSHMT full-length UTR (Lluc) or with the shorter alternatively spliced UTR (Sluc) as described in Materials and Methods (Figure 2). The relative translational efficiencies of the Sluc and Lluc messages were determined by measuring luciferase activity following in vitro protein synthesis over a range of mRNA concentrations (Figure 3). The translation efficiency, as represented by the luciferase activity generated, from the Sluc and Lluc mRNA templates is similar at mRNA concentrations of 7.0 and 10.0 $\mu$g/mL. Transcription from the Lluc template is 8-fold more efficient than that from the Sluc template at an mRNA concentration of 3.0 $\mu$g/mL, demonstrating that the alternative splicing of the cSHMT UTR can influence in vitro translation rates. Moreover, the translation efficiency from the Lluc mRNA template is nearly 2-fold higher compared to that of the luc mRNA template at all mRNA concentrations shown in Figure 3. This indicates that the cSHMT UTR stimulates translation in the in vitro assay. However, transient transfection of MCF-7 cells with the PU1 or PLU1 constructs (Figure 2) results in the same level of luciferase activity, indicating that alternative splicing of the 5′ UTR does not contribute to the regulation of cSHMT expression in this cell culture model (data not shown).

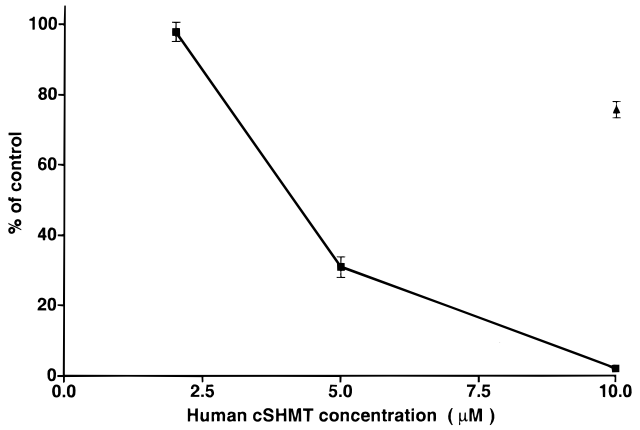


FIGURE 4: Effect of human cSHMT protein on Sluc in vitro translation. Squares represent the level of luciferase activity generated from 2 nM Sluc mRNA in a 30 min in vitro translation reaction in the presence of increasing concentrations of human cSHMT. The triangle represents the level of luciferase activity generated from 2 nM Sluc mRNA in a 30 min in vitro translation reaction in the presence of 10 $\mu$M human cSHMT protein that was boiled prior to addition to the translation reaction mixture. All values are relative to luciferase activity generated from an in vitro translation reaction mixture that lacks the cSHMT protein that has an arbitrary value of 100% with a SEM of 4%. All data are the mean of four measurements. Variance is expressed as the SEM.

*The cSHMT Protein Inhibits in Vitro Translation of the Sluc Construct*. The ability of the human cSHMT protein to inhibit the in vitro translation of Sluc was investigated. Figure 4 demonstrates that the addition of the cSHMT protein to the in vitro translation reaction mixture inhibits luciferase synthesis by 65% at a concentration of 5 $\mu$M, and by more than 95% at a concentration of 10 $\mu$M. The cellular concentration of SHMT in L1210 cells has been estimated to be 9 $\mu$M (*28*). To ensure that inhibition of translation is due to the cSHMT protein, 0.25 nmol of cSHMT enzyme (i.e., the amount of enzyme required to give a final concentration of 10 $\mu$M in the reaction mixture) was boiled for 5 min prior to addition to the assay. The inclusion of 10 $\mu$M denatured enzyme decreased the level of translation of Sluc mRNA by about 28% relative to a control reaction mixture containing 10 $\mu$M PLP. Therefore, only the active enzyme effectively inhibits translation by more than 95% when present at physiological concentrations.

*Specificity of cSHMT-Induced Translational Inhibition.* The specificity of the cSHMT-mediated inhibition of Sluc translation was investigated with respect to both the type of mRNA and the source of cSHMT protein. Figure 5A demonstrates that 10 $\mu$M cSHMT is effectively inhibiting in vitro translation of Sluc mRNA by more than 90% relative to those of reaction mixtures lacking the cSHMT protein. However, the cSHMT protein at 10 $\mu$M was not effective in inhibiting in vitro translation of the luc mRNA.

The cSHMT protein is highly conserved among species, with a degree of sequence similarity between rabbit and

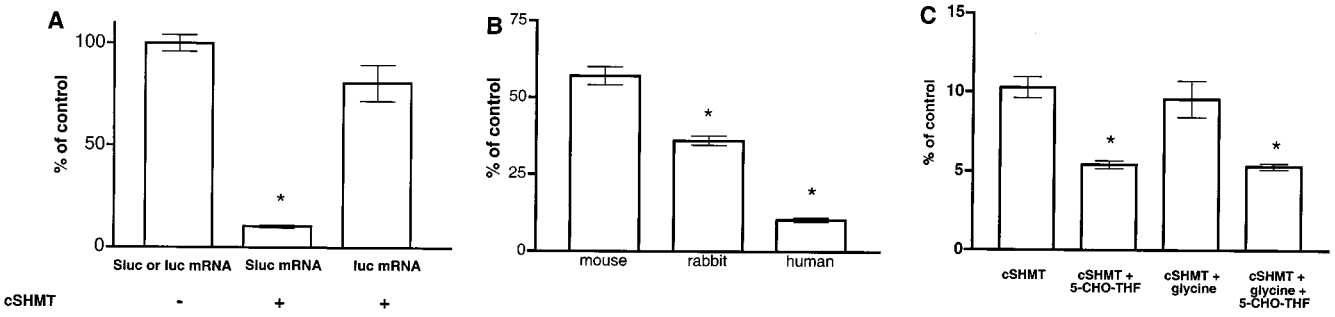


FIGURE 5: Effect of human the cSHMT protein on luc and Sluc in vitro translation. (A) The *Y*-axis shows the relative level of luciferase activity generated from 2 nM Sluc or luc mRNA in a 30 min in vitro translation reaction as described in Materials and Methods. Values are relative to luciferase activity generated from an in vitro translation using either luc or Sluc mRNA as a template in the absence of the cSHMT protein that has an arbitrary value of 100% with an SEM of 4%. All data are the mean of four measurements, and variance is expressed as the SEM. The effect of cSHMT on Sluc translation is significantly different from that from assays lacking cSHMT protein (the asterisk denotes $p < 0.002$, *t*-test assuming unequal variances). (B) Effect of murine, rabbit, and human cSHMT protein on Sluc in vitro translation. The *Y*-axis shows the level of luciferase activity generated from 2 nM Sluc mRNA in a 30 min in vitro translation reaction in the presence of 10 $\mu$M human, rabbit, or murine cSHMT protein. Values are relative to luciferase activity generated from an in vitro translation reaction mixture that lacks the cSHMT protein and has an arbitrary value of 100% with an SEM of 3%. All data are the mean of four measurements. Variance is expressed as the SEM. The effect of murine and rabbit cSHMT is significantly different from the effect of human cSHMT (the asterisk denotes $p < 0.04$, *t*-test assuming unequal variances). (C) Effect of cSHMT substrates on Sluc in vitro translation. The *Y*-axis represents the level of luciferase activity generated from 2 nM Sluc mRNA in a 30 min in vitro translation reaction in the presence of 10 $\mu$M human cSHMT protein and either glycine (2 mM), (6*R*,*S*)-5-formylTHF (200 $\mu$M), or both. All values shown are relative to control in vitro translation reaction mixtures using Sluc mRNA as a template in the absence of the cSHMT protein, but containing the folate or amino acid substrates, and these values have an arbitrary value of 100% with an SEM of 4%. The addition of glycine and/or 5-formylTHF alone inhibited the in vitro translation reactions less than 25%. Data are the mean of four measurements. Variance is expressed as the SEM. The effect of cSHMT with 5-formylTHF bound is significantly different from the effect of cSHMT alone (the asterisk denotes $p < 0.006$, *t*-test assuming unequal variances).

human enzymes of 92% (*13*). The cSHMT 5′ UTR nucleotide sequences of the mouse, rabbit, and human are also highly conserved near the start ATG codon. The first 11 nucleotides 5′ of the start ATG codon are 100% conserved between the mouse and human, and 73% conserved between the rabbit and human, suggesting a common and conserved function for this sequence. The ability of non-human cSHMT proteins to inhibit Sluc mRNA translation was investigated. Addition of purified rabbit liver cSHMT or purified mouse recombinant cSHMT to the in vitro translation reaction at concentrations of 10 $\mu$M inhibits translation of Sluc by 40 and 60%, respectively, while human cSHMT inhibited translation by more than 90% (Figure 5B). The ability of the murine and rabbit cSHMT protein to inhibit translation of Sluc suggests that both the cSHMT primary amino acid sequences and mRNA nucleotide 5′ UTR sequences have been conserved to permit mRNA binding by cSHMT. However, evolutionary drift has likely occurred since neither rabbit nor mouse cSHMT protein inhibits Sluc translation as effectively as the human enzyme.

The effect of folate and amino acid substrates on translational inhibition of Sluc was determined using 5-formylTHF to occupy the enzyme's folate binding site, and using glycine to occupy the amino acid binding site of cSHMT. (6*S*)-5-FormylTHF binds to rabbit cSHMT with a $K_d$ of 10 $\mu$M, while glycine binds to the enzyme with a $K_d$ of about 1.6 mM (*22*). The addition of 200 $\mu$M (6*R*,*S*)-5-formylTHF to the translation reaction mixture increases the efficacy of cSHMT inhibition in the presence or absence of glycine, while addition of 2 mM glycine alone has essentially no effect on translational inhibition by cSHMT (Figure 5C). Addition of (6*R*,*S*)-5-formylTHF alone to a final concentration of 200 $\mu$M in the absence of the cSHMT protein inhibits translation by less than 25%. These results indicate that occupancy of the folate binding site or the amino acid binding site of the cSHMT protein does not prevent translational inhibition. These studies were repeated using the Lluc mRNA template with similar results, suggesting that alternative splicing of the cSHMT 5′ UTR does not influence translational inhibition.

*Evidence for cSHMT as an mRNA Binding Protein*. To demonstrate that the cSHMT protein binds mRNA, gel mobility shift assays were performed (Figure 6). Figure 6A shows that the migration of $^{32}$P-labeled luc mRNA through a 3.5% nondenaturing polyacrylamide gel was restricted when the mRNA was preincubated with human cSHMT (Figure 6A, lanes 1 and 2), as was the migration of a $^{32}$P-labeled LUTR mRNA (lanes 3 and 4). These results indicate that the cSHMT protein can bind two unrelated mRNA species and therefore may display nonspecific mRNA binding properties. Figure 6B demonstrates that cSHMT also binds $^{32}$P-labeled Lluc fusion mRNA, and that unlabeled Lluc mRNA can effectively compete with labeled Lluc for binding to the cSHMT protein. Figure 6C demonstrates that unlabeled luc mRNA cannot effectively compete with Lluc for binding to the cSHMT protein, indicating that Lluc mRNA binds with greater affinity to cSHMT than luc mRNA. Additional studies have demonstrated that a 32-fold higher concentration of luc mRNA relative to $^{32}$P-labeled Lluc mRNA also does not diminish Lluc binding to cSHMT (data not shown). Figure 6D shows that Lluc mRNA can effectively compete with $^{32}$P-labeled luc mRNA for cSHMT binding, further demonstrating that the addition of the cSHMT UTR to the luc message increases the affinity of the luc message for the cSHMT protein. Figure 6E shows the effects of amino acid, folate, and folate polyglutamate substrates on mRNA binding. Lanes 2−4 demonstrate that the presence of saturating concentrations of cSHMT substrates does not inhibit the formation of the cSHMT−Lluc mRNA complex. These data confirm that an RNA−protein complex is formed between

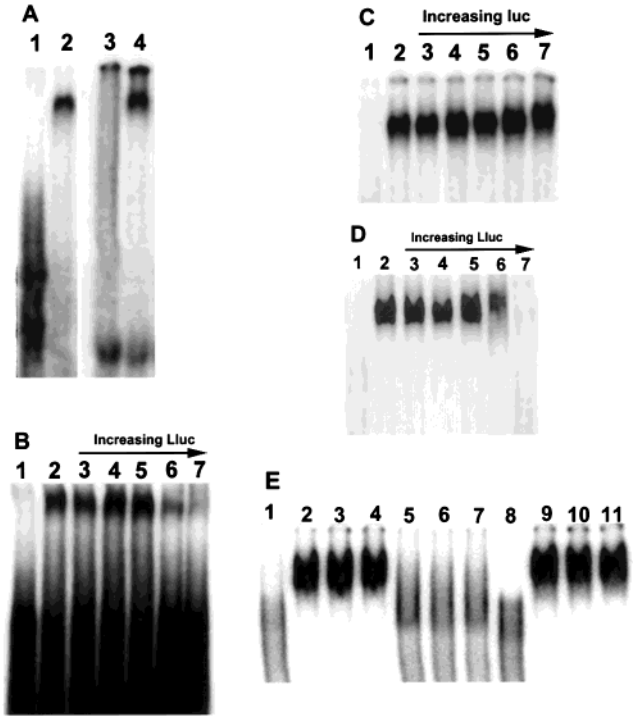


FIGURE 6: Gel mobility shift analyses demonstrating binding of Lluc mRNA to the human cSHMT protein. Lluc mRNA was incubated at 37 °C for 15 min in 1× binding buffer with 40 units of RNase Out, 3 $\mu$g of bovine serum albumin, 1.25 $\mu$g of yeast tRNA, 15% glycerol, and 8 mM DTT with and without cSHMT protein. (A) Migration of a $^{32}$P-labeled RNA probe in the presence and absence of human cSHMT: lane 1, 0.4 nM $^{32}$P-labeled luc mRNA; lane 2, 0.4 nM $^{32}$P-labeled luc mRNA and 10 $\mu$M human cSHMT protein; lane 3, 0.4 nM $^{32}$P-labeled LUTR mRNA; and lane 4, 0.4 nM $^{32}$P-labeled LUTR mRNA and 10 $\mu$M human cSHMT protein. (B) Competitive inhibition of $^{32}$P-labeled Lluc RNA binding to human cSHMT in the presence of unlabeled Lluc mRNA: lane 1, 0.4 nM $^{32}$P-labeled Lluc mRNA; lane 2, 0.4 nM $^{32}$P-labeled Lluc mRNA and 10 $\mu$M human cSHMT protein; and lanes 3−7, 0.4 nM $^{32}$P-labeled Lluc mRNA, 10 $\mu$M human cSHMT protein, and 0.4, 0.8, 1.6, 3.2, and 6.4 nM unlabeled Lluc mRNA, respectively. (C) Competitive inhibition of $^{32}$P-labeled Lluc RNA binding to human cSHMT by unlabeled luc mRNA: lane 1, 0.4 nM $^{32}$P-labeled Lluc mRNA; lane 2, 0.4 nM $^{32}$P-labeled Lluc mRNA and 10 $\mu$M human cSHMT protein; and lanes 3−7, 0.4 nM $^{32}$P-labeled Lluc mRNA, 10 $\mu$M human cSHMT protein, and 0.4, 0.8, 1.6, 3.2, and 6.4 nM unlabeled luc mRNA, respectively. (D) Competitive inhibition of $^{32}$P-labeled luc RNA binding to human cSHMT by unlabeled Lluc mRNA: lane 1, 0.4 nM $^{32}$P-labeled luc mRNA; lane 2, 0.4 nM $^{32}$P-labeled luc mRNA and 10 $\mu$M human cSHMT protein; and lanes 3−7, 0.4 nM $^{32}$P-labeled luc mRNA, 10 $\mu$M human cSHMT protein, and 0.4, 0.8, 1.6, 3.2, and 6.4 nM unlabeled Lluc mRNA, respectively. (E) Migration of the $^{32}$P-labeled RNA probe in the presence and absence of human cSHMT: lane 1, 0.4 nM $^{32}$P-labeled Lluc mRNA; lane 2, 0.4 nM $^{32}$P-labeled Lluc mRNA and 10 $\mu$M human cSHMT protein; lane 3, 0.4 nM $^{32}$P-labeled Lluc mRNA, 10 $\mu$M human cSHMT protein, 1 mM glycine, and 50 $\mu$M 5-formylTHF; lane 4, 0.4 nM $^{32}$P-labeled Lluc mRNA, 10 $\mu$M human cSHMT protein, 1 mM glycine, and 50 $\mu$M 5-formylTHF pentaglutamate; lane 5, 0.4 nM $^{32}$P-labeled Lluc mRNA and 10 $\mu$M murine cSHMT protein; lane 6, 0.4 nM $^{32}$P-labeled Lluc mRNA, 10 $\mu$M murine cSHMT protein, 1 mM glycine, and 50 $\mu$M 5-formylTHF; lane 7, 0.4 nM $^{32}$P-labeled Lluc mRNA, 10 $\mu$M murine cSHMT protein, 1 mM glycine, and 50 $\mu$M 5-formylTHF pentaglutamate; lane 8, 0.4 nM $^{32}$P-labeled luc mRNA; lane 9, 0.4 nM $^{32}$P-labeled luc mRNA and 10 $\mu$M human cSHMT protein; lane 10, 0.4 nM $^{32}$P-labeled luc mRNA, 10 $\mu$M human cSHMT protein, 1 mM glycine, and 50 $\mu$M 5-formylTHF; and lane 11, 0.4 nM $^{32}$P-labeled luc mRNA, 10 $\mu$M human cSHMT protein, 1 mM glycine, and 50 $\mu$M 5-formylTHF pentaglutamate.

Table 1: Effects of RNA on cSHMT Activity[a]

| experiment | RNA | $K_m$ for D,L-allothreonine (mM) | $k_{cat}$ ($s^{-1}$) |
|---|---|---|---|
| 1 | none | 4.2 ± 0.4 | 1.1 ± 0.2 |
| 2 | 40 $\mu$g/$\mu$L tRNA | 4.1 ± 0.3 | 0.9 ± 0.2 |
| 3 | 65 $\mu$M Sluc | 1.3 ± 0.3 | 0.7 ± 0.1 |
| 4 | 65 $\mu$M luc | 3.0 ± 0.4 | 1.1 ± 0.3 |

[a] Michaelis−Menten constants were determined for the recombinant human SHMT-catalyzed cleavage of allothreonine in the presence and absence of RNA. Reactions were performed at 25 °C in 50 mM HEPES buffer (pH 7.2). Values are the means of two independent experimental determinations, and variance represents the standard deviation of the mean.

the cSHMT protein and Lluc mRNA, and that the mRNA does not bind to the folate, amino acid, or polyglutamate binding sites on the enzyme. Formation of a complex between the murine cSHMT protein and Lluc mRNA (Figure 6E, lanes 5−7) is negligible or does not occur. However, the human cSHMT also binds luc mRNA in the presence and absence of substrates (Figure 6E, lanes 9−11). In summary, these data demonstrate that cSHMT exhibits nonspecific mRNA binding properties, but displays increased affinity and specificity for its 5′ UTR. These data support the results from the in vitro translation experiments (Figure 4). The lower-affinity binding of luc mRNA to the cSHMT protein is not sufficient to inhibit luc in vitro translation, while cSHMT binding to its 5′ UTR is sufficient to inhibit in vitro translation.

*Effect of mRNA Binding on cSHMT Activity*. The cSHMT enzyme contains an active site PLP cofactor, which has distinct spectral properties associated with specific reaction intermediates. Upon binding glycine and 5-formylTHF, the PLP cofactor exists in equilibrium between the external aldimine ($\lambda_{max}$ = 425 nm) and glycine quinonoid ($\lambda_{max}$ = 502 nm) (Figure 7A). Formation of the quinonoid structure from the external aldimine is associated with a conformational change in the enzyme (*22*). Figure 7B shows the effects of Sluc mRNA on the absorbance spectrum of the cSHMT·glycine·5-formylTHF ternary complex. Addition of Sluc mRNA (1 to 25 $\mu$M at 5 $\mu$M increments) to the ternary complex reduces the absorbance at 502 nm and increases the absorbance at 425 nm, indicating that Sluc mRNA binds to the cSHMT protein and shifts the equilibrium of the bound PLP to the external aldimine. The addition of 1 mM 5-formylTHF to cSHMT solutions containing Sluc mRNA does not restore quinonoid formation, indicating that the binding of mRNA to the enzyme is not competitive with 5-formylTHF and indicating a distinct binding site for mRNA. Analysis of the absorbance decrease at 502 nm ($\epsilon_{max}$ = 40 000 $mol^{-1}$ $cm^{-1}$ L) as a function of Sluc mRNA concentration by the method of Scatchard shows that Sluc mRNA binds to the cSHMT ternary complex with an apparent $K_d$ of 10 $\mu$M, with a single mRNA binding site per cSHMT monomeric subunit. The luc mRNA is not as effective in decreasing the absorbance at 502 nm when added to the cSHMT ternary complex, with 65 $\mu$M luc mRNA decreasing the absorbance at 502 nm by 50%. These data support and serve to quantify the results from the gel mobility shift assays and indicate that Sluc mRNA binds with higher affinity to the cSHMT protein than luc mRNA.

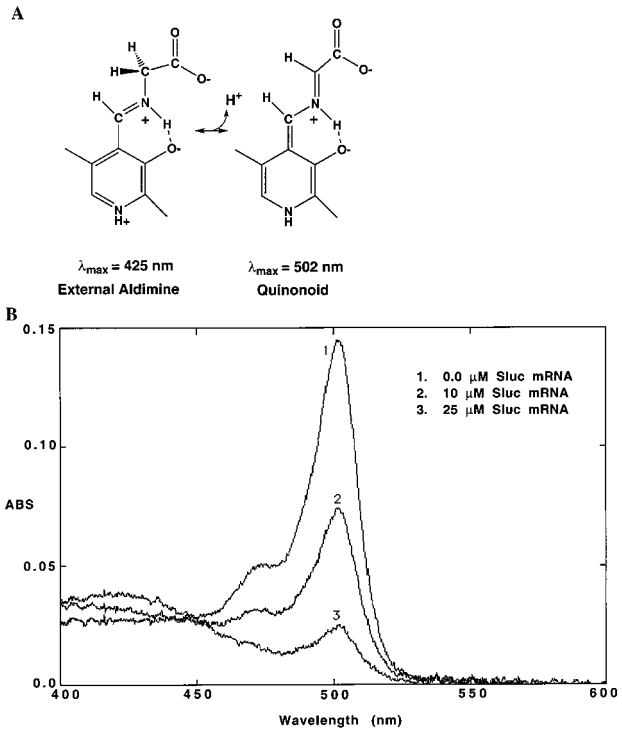


FIGURE 7: Effect of Sluc mRNA on the spectrum of the human cSHMT·glycine·5-formylTHF ternary complex. (A) The structure of reaction intermediates associated with cSHMT catalysis. (B) A solution containing 50 mM HEPES (pH 7.2), 5 mM glycine, 200 $\mu$M (6*R*,*S*)-5-formylTHF, and 4.0 $\mu$M human cSHMT was titrated with increasing concentrations of Sluc mRNA (1−25 $\mu$M). The absorbance spectrum of the enzyme was recorded from 550 to 450 nm after each addition of RNA, and the loss of absorbance at 502 nm was recorded. The amount of RNA bound was quantified by determining the loss of absorbance at 502 nm.

*Inhibition of cSHMT Activity by Sluc mRNA.* The cSHMT enzyme catalyzes the aldol cleavage of allothreonine. The influence of RNA on this cSHMT activity was investigated (Table 1). Linweaver−Burke plots demonstrate that Sluc mRNA is a partial mixed type inhibitor (*29*) of the cSHMT-catalyzed cleavage of allothreonine, and has the effect of lowering both $K_m$ (75%) and $k_{cat}$ (40%) at a Sluc concentration of 65 $\mu$M. At allothreonine concentrations of <1 mM, Sluc mRNA activates cSHMT activity by increasing the enzyme's affinity for allothreonine. At higher allothreonine concentrations, Sluc mRNA inhibits allothreonine cleavage by decreasing $k_{cat}$. Yeast tRNA at a concentration of 40 $\mu$g/$\mu$L (approximately 1.3 mM) has little influence on cSHMT activity. The luc mRNA, lacking the cSHMT UTR, does not activate or inhibit allothreonine cleavage activity, but does reduce the $K_m$ for allothreonine by 30%. These studies confirm that cSHMT does contain a distinct binding site for its 5′ UTR mRNA, and that this binding site is distinct from the substrate binding site.

## DISCUSSION

Folate metabolism is required for the synthesis of purines and thymidylate and the remethylation of homocysteine to methionine, processes that are fundamentally important for cell growth and proliferation (Figure 1). While a definitive metabolic role for the cSHMT enzyme has not been established, cSHMT is positioned to play key roles in regulating folate-dependent one-carbon metabolism. When catalyzing serine cleavage, cSHMT can serve to generate one-carbon units carried by THF and thereby regulate the supply of one-carbon units required for folate-dependent reactions. When catalyzing serine synthesis, cSHMT can regulate the flux of one-carbon units through the homocysteine remethylation pathway, and this has been demonstrated in cultured human cells (*20*). Therefore, regardless of the directionality of the enzyme reaction, changes in cSHMT expression and activity influence and possibly regulate metabolic pathways associated with folate-dependent one-carbon metabolism.

The isolation and sequencing of an unusually long, alternatively spliced 5′ UTR led to speculation that cSHMT is translationally autoregulated in a manner similar to that of TS and DHFR. In this study, we demonstrate that the cSHMT protein, when present at physiological concentrations, is an mRNA binding protein that can effectively bind to its 5′ UTR and inhibit translation in vitro. However, unlike the TS and DHFR mechanisms, mRNA binding by cSHMT appears to be independent of folate and amino acid substrate binding. Four independent experiments demonstrate that cSHMT contains an independent mRNA binding site. The cSHMT protein inhibits in vitro translation of Sluc and Lluc mRNA, but not luc mRNA, in the presence and absence of folate and amino acid substrates. Second, the absorbance spectra of the cSHMT·glycine·5-formylTHF ternary complex change in the presence of Sluc mRNA. Analysis of the absorbance changes by the method of Scatchard shows that each cSHMT monomer has a single mRNA binding site for Sluc with a $K_d$ of 10 $\mu$M. Third, the migration of Lluc mRNA is retarded during nondenaturing gel electrophoresis in the presence of human cSHMT protein bound with amino acid and folate substrates. Finally, Sluc mRNA is a partial mixed type inhibitor of the cSHMT-catalyzed cleavage of allothreonine. All studies suggest that the cSHMT enzyme contains a distinct binding site for mRNA. Additionally, results from the in vitro translation assays demonstrate that the cSHMT 5′ UTR mRNA is necessary for cSHMT-induced translational inhibition of luciferase protein synthesis, indicating that the cSHMT protein may autoregulate its translation in vivo. It is also possible that cSHMT binds to other regions of its mRNA as seen for TS.

The gel mobility shift assays demonstrate that cSHMT is an mRNA binding protein. The enzyme binds both luc and Lluc mRNA, but the studies also show that the cSHMT protein has a much higher affinity for Lluc (Figure 6). The enzyme clearly can distinguish between these two mRNA species. Additionally, Sluc mRNA can effectively inhibit the cSHMT-catalyzed cleavage of allothreonine (Table 1). The decrease in the $K_m$ of allothreonine for the cSHMT enzyme in the presence of Sluc suggests that this mRNA, but not tRNA, can induce a conformational change in the enzyme that influences the catalytic properties of the enzyme.

The cSHMT 5′ UTR may also play a role in altering cSHMT activity. The cSHMT enzyme activity is regulated by 5-formylTHF, and the polyglutamate forms of this folate are slow, tight-binding inhibitors. The binding of 5-formylTHF to cSHMT is a two-step process. 5-FormylTHF binds to and releases quickly from the cSHMT·glycine binary complex when the PLP is in its external aldimine form (Figure 7). The conversion of the external aldimine to the glycine quinonoid is slow, and this step is associated with a

conformational change in the enzyme (*22*). The off-rate of generation of 5-formylTHF from the cSHMT·glycine·5-formylTHF ternary complex is rate-limited by the slow conversion of the quinonoid ternary complex to the external aldimine ternary complex. The binding of cSHMT 5′ UTR mRNA shifts the equilibrium from the quinonoid ternary complex to the external aldimine ternary complex, and thereby permits rapid release of the inhibitor, 5-formylTHF, from the enzyme. Therefore, mRNA binding may also play a role in limiting the inhibitory effects of 5-formylTHF.

The inability of substrates to alter cSHMT-induced translational inhibition suggests an absence of metabolic control as seen for TS and DHFR. While TS and DHFR translational regulation appears to be poised to induce enzyme synthesis when metabolic conditions are appropriate, cSHMT in vitro translational regulation is substrate-independent. Therefore, these studies demonstrate that cSHMT, TS, and DHFR do not participate in a coordinately regulated metabolic pathway at the level of translation. There has been one previous report that rabbit cSHMT expression is translationally regulated. The rabbit cSHMT 5′ UTR contains an ORF within its 5′ UTR, and removal of this ORF is required for optimal expression of the cSHMT protein in overexpression systems (*30*). The authors of this study suggested that the ORF within the rabbit cSHMT 5′ UTR serves to regulate cSHMT translation in cells, and they suggested that this mechanism of cSHMT regulation is common to all mammals. However, this mechanism described for the rabbit enzyme is not applicable for the human or murine enzyme since neither contains an ORF within the 5′ UTR. Additional studies will be required to determine if cSHMT can regulate its translation in vivo.

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## REFERENCES

1. Curtis, D., Lehmann, R., and Zamore, P. D. (1995) *Cell 81*, 171−178.
2. Jansen, M., de Moor, C. H., Sussenbach, J. S., and van den Brande, J. L. (1995) *Pediatr. Res. 37*, 681−686.
3. Hentze, M. (1995) *Curr. Opin. Cell Biol. 7*, 393−398.
4. Chu, E., and Allegra, C. J. (1996) *BioEssays 18*, 191−198.
5. Melefors, O., and Hentze, M. (1993) *BioEssays 15*, 85−90.
6. Carey, J., Cameron, V., de Haseth, P. L., and Uhlenbeck, O. C. (1983) *Biochemistry 22*, 2601−2610.
7. Romaniuk, P. J., Lowary, P., Wu, H. N., Stormo, G., and Uhlenbeck, O. C. (1987) *Biochemistry 26*, 1563−1568.
8. Chu, E., Takimoto, C. H., Voeller, D., Grem, J. L., and Allegra, C. J. (1993) *Biochemistry 32*, 4756−4760.
9. Chu, E., Voeller, D. M., Koeller, D. M., Drake, J. C., Takimoto, C. H., Maley, G. F., Maley, F., and Allegra, C. J. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 517−521.
10. Chu, E., Koeller, D. M., Casey, J. C., Drake, B. A., Chabner, B. A., Elwood, P. C., Zinn, S., and Allegra, C. J. (1993) *Proc. Natl. Acad. Sci. U.S.A. 88*, 8977−8981.
11. Chu, E., Voeller, D. M., Morrison, P. F., Jones, K. L., Takechi, T., Maley, G. F., Maley, F., and Allegra, C. J. (1994) *J. Biol. Chem. 269*, 20289−20293.
12. MacKenzie, R. E. (1984) in *Folates and Pterins* (Blakely, R. L., and Benkovic, S. J., Eds.) Vol. 1, pp 255−306, Wiley, New York.
13. Garrow, T. A., Brenner, A. A., Whitehead, V. M., Chen, X., Duncan, R. G., Korenberg, J. R., and Shane, B. (1993) *J. Biol. Chem. 268*, 11910−11916.
14. Appling, D. R. (1991) *FASEB J. 5*, 2645−2651.
15. Shane, B. (1989) *Vitam. Horm. 45*, 273−335.
16. Schirch, V., and Strong, W. B. (1989) *Arch. Biochem. Biophys. 269*, 371−380.
17. Nasrallah, I. M., Girgis, S., Suh, J. R., Oppenheim, E., Mowry, K. Z., Reig, B., and Stover, P. J. (1997) in *Chemistry and Biology of Pteridines and Folates* (Pfleiderer, W., and Rokos, H., Eds.) pp 377−380, Blackwell Science, Berlin.
18. Pfendner, W., and Pizer, L. I. (1980) *Arch. Biochem. Biophys. 200*, 503−512.
19. Stover, P. J., Chen, L., Suh, J. R., Stover, D., Keyomarsi, K., and Shane, B. (1997) *J. Biol. Chem. 272*, 1842−1848.
20. Girgis, S., Suh, J., Jolivet, J., and Stover, P. (1997) *J. Biol. Chem. 272*, 4729−4733.
21. Fowler, B., Whitehouse, C., Wenzel, F., and Wraith, J. E. (1997) *Pediatr. Res. 41*, 145−151.
22. Stover, P., and Schirch, V. (1991) *J. Biol. Chem. 266*, 1543−1550.
23. Girgis, S., Nasrallah, I. M., Suh, J. R., Oppenheim, E., Zanetti, K. A., Mastri, M. G., and Stover, P. J. (1998) *Gene 210*, 315−324.
24. Schirch, L. G., and Gross, T. (1968) *J. Biol. Chem. 243*, 5651−5655.
25. Kruschwitz, H., Ren, S., Di Salvo, M., and Schirch, V. (1995) *Protein Expression Purif. 6*, 411−416.
26. Stover, P., and Schirch, V. (1992) *Anal. Biochem. 202*, 82−88.
27. Ausubel, F. M. (1998) *Current Protocols in Molecular Biology*, Vol. 1, Section 9.1, Wiley, New York.
28. Strong, W. B., Tendler, S. J., Seither, R. L., Goldman, I. D., and Schirch, V. (1990) *J. Biol. Chem. 265*, 12149−12155.
29. Segal, I. H. (1975) in *Enzyme Kinetics*, pp 189−191, Wiley, New York.
30. Byrne, P. C., Sanders, P. G., and Snell, K. (1995) *Biochem. Biophys. Res. Commun. 214*, 496−502.
31. Nakshartri, H., Bouillet, P., Bhat-Nakshartri, P., and Chambon, P. (1997) *Gene 174*, 79−84.
32. Byrne, P. C., Sanders, P. G., and Snell, K. (1992) *Biochem. J. 286*, 117−123.

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