Ligand-mediated Induction of Thymidylate Synthase Occurs by Enzyme Stabilization

IMPLICATIONS FOR AUTOREGULATION OF TRANSLATION*

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Thymidylate synthase (TS) is indispensable in the de novo synthesis of dTMP. As such, it has been an important target at which anti-neoplastic drugs are directed. The fluoropyrimidines 5-fluorouracil and 5-fluoro-2'-deoxyuridine are cytotoxic as a consequence of inhibition of TS by the metabolite 5-fluoro-2'-deoxyuridine 5'-monophosphate (FdUMP). This inhibition occurs through formation of a stable ternary complex among the enzyme, the nucleotide analog, and the co-substrate N5,N10-methylene tetrahydrofolate. Numerous studies have shown that cellular concentrations of TS undergo about a 2–4-fold induction following treatment with TS inhibitors. An extensive body of in vitro studies has led to the proposal that this induction occurs because of relief of the translational repression brought on by the binding of TS to its own mRNA. In the current study, we have tested several predictions of this autoregulatory translation model. In contrast to expectations, we find that fluoropyrimidines do not cause a change in the extent of ribosome binding to TS mRNA. Furthermore, mutations within the mRNA that abolish its ability to bind TS have no effect on the induction. Finally, enzyme turnover measurements show that the induction is associated with an increase in the stability of the TS polypeptide. Our results, in total, indicate that enzyme stabilization, rather than translational derepression, is the primary mechanism of TS induction by fluoropyrimidines and call into question the general applicability of the autoregulatory translation model.

Thymidylate synthase (TS, EC 2.1.1.45) catalyzes the reductive methylation of dUMP by CH3H2PteGlu, generating dTMP and dihydrofolate (for a review, see Ref. 1). Because the enzyme is indispensable in the de novo synthesis of dTMP, it plays an important role in DNA replication in actively dividing cells and has been an attractive target at which anti-neoplastic agents are directed. Fluoropyrimidines (e.g. 5-fluorouracil and FdUrd) and, more recently, anti-folates (e.g. AG337, ZD1694, BW1843U89) have been useful in the clinical management of tumors of the breast, colon, stomach, and head and neck (2–5). Fluoropyrimidines exert their effects through formation of the nucleotide analog FdUMP, which inhibits TS via formation of a covalent complex containing the analog CH3H2PteGlu and the enzyme (1). This complex, which is termed the inhibitory ternary complex, is quite stable and leads to prolonged inhibition of the enzyme, depletion of dTMP pools, and thymineless death.

A number of studies with cultured cell lines, tumor models, and clinical specimens have shown that TS inhibitors induce enzyme levels by about 2–4-fold (6–8). Because response to TS-directed chemotherapy is dependent upon the enzyme concentration, such induction has been viewed as a potential barrier to successful therapeutic outcomes. As a result, there has been a great deal of interest in the mechanism of the induction and in strategies to ameliorate its effects. The increases in TS levels do not involve changes in mRNA concentrations, indicating that induction occurs at the translational or post-translational level. Recent studies have shown that TS binds to its mRNA in vitro and inhibits the translational efficiency of the mRNA (9); TS ligands disrupt this complex and restore translation (9–12, see Ref. 13 for a review). These findings have provided the basis for the so-called translational autoregulation model, which postulates that ligand-mediated induction of TS occurs through destabilization of the TS protein-mRNA complex, followed by relief of translational repression (13).

The autoregulatory translation model is based upon an extensive body of in vitro studies that are both elegant and persuasive. However, several predictions of the model remain untested. For example, because the binding of ribosomes to mRNA is rate-limiting in eukaryotic translation (14), the model predicts that the number of ribosomes/TS mRNA molecule will increase in cells treated with TS inhibitors. In addition, it is expected that mutant TS mRNAs that have lost the ability to bind the enzyme (e.g. see Ref. 11) will be constitutively translated so that enzyme levels will be resistant to the inductive effects of inhibitors. In the present study, we have tested these predictions in human colon tumor cell lines. Our results do not support the autoregulatory translation model; rather, we find that TS induction can be accounted for by changes in the stability of the TS polypeptide. Thus, the translation model may not be a universal explanation for ligand-mediated induction of TS.

EXPERIMENTAL PROCEDURES

Cell Lines—Human colon tumor cell line HCT15 was purchased from the American Type Type Collection. A FdUrd-resistant derivative of this line, which expresses the P303L mutant of TS, is described else-
Fig. 1. Polysome profiles for TS mRNA. Polysomes prepared from HCT15 and HCT15/200 cells were fractionated by sucrose gradient centrifugation and analyzed for TS mRNA by Northern blotting. Panel A shows the distribution of total RNA in the gradient as monitored by the absorbance at 254 nm (A254). Panels B and C show the distribution of TS mRNA in control and FdUrd-treated HCT15 cells, respectively. Panels D and E show the distribution of TS mRNA in control and FdUrd-treated HCT15/200 cells, respectively. The locations of the 40 S, 60 S, 80 S, and polysomal RNAs are indicated. The numbers of ribosomes/mRNA molecule are shown beneath the peaks in the polysome region of the gradient. The size of TS mRNA is shown to the left of the Northern blots. kb, kilobases.

where. Cells were typically grown at 37 °C in RPMI 1640 medium containing 10% fetal bovine serum (Life Technologies, Inc.) in a humidified 5% CO2 atmosphere.

Measurement of TS Levels—Extracts of logarithmically growing cells were prepared by sonication and centrifugation at 100,000 × g for 1 h. The concentration of TS in the supernatants was determined by either the FdUMP binding assay (16) or by Western blotting (17). A monoclonal antibody against human TS, which was provided by Dr. S. Berger, served as the probe for the Western blot analyses. The TS-bound antibody was detected by chemiluminescence using the ECL-PLUS kit from Amersham Pharmacia Biotech; signals were quantitated on a Storm PhosphorImager.

Polysome Analysis—Polysomes were isolated from cells using the methods of Palacios et al. (18) with minor modifications (19). Briefly, cycloheximide (90 μg/ml) was added to the culture medium. At various times, cells were washed and homogenized at 4 °C in 25 mM Tris-Cl, pH 7.5, containing 25 mM NaCl, 5 mM MgCl2, 250 mM sucrose, 1 mg/ml heparin, and 90 μg/ml cycloheximide. The homogenate was centrifuged at 10,000 rpm for 10 min, and the supernatant was layered onto a 4.2-ml sucrose gradient (0.5–1.5M) containing a 1-ml 2.5 M sucrose cushion. Fractions of 0.3 ml were collected, and RNA was extracted from each fraction and analyzed by Northern blotting. To verify that RNA sedimenting in the polysomal region of the gradient is ribosome-bound, controls in which Na2EDTA was added to the extraction buffer prior to labeling on the sucrose gradient were included.

Site-directed Mutagenesis—A GCC→AAA substitution was introduced immediately upstream of the translational initiation codon of TS mRNA by polymerase chain reaction mutagenesis. The template for mutagenesis was plasmid pKB169, which contains a cDNA copy of the TS mRNA between nucleotides 25 and 1354 (20). The mutagenic primers corresponded to nucleotides 81–110 within the cDNA; the forward mutagenic primer was 5'-CGCCCCCGCCGAAAAATGCTTGCGCAGCCGCTC-3' (the mutant nucleotides are underlined), whereas the reverse mutagenic primer was 5'-GAGCCGCGCCACAGGCCGTTTGGCCGCCG-3'. In one polymerase chain reaction, the reverse mutagenic primer and an upstream flanking primer (5'-GTAATACGACTCACTATAGGG-C-3'), corresponding to the T7 promoter region of the vector, were used; in a second reaction, the forward mutagenic primer and a downstream primer (5'-CTGCGATGCGAATACGAG-3'), corresponding to nucleotides 260–279 of the cDNA, were used. Reaction mixtures contained, in a total volume of 100 μl, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2 mM MgCl2, 250 μM dNTPs, 5% (v/v) formamide, and 5 units of AmpliTaq DNA polymerase. Polymerase chain reaction was carried out for 30 cycles (94 °C for 1.5 min, 52 °C for 2.5 min, and 72 °C for 3 min) followed by a 7-min extension at 72 °C. The DNA products from the two reactions were combined and co-amplified under the same conditions but using the T7 and the downstream primers.

The final product was digested with HindIII and PstI and cloned in place of the corresponding fragment in pKB169; the insert of the resulting plasmid, pTF528, was sequenced to verify the presence of the GCC→AAA substitution. pTF528 was digested with HindIII and BgIII, and the TS cDNA-containing fragment was cloned in place of the corresponding fragment in the expression construct pJZ205, which contains the wild-type TS cDNA under control of the SV40 promoter; the final construct was denoted pTF530.

DNA Transfection—Plasmids pJZ205 and pTF530 were stably transfected into a TS-deficient Chinese hamster lung cell line (RJK88.13, Ref. 21) by calcium phosphate-DNA co-precipitation in medium containing 20 μg/ml thymidine; 16–25 μg of plasmid DNA were added per culture. After 48 h, the cells were placed in selective medium lacking thymidine, and surviving colonies were pooled, passed several times through selective medium, and stored until further use. Cells transfected with the wild-type cDNA plasmid (i.e. pJZ205) were denoted

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RESULTS

Analysis of Polysomal TS mRNA—The binding of ribosomes to mRNA is rate-limiting in eukaryotic translation (14). As a consequence, changes in translational efficiency are generally associated with alterations in the number of ribosomes bound to a particular mRNA. The translational model of TS regulation predicts that in the presence of ligands, the number of ribosomes/TS mRNA molecule will increase. To test this prediction, we examined the polysome distribution profiles for TS mRNA both prior to and following treatment with FdUrd.

Human colon tumor cell line HCT15 was treated for 24 h with 6 nM FdUrd, which corresponds to the ID$_{50}$ for this cell line.$^2$ Under these conditions, TS levels increase about 2–3-fold with no change in mRNA concentrations (data not shown). Cellular extracts were fractionated by sucrose gradient centrifugation to separate various size classes of polysomes (Fig. 1A) and the amounts of TS-specific mRNA in the gradient fractions were assessed by Northern blotting (Fig. 1, B and C). Polysome profiles were virtually identical for both control and FdUrd-treated cells. In each circumstance, nearly all of the TS mRNA sediments in the polysome region of the gradient with little, if any, mRNA in nonpolysomal fractions; the polysomal TS mRNA contained approximately 4–7 ribosomes/molecule (Fig. 1). The addition of Na$_2$EDTA to the extracts prior to centrifugation resulted in the TS mRNA being exclusively nonpolysomal, indicating that the mRNA is indeed ribosome-bound (data not shown).

It could be argued that the modest extent of enzyme induction (i.e. 2–3-fold) is too small to be manifested as a detectable change in the polysome profile. We therefore made use of a mutant cell line in which the FdUrd-mediated induction of TS is amplified relative to that in HCT15. Cell line HCT15/200 is a FdUrd-resistant derivative of HCT15 that expresses wild-type TS mRNA and the GCC$^3$AAA mutant, respectively. Lane A, control RJK88.13/TS(wt); lane B, RJK88.13/TS(wt) treated for 24 h in 6 nM FdUrd; lane C, control RJK88.13/TS(GCC$^3$AAA); lane D, RJK88.13/TS(GCC$^3$AAA) treated for 24 h in 6 nM FdUrd. The size of TS (37 kDa) is indicated. The retarded migration of ternary complexes in FdUrd-treated cells has been observed previously (9–11).

The concentration of TS was determined by Western blotting at various times after the addition of cycloheximide to the media of control and FdUrd-treated cells. The fraction of enzyme remaining was plotted as a function of time after cycloheximide addition. Results of a typical experiment, which was carried out three times, are shown. Panel A, cell line HCT15, control (○) and treated for 24 h with 6 nM FdUrd (■); panel B, cell line HCT15/200, control (○) and treated for 24 h with 200 nM FdUrd (■).

Binding to TS mRNA.

Identical results were obtained with several other colon tumor cell lines (data not shown). Thus, the absence of an effect of FdUrd on the polysomal distribution of TS mRNA is not specific to cell line HCT15.

Effects of Mutations That Abolish TS Binding to mRNA—RNA/protein binding experiments have shown that the translational initiation region of TS mRNA, particularly the AUG initiation codon and the three bases preceding it, are critical determinants of TS binding to its mRNA (11). It has been suggested that TS recognizes and binds a stem loop that is 30 bases in length and encompasses the AUG codon (11). If destabilization of the TS-TS mRNA complex and subsequent derepression of translation are central to fluoropyrimidine-mediated enzyme induction, then the translatability of a mRNA that is incapable of binding the enzyme should be resistant to FdUrd; enzymes encoded by such a mutant mRNA should not undergo induction in response to drugs.

To test this notion, we made use of the findings of Chu et al. (11), who reported that a mutant oligoribonucleotide containing AAA in place of GCC immediately upstream of the translational initiation codon of TS mRNA failed to compete with full-length mRNA for binding to TS; a wild-type oligoribonucleotide fully competed (11). We introduced the GCC$^3$AAA substitution into a full-length TS cDNA expression plasmid, and the resulting construct was stably transfected into a TS-deficient Chinese hamster lung cell line (RJK88.13, Ref. 21), resulting in cell line RJK88.13/TS(GCC$^3$AAA); TS induction in response to FdUrd was measured and compared with that in cell line RJK88.13/TS(wt), which is a stably transfected line

\[ \text{37kD} \]

\[ \text{B} \]

\[ \text{C} \]

\[ \text{D} \]

Fig. 2. Effect of a mutant TS mRNA lacking the ability to bind TS. FdUrd-mediated induction of TS was measured by Western blot analysis of stably transfected cell lines RJK88.13/TS(wt) and RJK88.13/TS(GCC$^3$AAA), which express wild-type TS mRNA and the GCC$^3$AAA mutant, respectively. Lane A, control RJK88.13/TS(wt); lane B, RJK88.13/TS(wt) treated for 24 h in 6 nM FdUrd; lane C, control RJK88.13/TS(GCC$^3$AAA); lane D, RJK88.13/TS(GCC$^3$AAA) treated for 24 h in 6 nM FdUrd. The size of TS (37 kDa) is indicated. The retarded migration of ternary complexes in FdUrd-treated cells has been observed previously (9–11).

Fig. 3. Measurement of the half-life of the TS polypeptide. The binding of ribosomes to mRNA is rate-limiting in eukaryotic translation (14). As a consequence, changes in translational efficiency are generally associated with alterations in the number of ribosomes bound to a particular mRNA. The translational model of TS regulation predicts that in the presence of ligands, the number of ribosomes/TS mRNA molecule will increase. To test this prediction, we examined the polysome distribution profiles for TS mRNA both prior to and following treatment with FdUrd. Human colon tumor cell line HCT15 was treated for 24 h with 6 nM FdUrd, which corresponds to the ID$_{50}$ for this cell line.$^2$ Under these conditions, TS levels increase about 2–3-fold with no change in mRNA concentrations (data not shown). Cellular extracts were fractionated by sucrose gradient centrifugation to separate various size classes of polysomes (Fig. 1A) and the amounts of TS-specific mRNA in the gradient fractions were assessed by Northern blotting (Fig. 1, B and C). Polysome profiles were virtually identical for both control and FdUrd-treated cells. In each circumstance, nearly all of the TS mRNA sediments in the polysome region of the gradient with little, if any, mRNA in nonpolysomal fractions; the polysomal TS mRNA contained approximately 4–7 ribosomes/molecule (Fig. 1). The addition of Na$_2$EDTA to the extracts prior to centrifugation resulted in the TS mRNA being exclusively nonpolysomal, indicating that the mRNA is indeed ribosome-bound (data not shown).

It could be argued that the modest extent of enzyme induction (i.e. 2–3-fold) is too small to be manifested as a detectable change in the polysome profile. We therefore made use of a mutant cell line in which the FdUrd-mediated induction of TS is amplified relative to that in HCT15. Cell line HCT15/200 is a FdUrd-resistant derivative of HCT15 that expresses high concentrations of a mutant TS molecule containing a Pro $\rightarrow$ Leu substitution at residue 303.$^2$ This amino acid substitution renders the polypeptide relatively unstable, as compared with the wild-type enzyme.$^5$ Treatment of HCT15/200 cells for 24 h in 200 nM FdUrd results in a 10–15-fold induction of TS with no change in mRNA concentrations (data not shown). The polysome profiles for TS mRNA from this cell line are presented in Fig. 1, D and E. The high mRNA concentrations in the gradient fractions reflect the 40-fold overproduction of mRNA in HCT15/200 as compared with HCT15.$^5$ The vast majority of TS mRNA was polysomal and sedimented at a region corresponding to about 4–7 ribosomes/mRNA. Importantly, the polysome distribution pattern was identical in both control and FdUrd-treated cells (Fig. 1, D and E). Thus, even with a 10–15-fold induction of TS, there is no detectable change in the extent of ribosome
containing the wild-type TS cDNA. Fig. 2 shows that TS levels in cells expressing the mutant mRNA are induced to the same extent (i.e., 2–3-fold) as cells expressing the wild-type mRNA. Thus, the GCC→AAA mutation had no detectable effect on enzyme inducibility, suggesting that binding of TS to the initiation region of the mRNA is not required for the induction.

**Stabilization of the TS Polypeptide by FdUrd**—The results presented above do not support the autoregulatory translation model of TS induction. This leaves enzyme stability as a possible mechanism. We therefore measured the half-life of the TS protein in HCT15 cells both prior to and following treatment with FdUrd. Cycloheximide was added to cells, and the concentrations of TS protein were assayed by Western blotting at various times thereafter. The half-life of TS was 7.3 ± 0.3 h in control cells and 25 ± 1.0 h in FdUrd-treated cells (Fig. 3A). This 3–4-fold stabilization of the TS polypeptide readily accounts for the approximately 2–3-fold induction of enzymes in response to fluoropyrimidines.

In HCT15/200 cells, where the FdUrd-mediated induction of TS is amplified, the half-life of the enzyme was 2.3 ± 1.7 h, indicating that the mutant P303L polypeptide expressed in these cells is significantly less stable than the wild-type polypeptide (Fig. 3B). Treatment with FdUrd increased the half-life to 18 ± 1.4 h (Fig. 3B), a degree of enzyme stabilization that fully accounts for the induction.

Thus, TS induction by FdUrd appears to result from an increase in the stability of the TS polypeptide. We find no evidence for a translational level control mediated by TS binding to its mRNA.

**DISCUSSION**

A large body of *in vitro* studies has clearly shown that free TS has an affinity for its own mRNA and that this affinity is decreased by TS ligands (9–13). The binding of TS to mRNA represses the translational efficiency of the latter and inhibits enzyme synthesis (9–13). TS inhibitors, therefore, have an impact on TS production, as well as on enzyme activity. These findings, in total, have led to the notion that derepression of TS mRNA translation, which occurs via disruption of the TS-TS mRNA complex, is a central mechanism underlying ligand-mediated induction of enzyme concentrations in cells (15). The experiments reported in the current paper are not consistent with such a model. First, the polynucleotide distribution patterns for the mRNA were virtually identical in control and FdUrd-treated cells. If TS induction occurs via a translational mechanism, then an increase in the number of ribosomes/mRNA would be expected in the presence of drugs. Second, a 3-base substitution in TS mRNA that abolishes its ability to bind the enzyme had no observable effect on induction. Thus, interactions between TS and its mRNA do not appear to be required for the drug-mediated increases in cellular TS concentrations. Finally, direct biochemical measurements showed that treatment with FdUrd stabilizes the TS molecule to an extent that fully accounts for the induction. We conclude that fluoropyrimidine-mediated increases in TS levels occur through an effect on enzyme stability, with no effect on mRNA translation. This conclusion is similar to that made by Washbien (22) many years ago, following the analysis of human gastrointestinal cell lines.

The finding that ligands stabilize TS is entirely consistent with current views on TS structure and function. It is well documented that upon formation of either the catalytic or the inhibitory ternary complex, the enzyme, particularly its C-terminal residues, undergoes a major conformational shift that is stabilized by a hydrogen bonding network involving the folate co-substrate (1). Indeed, this shift alters the susceptibility of TS to proteolytic enzymes *in vitro* (15). It is therefore not surprising that the conformational change stabilizes the enzyme *in vivo*.

It has been suggested that increases in TS levels cause resistance to TS-directed agents and may represent a significant barrier to successful cancer chemotherapy (13). Distinguishing between mRNA translation and enzyme stabilization as mechanisms for TS induction has implications with regard to how such resistance occurs. In the translation model, increases in the TS level are derived from a newly synthesized enzyme, which confers resistance by enhancing the rate of dTMP synthesis. In contrast, in the enzyme stabilization model, the induced enzyme is bound into the inhibitory ternary complex; resistance in this model is conferred by “titrating out” FdUMP, thereby lowering the effective concentration of free nucleotide analog available for inhibition of newly synthesized enzyme. Clearly, further insights will require sorting out the relative importance of enzyme stability and mRNA translation in the regulation of cellular TS levels, particularly in response to TS-directed anti-metabolites.

The present results, coupled with the absence of rigorous experiments testing the *in vivo* relevance of TS binding to its mRNA, raise questions concerning the general applicability of the translational model of TS induction. Of course, it is formally possible that cell lines other than those focused upon here may exhibit a translational derepression mechanism. In the least, it seems reasonable to conclude that the translation model cannot be invoked as a universal explanation for the induction of TS by its inhibitors.

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