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Regulation of Thymidylate Synthase in Human Colon Cancer Cells Treated with 5-Fluorouracil and Interferon-γ

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SUMMARY

The effects of fluorouracil (5-FU) and interferon-γ (IFN-γ) on the regulation of thymidylate synthase (TS) gene expression were investigated in the human colon cancer H630 cell line. By Western immunoblot analysis, TS protein levels in H630 cells were increased 3-, 5.5-, 5-, and 2.5-fold after 8-, 16-, 24-, and 36-hr exposure to 1 μM 5-FU, respectively. When H630 cells were exposed to varying concentrations of 5-FU (0.3–10 μM) for 24 hr, increases in TS protein up to 5.5-fold were observed. A 24-hr exposure to 1 μM 5-FU resulted in a 4.5-fold increase in the level of TS protein, whereas in 5-FU/IFN-γ-treated cells TS protein was increased by only 1.8-fold, compared with control cells. IFN-γ treatment alone did not affect TS protein levels, relative to control. Northern blot analysis revealed no changes in TS mRNA levels when H630 cells were exposed either to 1 μM 5-FU for 8–36 hr, to varying concentrations of 5-FU (0.3–10 μM) for 24 hr, or to the combination of 5-FU and IFN-γ. Pulse-labeling studies with [^{35}S]methionine demonstrated a 3.5-fold increase in net synthesis of TS in cells treated with 1 μM 5-FU, whereas the level of newly synthesized TS increased only 1.5-fold in cells treated with 5-FU/IFN-γ, compared with control cells. Pulse-chase studies revealed that the half-lives of TS protein in control and 5-FU-treated cells were equivalent. These findings demonstrate that the increase in TS protein after 5-FU exposure and the subsequent inhibitory effect of IFN-γ on TS protein expression are both regulated at the post-transcriptional level.

TS (EC 2.1.1.45) catalyzes the conversion of dUMP and 5,10-methylenetetrahydrofolate to dTMP and dihydrofolate (1). This reaction provides the sole intracellular *de novo* source of dTMP, a necessary precursor of DNA, and for this reason TS is a critical target enzyme in cancer chemotherapy (2, 3).

Most of the initial studies examining regulation of TS enzyme expression focused on cell cycle-directed events. TS is present at significantly higher levels (20-fold) in proliferating cells that are engaged in DNA replication, compared with resting cells (4–8). The increase in TS enzyme activity that accompanies entry into the S phase of the cell cycle is regulated at both the transcriptional and post-transcriptional levels (9–13). Jenh *et al.* (10) demonstrated that TS enzyme activity increased 8–9-fold in a 5-fluorodeoxyuridine-resistant cell line (LU3-7) after serum stimulation. This increase was prevented by the addition of inhibitors of protein and RNA synthesis, suggesting that synthesis and translation of TS mRNA are required. Further studies revealed that the increase in TS activity was primarily due to an increase in the rate of synthesis of the enzyme. More recently, a number of investigators have described acute increases in TS levels in the *in vitro*, *in vivo*, and clinical settings after short term exposure to the fluoropyrimidines (14–18). This increase in TS protein expression after fluoropyrimidine exposure appears to be an important mechanism by which malignant cells develop resistance to these antineoplastic agents (14–18). Although the underlying mechanisms for this enhanced expression of enzyme after exposure of cells to fluoropyrimidines have not been elucidated, several possibilities exist, including increased transcription of TS-specific sequences, increased stability of TS mRNA, increased efficiency of TS mRNA translation, and increased stability of TS protein.

A number of recent reports have documented synergistic antiproliferative interactions between the IFNs and 5-FU in a number of murine and human cancer cell lines (19–23). Although some studies have suggested that the mechanism of synergy may involve enhanced inhibition of TS (23, 24), the precise mechanism remains poorly defined. In an attempt to understand the biochemical interaction between 5-FU and IFN-γ, we examined the biological effects of this combination in the human colon cancer H630 cell line (18). IFN-γ, at non-growth-inhibitory concentrations, significantly enhanced the 5-FU-associated cytotoxicity. Those studies demonstrated that exposure of H630 cells to 5-FU alone resulted in a 3-fold increase in both TS enzyme binding and catalytic activity. Treatment of these cells with IFN-γ repressed the 5-FU-associated increase in TS enzyme activity, thereby enhancing the sensitivity of these cells to 5-FU by 18-fold. Thus, this increase in TS

ABBREVIATIONS: TS, thymidylate synthase; 5-FU, 5-fluorouracil; IFN, interferon; DHFR, dihydrofolate reductase; SDS, sodium dodecyl sulfate; FdUMP, fluorodeoxyuridine monophosphate; 5,10-methylene-H_4PteGlu, 5,10-methylenetetrahydrofolate.

enzyme activity appeared to correlate with the development of resistance to 5-FU (18).

In the present report, we extended our studies to determine the underlying molecular basis for the interaction between 5-FU and IFN-γ in the human colon cancer H630 cell line. Our findings demonstrate that the increase in TS protein expression after 5-FU exposure and the subsequent inhibitory effects of IFN-γ on TS induction are both regulated at a translational level.

Materials and Methods

Cell culture. The human colon adenocarcinoma H630 cells were kindly provided by Drs. Adi Gazdar and John Minna of the NCI-Navy Medical Oncology Branch (National Cancer Institute, Bethesda, MD). The origin and characteristics of this cell line have been described previously (25, 26). These cells were grown in 75-cm^2 plastic tissue culture flasks (Falcon Labware, Oxnard, CA), in growth medium consisting of RPMI 1640 with 10% dialyzed fetal bovine serum (GIBCO, Grand Island, NY) and 2 mM glutamine, and were maintained in a humidified incubator at 5% CO_2 and 37°. All other medium components were obtained from Biofluids Co. (Rockville, MD).

Western immunoblot analysis. H630 colon cancer cells were washed three times with PBS, harvested, and resuspended in 0.1 M KH_2PO_4, pH 7.2. Cell lysis was accomplished by sonication, using three 2–3-sec bursts. The extracts were centrifuged at 10,000 × *g* for 30 min, and protein concentrations were determined by the Bio-Rad protein assay. Equivalent amounts of protein (300 μg) from each sample were resolved by SDS-polyacrylamide gel electrophoresis, using 15% acrylamide gels, according to the method of Laemmli (27). The gels were then electroblotted onto nitrocellulose membranes. Antibody staining was performed using a chemiluminescence detection system (Tropix Inc., Bedford, MA) with a mouse monoclonal TS primary antibody and an alkaline phosphatase-conjugated secondary antibody (1/10,000 dilution). The mouse 106 TS primary monoclonal antibody was developed against purified human recombinant TS protein, and its origin and characteristics have been described previously (28). The membranes were stripped and reprobed with a mouse α-tubulin primary monoclonal antibody (1/500 dilution; Amersham, Arlington Heights, IL) and a horseradish peroxidase-conjugated secondary antibody (1/1000 dilution; Bio-Rad Laboratories, Richmond, CA).

Isolation of total RNA and RNA blot hybridization (Northern analysis). H630 colon cancer cells were washed three times with ice-cold phosphate-buffered saline and harvested from 150-cm^2 tissue culture flasks with a rubber policeman. Total RNA was extracted according to the method of Chomczynski and Sacchi (29). For Northern blot analysis, total cellular RNA (30 μg/sample) was denatured, fractionated on a 1% formaldehyde-agarose gel, and transferred to a Nytran filter membrane as described previously (30). The cDNA for human TS (pcHTS-1) was a generous gift from Dr. T. Seno (Saitama Cancer Center Research Institute, Saitama-Ken, Japan) (31).

Measurement of TS biosynthesis. H630 cells were treated for 22 hr with no drug, 5-FU, IFN-γ, or the combination of 5-FU and IFN-γ, in medium containing 10% dialyzed fetal bovine serum. The medium was changed and cells were washed with methionine-free medium and then incubated for an additional 2 hr in 3 ml of methionine-free medium plus 10% dialyzed fetal bovine serum containing 100 μCi/ml methionine (Trans ^{35}S-label; specific activity, 1013 Ci/mmol; ICN Biomedicals). Cells were then washed twice with 10 ml of ice-cold phosphate-buffered saline and scraped into ice-cold cell lysis buffer (0.5% Triton X-100, 300 mM NaCl, 50 mM Tris·HCl, pH 7.4, 10 μg/ml leupeptin, 0.1 mM phenylmethylsulfonyl fluoride). For experiments measuring the effect of 5-FU and IFN-γ on TS enzyme stability, H630 cells were treated under the same conditions as stated above. Radiolabel was removed after a 2-hr incubation, and fresh methionine-containing medium was added to the cell cultures. Cells were then harvested at 24 and 48 hr. Immunoprecipitation of TS protein was performed using a TS polyclonal antibody (30) together with Protein-A agarose (Bethesda Research Laboratories), according to the method of Harford (32). Each sample contained 40 × 10^6 cpm of trichloroacetic acid-insoluble radioactivity. The relative amounts of TS were determined by densitometric scanning using a Beckman DU-65 spectrophotometer. Samples were analyzed by autoradiography after electrophoresis in a 15% SDS-polyacrylamide gel with the buffer system of Laemmli (27).

Quantitation of TS protein and TS mRNA from autoradiographs. The levels of TS protein and TS mRNA were determined by densitometric scanning of the autoradiographs using a Beckman DU-65 spectrophotometer. Densitometry values for TS mRNA were normalized to the corresponding level of human β-actin mRNA.

Results

Effect of 5-FU on TS protein. We previously observed a 3.1-fold increase in TS enzyme activity after a 24-hr exposure of H630 cells to 1 μM 5-FU (18). This increase in TS activity was measured using either the radioenzymatic FdUMP binding assay or the TS catalytic assay. To demonstrate that these changes in TS enzyme activity reflected changes in the absolute level of TS protein expression, TS protein levels in H630 cell extracts after exposure to 5-FU were measured by means of Western immunoblot analysis. A representative Western blot (one of three separate experiments) is presented in Fig. 1. As seen in both Fig. 1, A and B, 5-FU treatment resulted in the

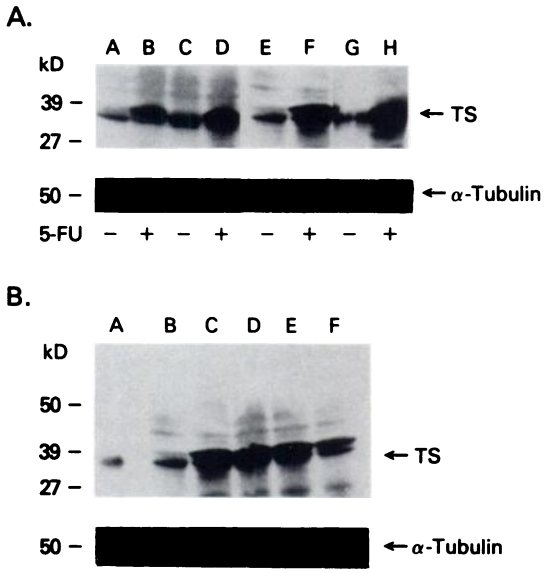

Fig. 1. Western immunoblot analysis of TS protein levels in H630 cells treated with 5-FU. A, H630 cells were incubated with 1 μM 5-FU, where indicated, for varying time periods (8–36 hr). At each time point, cytosolic extracts were prepared, and equal amounts of protein (300 μg) were loaded onto each lane. TS protein was detected by immunoblot analysis with a mouse TS monoclonal antibody as described in Materials and Methods. Proteins were from H630 cultures treated for 8 (*lanes A* and *B*), 16 (*lanes C* and *D*), 24 (*lanes E* and *F*), or 36 hr (*lanes G* and *H*). B, H630 cells were incubated for 24 hr with varying concentrations of 5-FU. After this time period, cells were harvested and cytosolic extracts were prepared. Equal amounts of protein (300 μg) were loaded onto each lane, and TS protein was detected by immunoblot analysis as outlined in Materials and Methods. Proteins were from cultures exposed to no drug (*lane B*) or 0.3 (*lane C*), 1 (*lane D*), 3 (*lane E*), or 10 μM 5-FU (*lane F*). Human recombinant TS protein was included as a standard (*lane A*). The membranes were stripped and reprobed with a mouse α-tubulin primary monoclonal antibody (1/500 dilution) to control for loading and integrity of protein. Quantitation of signal intensities was performed by densitometric scanning (Beckman DU-65 spectrophotometer).

formation of two distinct bands, one resolving at 35 kDa and the other at approximately 38 kDa. The higher molecular mass band represents TS protein bound in a covalent complex to the nucleotide FdUMP. Once formed, the complex is not subject to dissociation, even under denaturing conditions. This same observation has been made by several investigators (15, 28, 33). Because these two bands represent different forms of TS, measurement of both bands by densitometry was performed to determine total levels of TS protein. Densitometric analysis of the Western immunoblots revealed a 3- ± 0.4-, 5.5- ± 1-, 5- ± 0.6-, and 2.5- ± 0.2-fold increase in TS protein when H630 cells were treated with 1 μM 5-FU for 8, 16, 24, and 36 hr, respectively (Fig. 1A). When the concentration of 5-FU was varied for a 24-hr exposure, similar increases in TS protein were observed at 0.3 (5.5- ± 1-fold), 1 (5.5- ± 0.8-fold), 3 (4.5- ± 0.2-fold), and 10 μM (2- ± 0.3-fold) (Fig. 1B). The time- and dose-dependent increases in TS protein expression are not the result of loading artifact, given that the expression of an unrelated protein, α-tubulin, remains unchanged throughout.

Effect of 5-FU on TS mRNA. To identify more precisely the molecular events underlying the regulation of TS gene expression, the levels of TS mRNA in response to 5-FU drug treatment were measured. When H630 cells were treated with 5-FU (1 μM) for 6–36 hr, the amount of TS mRNA remained unchanged (Fig. 2A). Similarly, there were no apparent differences in TS mRNA levels in H630 cells treated with varying concentrations of 5-FU (0.3, 1, 3, and 10 μM, respectively), compared with control untreated cells (Fig. 2B). When each of these Northern blots were stripped and reprobed for human β-actin mRNA expression, there were no changes in human β-actin mRNA levels in response to 5-FU exposure (Fig. 2). However, there was a slight alteration in the mobility of β-actin mRNA after exposure to increasing concentrations of 5-FU. Although this result was not consistently observed in our experiments, this difference in mobility may be due to either incorporation of 5-FU metabolites into RNA, limited nuclease digestion, or an artifact. When the changes in TS protein and TS mRNA over a 36-hr time period after exposure to 1 μM 5-FU were compared, the increased expression of TS protein was independent of the levels of TS mRNA (Fig. 3). These findings suggest that the increase in TS protein after 5-FU treatment is regulated at the post-transcriptional level.

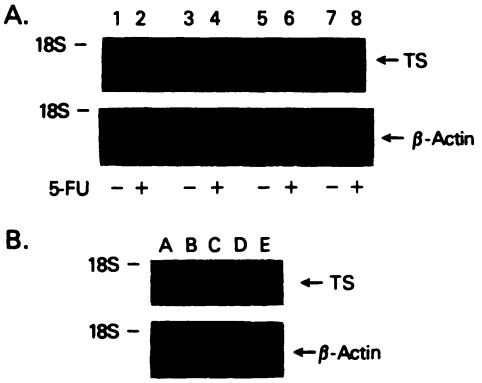

Fig. 2. Northern blot analysis of TS mRNA levels in H630 cells treated with 5-FU. A, H630 cells were treated in the absence or presence of 1 μM 5-FU, where indicated, for varying time periods (8–36 hr). At each time point, cells were harvested and total cytoplasmic RNA was prepared. Equal amounts (30 μg) of total RNA were electrophoresed, transferred, and hybridized with a ^{32}P-radiolabeled human TS cDNA as outlined in Materials and Methods. RNA was from cells treated for 8 (*lanes 1* and *2*), 16 (*lanes 3* and *4*), 24 (*lanes 5* and *6*), or 36 hr (*lanes 7* and *8*). B, H630 cells were incubated for 24 hr with varying concentrations of 5-FU. Total RNA was isolated, and equal amounts (30 μg) of RNA were electrophoresed, transferred, and hybridized with a ^{32}P-radiolabeled human TS cDNA as described in Materials and Methods. RNA was from H630 cultures treated with no drug (*lane A*) or 0.3 (*lane B*), 1 (*lane C*), 3 (*lane D*), or 10 μM 5-FU (*lane E*). The filters were then stripped of the TS probe and rehybridized with a human β-actin probe to control for loading and integrity of mRNA. Quantitation of signal intensities was performed by densitometric scanning (Beckman DU-65 spectrophotometer).

Effect of 5-FU and IFN-γ on TS protein and on TS mRNA. We next determined the combined effects of 5-FU and IFN-γ on TS protein expression. H630 cells were treated for 24 hr with no drug, 5-FU (1 μM), IFN-γ (30 units/ml), or the combination of 5-FU/IFN-γ. The Western immunoblot shown in Fig. 4A is representative of four separate experiments. Exposure to 5-FU (1 μM) resulted in a 4.0- ± 0.7-fold increase in TS protein. As was seen in Fig. 1, treatment with 5-FU resulted in two bands, with the one at 35 kDa representing free TS and the 38-kDa band representing TS bound in the covalent ternary complex. IFN-γ treatment alone did not change the level of TS protein, compared with control. However, the simultaneous addition of IFN-γ to cells treated with 5-FU inhibited the 5-FU-mediated induction of TS by 58 ± 7%. No changes in α-tubulin protein expression were observed when H630 cells were treated under identical drug conditions.

As shown in Fig. 4A, the simultaneous presence of IFN-γ produced an inhibitory effect on the 5-FU-mediated induction of TS protein. When TS mRNA levels were measured under the same conditions, there were no apparent differences in TS mRNA steady state levels (Fig. 4B). Thus, the changes in TS enzyme activity and TS protein that occur after 5-FU and 5-FU/IFN-γ treatment are independent of TS mRNA levels. This finding suggests that the effects of 5-FU and IFN-γ on TS protein expression are both regulated at the post-transcriptional level.

Effects of 5-FU and IFN-γ on TS synthesis. The effects of 5-FU and IFN-γ on TS biosynthesis were subsequently determined. To do these studies, a TS polyclonal antibody was

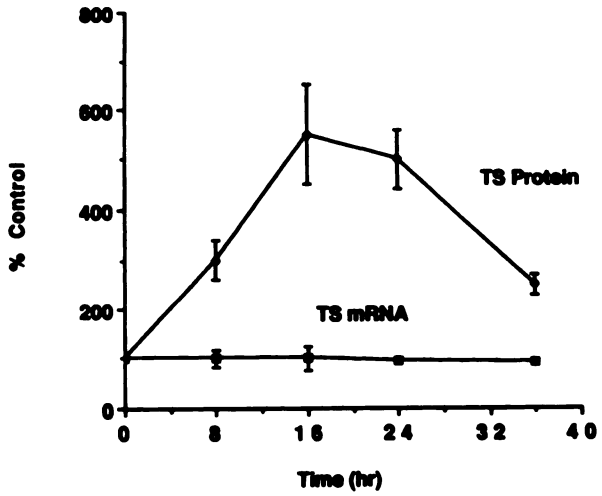

Fig. 3. Correlation of the level of TS protein with the level of TS mRNA after 5-FU exposure. The relative amount of TS protein (♦) and TS mRNA (□) after 1 μM 5-FU exposure exposure over 8–36 hr was quantitated by densitometry of the autoradiographs shown in Fig. 1A and Fig. 2A, respectively.

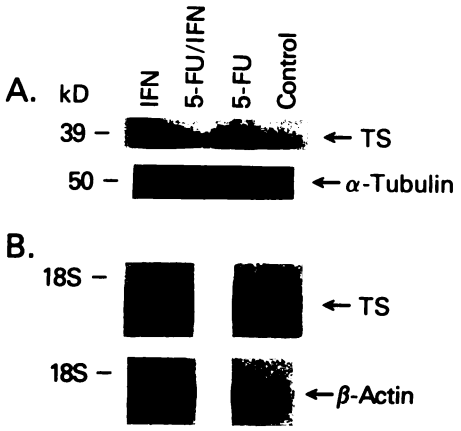

Fig. 4. A, Effect of 5-FU and IFN-γ on TS protein. H630 cells were incubated for 24 hr at 37° under the following conditions: no drug, 1 μM 5-FU, 30 units/ml IFN-γ, or 1 μM 5-FU plus 30 units/ml IFN-γ. Total protein (300 μg) was resolved on a 15% SDS-polyacrylamide gel. Western immunoblot analysis was performed with a chemiluminescent detection system, using a mouse TS primary monoclonal antibody and an alkaline phosphatase-conjugated secondary antibody (1/10,000 dilution). The membranes were stripped and reprobed with a mouse α-tubulin primary monoclonal antibody (1/500 dilution) to control for loading and integrity of protein. B, Effect of 5-FU and IFN-γ on TS mRNA levels. H630 cells were treated under the same conditions as described for A. Total RNA was extracted from cells, and 30 μg of RNA were analyzed for each of the following conditions: no drug, 5-FU, IFN-γ, and 5-FU/IFN-γ. The filters were then stripped of the TS probe and rehybridized with a human β-actin probe to control for loading and integrity of mRNA.

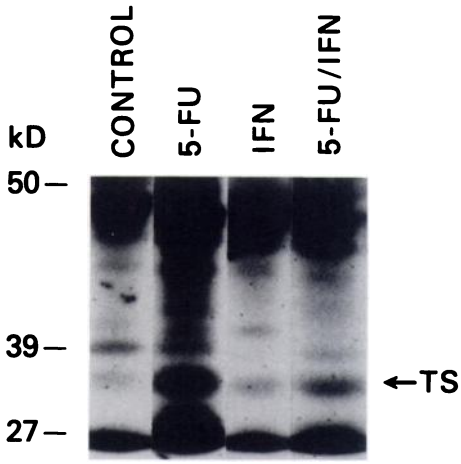

Fig. 5. Electrophoretic analysis of labeled proteins isolated from 5-FU-treated cells. H630 cells were treated with no drug, 1 μM 5-FU, 30 units/ml IFN-γ, or the combination of 5-FU and IFN-γ for 22 hr. They were then washed twice with serum-free, methionine-free medium and labeled for an additional 2 hr with 100 μCi/ml [^{35}S]methionine in 3 ml of methionine-free medium containing 10% dialyzed fetal bovine serum. Cytoplasmic extracts were prepared and electrophoresed as described in Materials and Methods.

used (30). Control experiments demonstrated that this antibody was capable of immunoprecipitating both free and bound TS enzyme with similar efficiency (Fig. 5). With the use of radiolabel, however, the difference between the free and bound forms of TS cannot be easily resolved (data not shown). Thus, the ^{35}S-labeled TS protein observed in response to 5-FU treatment contains both free and bound TS and represents total TS. The level of total TS synthesis was increased 3.5-fold in 5-FU-treated cells, compared with control untreated cells (Fig. 5; Table 1). IFN-γ (Fig. 5; Table 1) had no apparent effect on TS

TABLE 1
Effect of 5-FU and IFN-γ on TS synthesis

Treatment[a]	Total Protein[b]	TS[c]
	cpm × 10^{-6}	*arbitrary units*
Control	6.2 ± 0.3	1.9 ± 0.2
5-FU	5.2 ± 0.3	6.8 ± 0.7
IFN-gg	5.3 ± 0.4	2.5 ± 0.1
5-FU/IFN-γ	5.1 ± 1.3	2.9 ± 0.4

[a] H630 cells were treated for 22 hr with drug, labeled for an additional 2 hr with [^{35}S]methionine, and analyzed as described for Fig. 5.
[b] The level of incorporation of [^{35}S]methionine into total cellular protein, determined as total cellular trichloroacetic acid-precipitable radioactivity. Values represent the mean ± standard error of four separate experiments.
[c] Autoradiograms were scanned with a densitometer. The amount of radioactivity in the TS peak was determined by measuring the intensity of the TS peak and subtracting a background value consisting of the average intensity of regions adjacent to the TS peak. Values represent the mean ± standard error of four separate experiments.

synthesis, compared with control conditions. In contrast, the presence of IFN-γ in cells treated simultaneously with 5-FU (Fig. 5; Table 1) resulted in a 66% decrease in the relative amount of newly synthesized TS, compared with cells treated with 5-FU alone.

Treatment with 5-FU resulted in an approximately 20% decrease in total cytoplasmic protein labeling (Table 1). Previous studies demonstrated that exposure to 1 μM 5-FU resulted in 35% inhibition of cell growth (18). Thus, this inhibition of overall protein synthesis may be the result of inhibitory effects of 5-FU on cellular synthesis and function. In contrast, synthesis of TS and other proteins was significantly increased after 5-FU exposure (Fig. 5). There is a prominent protein product that resolves at approximately 27 kDa. Using an *in vitro* translation system, it was shown that translation of human TS mRNA results in a predominant product of 35 kDa and another of 27 kDa (34). Although the etiology of this lower molecular mass species remains unclear, it may represent either a TS protein degradation product, the result of an internal translational start site, or the product of premature termination of translation. The increased synthesis of the 27-kDa protein seen in Fig. 5 is consistent with this, as yet uncharacterized, "TS-associated product." Although 5-FU exposure is associated with altered expression of other unrelated proteins, as shown in Fig. 5, it is not clear whether these responses represent specific 5-FU effects. However, it is conceivable that, in response to this cytotoxic stress, the synthesis of a host of proteins is altered as an adaptive mechanism.

To demonstrate that the increase in labeling of TS after 5-FU exposure was not the result of a loading artifact, control experiments were performed in which immunoprecipitation of human α-tubulin protein was performed using a mouse α-tubulin monoclonal antibody. There were no differences in the levels of α-tubulin protein between control and 5-FU-treated cells (data not shown).

Effect of 5-FU and IFN-γ on TS enzyme stability. The stability of TS enzyme in 5-FU-treated H630 cells, compared with untreated cells, was subsequently determined. The half-life of TS enzyme was 28 hr in control untreated cells (Fig. 6). This value is consistent with previous studies that have reported the half-life of TS to be >24 hr (10). Treatment with 5-FU did not significantly alter the half-life of TS ($t_{½}$ = 24 hr), compared with control (Fig. 6). The half-lives of TS in cells treated with either IFN-γ alone or the combination of 5-FU and IFN-γ were equivalent to that in control H630 cells ($t_{½}$ =

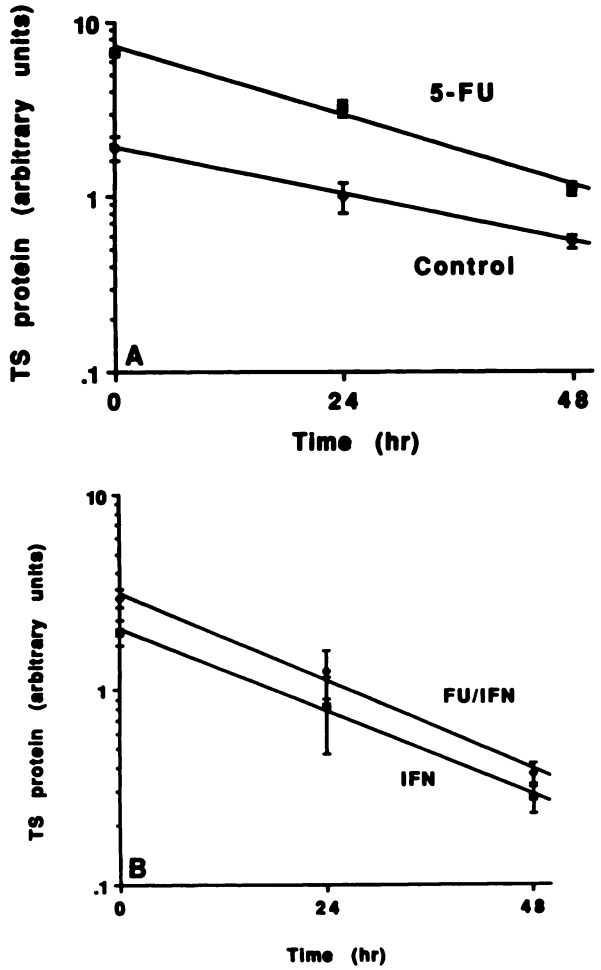

Fig. 6. TS stability in 5-FU-treated H630 cells. Cells were incubated in the absence (control) or presence of 1 μM 5-FU for 22 hr. They were then incubated with [^{35}S]methionine for an additional 2 hr. The medium was removed and the cells were incubated for 24 or 48 hr in fresh medium containing excess unlabeled methionine. The *lines* represent TS half-lives of 28 hr for control, 24 hr for 5-FU-treated, 28 hr for IFN-γ-treated, and 28 hr for 5-FU/IFN-γ-treated cells. Each *point* represents the mean ± standard error of at least three separate experiments.

28 hr) (Fig. 6).

The value determined for the half-life of TS protein represents the combined turnover of both free and bound TS. However, because the majority (~80%) of TS in the 5-FU-treated cells represents the bound form, the measured half-life principally reflects that of bound TS. Because there is no difference in the turnover of total TS protein in control untreated versus 5-FU-treated cells, this finding suggests that the increase in TS protein results from newly synthesized TS and not from decreased enzyme degradation.

Discussion

Several investigators have described elevations in TS enzyme levels after short term exposure to the fluoropyrimidines in *in vitro* and *in vivo* model systems and in clinical tumor specimens (14–18). However, to date, the molecular processes underlying this event have not been well defined (14–18). We previously reported that 24-hr exposure to 5-FU results in an acute 3-fold increase in TS enzyme activity in H630 human colon cancer cells (18). The presence of IFN-γ in cells treated with 5-FU inhibited the 5-FU-induced expression of TS enzyme activity, resulting in enhanced cellular sensitivity to 5-FU. This induction of TS enzyme activity appeared to represent one mechanism whereby the H630 colon cancer cells rapidly develop resistance to 5-FU.

The present study was undertaken to elucidate the regulatory elements controlling the acute induction of TS. Our findings demonstrate that the changes in the level of TS protein in H630 cells in response to 5-FU treatment closely parallel the changes previously observed with TS enzyme activity. There was a 2.5–5.5-fold increase in TS protein expression in response to 5-FU that was both time and dose dependent. Analysis of the Western immunoblots revealed an approximately 30–40% increase in levels of free TS protein. This small increase in free TS is consistent with previous results that revealed a 40% increase in free TS enzyme activity in 5-FU-treated cells (18). Thus, the increase in TS protein in response to 5-FU is primarily due to an increase in TS enzyme in the drug-bound form. The induction of TS protein after 5-FU exposure allows free TS to remain at approximately 30–40% above base-line levels, such that thymidylate and DNA biosynthesis can be maintained in the face of cytotoxic 5-FU treatment. It is maintenance of free TS that is especially relevant, because it is free TS that is directly responsible for maintaining normal levels of thymidylate within the cell.

The increases in both TS enzyme activity and TS protein do not parallel TS mRNA levels after 5-FU exposure, inasmuch as the amount of TS mRNA remains unaltered. We have determined that a 24-hr exposure to 5-FU results in a 3.5-fold increase in the level of TS biosynthesis. Furthermore, pulse-chase experiments demonstrated that the stability of TS protein after 5-FU exposure is approximately the same, compared with control non-drug-treated conditions. Thus, the enhanced expression of TS in H630 cells treated with 5-FU is regulated at the post-transcriptional level and appears to result from an absolute increase in the level of TS biosynthesis.

We previously showed that addition of IFN-γ to H630 cells treated with 5-FU ablated the 5-FU-mediated increase in TS enzyme activity (18). The results of this study demonstrate that IFN-γ has a similar inhibitory effect on TS protein, as determined by Western immunoblot analysis. Moreover, these alterations in TS protein levels are not associated with corresponding changes in TS mRNA levels. Further work revealed that the 5-FU-mediated increase in TS biosynthesis was markedly reduced in the presence of IFN-γ. These findings suggest that IFN-γ regulates the 5-FU-mediated expression of TS at a translational level. This effect of IFN-γ on TS expression appears to have biological relevance. With addition of IFN-γ, TS protein levels were inhibited by nearly 60%, compared with cells treated with 5-FU alone. We previously observed that the combination of 5-FU and IFN-γ, at the same concentrations used in the present experiments, inhibited the growth of H630 cells by 60%. Thus, inhibition of the 5-FU-associated induction of TS expression by IFN-γ significantly enhanced the cytotoxic effects of 5-FU against this particular cell line.

Previous studies have shown that the IFNs regulate gene expression at both the transcriptional and post-transcriptional levels (35–37). For example, both regulation of type I and II procollagen mRNA levels by IFN-γ and that of c-*myc* mRNA levels by IFN-β are at a post-transcriptional level. The IFNs have also been shown to induce an RNA-dependent protein kinase that is responsible for phosphorylation of eukaryotic

initiation factor (elF-2) (38–40). The functional activity of elF-2 is down-regulated once its α subunit is phosphorylated. Thus, this protein kinase-dependent process represents one mechanism for the regulation of eukaryotic protein synthesis. Because IFN-γ inhibits the 5-FU-associated increase in TS protein expression, one possibility is that the regulatory effects of IFN-γ on TS biosynthesis are mediated through this specific pathway. Additional studies will be required to determine the underlying molecular mechanism by which IFN-γ controls TS mRNA translation.

In their initial report describing the sequence of the human TS cDNA clone, Takeishi *et al.* (31) suggested the possibility of translational regulation of TS expression, given the theoretical potential of three interconvertible secondary structures, each containing a stem-loop structure in the 5′ untranslated region of the human TS mRNA. We recently reported that translation of human TS mRNA is controlled by TS, its own protein end product, in an autoregulatory manner (34). Using a cell-free rabbit reticulocyte model system, we observed that translation of TS mRNA was inhibited in the presence of human recombinant TS protein. When substrates for TS, including the nucleotides dUMP and FdUMP, and the reduced folate substrate 5,10-methylene-H_4PteGlu were included in translation reactions containing TS mRNA and TS protein, inhibition of TS mRNA translation by TS protein was completely relieved. These results suggested that either the native conformational state or active site occupancy of TS may be a critical determinant of TS mRNA translation and that changes in the levels of the physiological ligands dUMP and 5,10-methylene-H_4PteGlu may play a role in regulating the intracellular levels of TS. Recent studies suggest that the levels of TS enzyme during the cell cycle are controlled at a post-transcriptional level (41). It is conceivable that regulation of TS in this setting may be a consequence of alterations in dUMP and/or 5,10-methylene-H_4PteGlu pools that occur during the cell cycle. With regard to the *in vitro* cellular system, exposure of cells to 5-FU results in the intracellular accumulation of the nucleotides FdUMP and dUMP. Each of these nucleotides would interact with TS and inhibit its subsequent binding to TS mRNA, thereby resulting in enhanced efficiency of TS mRNA translation. The end result would be an increased intracellular level of TS protein. This study provides support for the proposed model of TS translational autoregulation and suggests that this regulatory process is biologically relevant. Moreover, malignant cells that are capable of acutely increasing TS protein expression would appear to have a protective mechanism by which they rapidly become resistant to the cytotoxic stress of an antineoplastic agent such as 5-FU.

In addition to the increase in TS protein synthesis after 5-FU exposure, there are alterations in labeling of unrelated protein products. It is conceivable that synthesis of a host of proteins may be altered in response to exposure to a cytotoxic agent, as an adaptive mechanism. However, the regulatory mechanisms underlying their expression may be quite different from those observed for TS and may involve alterations in transcription, mRNA stability, protein degradation, etc. Although the specificity of these processes remains unclear, altered expression of these unrelated proteins may represent a broad range of cellular responses that allow the cell to maintain its proliferative capacity in the presence of a cytotoxic stress, and it appears to be 5-FU mediated.

Recent studies have shown that the increased accumulation of thymidine kinase protein during the S phase of the cell cycle results from enhanced efficiency of translation of thymidine kinase mRNA (42, 43). Several investigators have suggested that expression of DHFR in response to methotrexate exposure may also be regulated at the translational level (44–46). Furthermore, Bastow *et al.* (45) postulated that the intracellular levels of DHFR protein may control the efficiency of DHFR mRNA translation in an autoregulatory manner. All three of these enzymes, TS, thymidine kinase, and DHFR, catalyze reaction pathways critical for DNA biosynthesis. Thus, the ability to regulate the expression of each of these respective protein products at a translational level in the setting of acute cellular stress may represent one mechanism by which normal cellular synthetic function is maintained.

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