Increased thymidylate synthase protein levels are principally associated with proliferation but not cell cycle phase in asynchronous human cancer cells

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Summary. We have analysed cell cycle variations in thymidylate synthase (TS) protein in asynchronously growing NCI H630 and HT 29 colon cancer and MCF-7 breast cancer cell lines. Western immunoblot analysis using the TS 106 monoclonal antibody revealed a 14- to 24-fold variation in TS levels between the peak exponential and confluent growth phase in the three cell lines. Similar variations in TS levels and TS activity were detected using the 5-fluorodeoxyuridine monophosphate and deoxyuridine monophosphate biochemical assays. The percentage of cells in S-phase, which paralleled changes in TS levels, reached a maximum of 38-60% in asynchronous exponentially growing cells compared with 5-10% in confluent cells. In asynchronous exponential cell analysis of TS levels in each cell cycle phase using two-parameter flow cytometric analysis revealed that TS protein levels were 1.3- to 1.5-fold higher in S than in G0, G1, phase cells, and 1.5- to 1.8-fold higher in G0, M than G0, G1 cells. Similar differences of 1.1- to 1.5-fold between G0, G1 and S-phase and 1.6- to 1.9-fold between G0, G1 and G0, M-phase were detected by Western immunoblot and biochemical assays. TS protein was not detectable by Western blot analysis, flow cytometry or biochemical analysis in the G0, G1 population of confluent cells. Twenty-six percent of cells in this population were G0, G1 cells compared with 2% in exponentially growing cells. In contrast to TS, a 4-fold difference in thymidine kinase (TK) was detected between G0, G1 and S-phase cells in exponentially growing MCF-7 cells. The level of TS enzyme is associated with cellular proliferation and the percentage of cells in S-phase; however, TS protein is not exclusively associated with S-phase in asynchronously growing cells. The variation in TS levels between exponentially growing and confluent cell population appears to be due to differences in TS levels between G0, G1 and G0, G1 cells.

Keywords: cell cycle; TS

Thymidylate synthase (TS; EC 2.1.1.45) catalyses the reductive methylation of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP). This reaction is an essential step in DNA biosynthesis, since it provides the only de novo source of thymidylate. TS is also a critical target for the fluoropyrimidine drugs that are widely used in the treatment of breast cancer, as well as tumours of the gastrointestinal and upper aerodigestive tracts. In tumour cells, 5-fluorouracil (5-FU) and fluorodeoxyuridine (FdUrd) are converted to 5-fluoro-2-deoxyuridine monophosphate (FdUMP), which forms a covalent ternary complex with TS in the presence of the folate co-factor 5, 10-methylene tetrahydrofolate (5, 10-CH₂H₂PteGlu).

Previous studies have demonstrated in both mammalian and yeast systems that TS activity is higher during DNA replication and decreases when cells are non-dividing (Conrad, 1971; Maley and Maley, 1960; Navalgun et al., 1980; Storms et al., 1984). Other studies have shown that, while increased TS activity correlates with the DNA synthetic phase, this increase is not blocked by inhibitors of DNA synthesis (Jenh et al., 1985). This suggests that, while TS activity may be associated with proliferation, its regulation may be independent of DNA synthesis and cell cycle phase. More recent studies have demonstrated that TS enzyme levels rise acutely when cells are exposed to cytotoxic agents such as 5-FU (Chu et al., 1990). Thus, in addition to changes in TS related to the cell cycle, neoplastic cells may increase TS levels as a protective mechanism against cytotoxic stress. This acute induction of TS protein may represent an important mechanism in the development of tumour resistance.

Recently, we have developed several monoclonal antibodies to human TS that are highly specific and detect TS in the cytoplasm of tumour cells and tissue (Johnston et al., 1991, 1992). These antibodies have facilitated the study of TS in cell lines and human tissues and have allowed TS to be measured within individual cells. We have also demonstrated that increased TS protein levels predict for poor clinical outcome in patients with rectal cancer (Johnston et al., 1994). This may be the result of the association of TS protein levels with cellular proliferation.

The purpose of this study was to analyse cell cycle variations in TS levels during the various cell cycle phases and proliferation to determine its association with DNA synthesis and S-phase in asynchronously growing tumour cells.

Materials and methods

Chemicals

Dextran (clinical grade), 5-FU, acid-washed activated charcoal, fluorescein isothiocyanate (FITC)-labelled goat antimouse conjugate, propidium iodide, cycloheximide, thimerosal and non-specific murine ascitic fluid were all purchased from Sigma (St Louis, MO, USA). [6-3H]FdUMP (specific activity 23 Ci mmol⁻¹ and [5-3H]dUMP (sp. act. 22 Ci mmol⁻¹) were obtained from Moravek Biochemicals (Brea, CA, USA). The ECL-enhanced chemiluminescence kit was obtained from Amersham (Buckinghamshire, UK). Nitrocellulose membranes were purchased from Schleicher & Schuell (Keene, NH, USA). The monoclonal antibody to α-tubulin was obtained from Oncogene Science (Uniondale, NY, USA). Ki-67 monoclonal antibody was obtained from Dako (Carpinteria, CA, USA). Goat anti-mouse horseradish...
peroxidase conjugate. BioRad protein assay, Tween 20 and SDS were obtained from Bio-Rad Laboratories (Richmond, CA, USA). Tris and glycine were purchased from Schwarz/Mann Biotech, ICN Biomedicals (Cleveland, OH, USA). Phosphate-buffered saline (PBS) was obtained from Digeene Diagnostics (Beltsville, MD, USA). Acrylamide was purchased from National Diagnostics (Atlanta, GA, USA). Ammonium persulfate was bought from BRL Life Technologies (Gaithersburg, MD, USA). Carnation non-fat milk was obtained from Carnation (Los Angeles, CA, USA).

Cell culture

The characteristics of the human colon cancer cell lines NCI-H693, T47D, HT-29 and the human breast cancer cell line MCF-7 have been previously described (Park et al., 1987; Soule et al., 1973). All cells were maintained in RPMI-1640 (Gibco, Grand Island, NY) with 10% heat-inactivated fetal calf serum (FCS) plus 2 mM glutamine. For proliferation assays, cells were seeded on day 0 at a density of 2 × 10^6 cells cm^-2. Doubling times were calculated over the first 72 h of cell growth using linear regression analysis.

Cell harvest

Cells were trypsinised, resuspended in RPMI medium containing 10% FCS, and an aliquot was counted using a ZF Coulter Counter (Coulter Electronics, Hialeah, FL, USA). Cells were washed twice in ice-cold PBS and stored as pellets at -80°C. Before analysis, cells were lysed by sonication in 0.1 M potassium dihydrogen phosphate pH 7.4. Sonication of 1 × 10^6 cells ml^-1 was performed with four 2-s bursts from a Vibra cell sonifier from Sonics and Materials (Danbury, CT, USA). The cellular extracts were centrifuged at 5000 g for 15 min, and the supernatants collected. Protein concentrations were determined using the BioRad protein assay (Bradford, 1976).

Western blot analysis

Equivalent amounts of protein (400 µg) from each cellular lysate were resolved by polyacrylamide gel electrophoresis using 12.5% acrylamide, according to the method of Laemmli (1970). The gels were electroblotted onto a nitrocellulose membrane in transfer buffer (48 mM Tris, 39 mM glycine, 0.5 M EDTA in 20% methanol) for 2 h. The nitrocellulose blots were treated at room temperature with blocking solution (blotto: 5% Carnation non-fat milk, 10 mM Tris, 0.01% thimerosal) for 45 min. After washing with PBS-T (PBS with 0.1% Tween 20), primary antibody (TS 106, ascitic fluid, 1:100 in blotto) was added for 90 min. After two washes with PBS-T and three washes with blotto, secondary antibody (goat anti-mouse horseradish peroxidase, BioRad, 1:1000 in blotto) was used for 60 min. After another four washes with PBS-T, the chemiluminescent substrate (luminol, plus enhancer, according to the ECL method of Amersham) was applied for 1 min. Blots were then air dried, covered by a plastic foil and exposed to film (Kodak, X-OMat AR) for 5–60 s. Denitrosiency scanning of the film was performed using a Hewlett-Packard Scan Jet Plus and analysed using an image analysis software program (NIH Image v.1.40, provided by Wayne Rasband, NIMH, NIH, Bethesda, MD, USA).

FdUMP binding assay

Equivalent volumes of cytosolic extracts (50 µl) were assayed in duplicate. The assay was performed in a total volume of 200 µl containing 75 µM 5-10-CH_2H_2PteGlu, 3 pmol of [3H]FdUMP, 100 mM 2-mercaptoethanol and 50 mM potassium dihydrogen phosphate pH 7.4 as has been previously described (Moran et al., 1979; Johnston et al., 1991).

dUMP catalytic activity

Equivalent volumes of cytosolic extracts (50 µl) were assayed in duplicate. The assay was performed in a final volume of 200 µl containing 100 pmol [5-3H]FdUMP, 100 mM 2-mercaptoethanol, 50 mM potassium dihydrogen phosphate pH 7.2 and 50 µl (or 5 µl) of cellular extracts as previously described (Roberts, 1966).

Thymidine kinase assay

Thymidine kinase was assayed as previously described by Ives et al. (1969). The reaction mixture consisted of 10 mM ATP, 10 mM magnesium chloride 50 mM Tris-HCl pH 7.5, 15 mM sodium fluoride 0.1 µCi [3H]thymidine (Moravek 20 Ci mmol^-1), 5 µM unlabelled thymidine. 1–20 µl of cell lysate in a total volume of 50 µl. The reaction was allowed to proceed for 30 min at room temperature and stopped by boiling for 60 s. The reaction as measured was linear with time, and the rate was proportional to the lysate volume used. The assay mixture was spotted onto a 2.5 cm DE 81 ion-exchange disc (Whatman). After 10 min the disc was washed with three changes of distilled water. 30 µl per disc, and then placed in scintillation fluid containing 1 ml of 0.1 M hydrochloric acid–0.2 M potassium chloride, and the vials were gently shaken for 20 min. Scintillation fluid was added and samples counted in a scintillation counter. The values were expressed as pmol min^-1 mg^-1 cytosol protein.

Cell cycle distribution

One to two million cells were resuspended in 0.7 ml of ice-cold PBS and fixed by adding 1.3 ml of 95% ethanol with 0.5% Tween 20 drop wise to the cell suspension with gentle vortex mixing. The cell suspension was kept at 4°C overnight. After washing, cells were resuspended in 200 µl of PBS-TB [PBS with 0.1% (w/v) bovine serum albumin and 0.5% Tween 20] containing 10 µg ml^-1 RNAse and incubated at 37°C for 20 min. Cells were then pelleted and resuspended in 0.5 ml of PBS–TB containing 50 µg ml^-1 propidium iodide (PI). Cell cycle data were acquired using a Becton-Dickinson FACScan with 15 mW excitation at 488 nm. The PI signal was assessed through a 650 long-pass filter on FL3. The software used for acquisition and analysis was the LYSYS II software version 1.1 from Becton-Dickinson Immucytometry Systems (San Jose, CA, USA).

Two-parameter flow cytometry with TS and PI

Aliquots of two million cells were harvested by centrifugation at 500 g, washed and fixed as above. Cells were placed in blocking buffer BSA-T (3% BSA, 0.2% Tween 20 in PBS) at 4°C for 30 min. After pelleting, cells were incubated with 200 µl of the primary antibody (TS 106 monoclonal or non-specific ascitic fluid as a control at 1:100 dilution in PBS) for at least 1 hr at 4°C. After one wash, cells were incubated with 200 µl of the secondary antibody: goat anti-mouse immuno-globulin–fluorescein isothiocyanate (FITC) conjugate diluted 1:50 in PBS-TG [PBS with 0.1% (v/v) goat serum and 0.5% Tween 20]. After two washes, samples were resuspended in 0.5 ml of PBS-TB containing 50 µg ml^-1 of PI. The PI signal was assayed as described above. The FITC signal was collected through a 530/30 bandpass filter on FL1. Analysis was performed using the LYSYS II software version 1.1 from Becton-Dickinson. The analysis was restricted to singlets using pulse area vs pulse width gating of the PI signal. A DNA histogram was obtained from each sample and boundaries were established based on the mean channel of the G1 and G2/M peaks to define G1, G2, S-phase and G2-M regions. In both the control population (ascitic fluid diluted 1:100 in PBS) and the population incubated with TS 106, the mean fluorescent intensity (MFI) of the FITC signal was determined from each phase of the cell cycle. Ratios of relative TS
staining intensities between different cell cycle phases were then calculated, after correcting for non-specific staining.

**Cell cycle sorting using Hoechst 33342**

Unfixed cells were stained with the superviral stain Hoechst 33342 according to the method of Crissman et al. (1990). Cells in G0, G1, S and G2-M phase were then sorted according to their DNA content, using a Becton-Dickinson FACStar Plus flow cytometer with 100 mW excitation at 351–465 nm. The Hoechst 33342 signal was assessed through a 400 long-pass filter into three distinct populations: G0, G1 phase, S-phase and G2, M-phase cells. Pellets were stored at −80°C until assayed for TS protein levels and TS biochemical activity. Sorted populations were confirmed to contain only G0, G1, S or G2-M cells by subsequent cell cycle analysis with PI.

![Figure 1](1) TS levels in asynchronous populations of three human cancer cell lines. TS levels were measured by three different methods as a function of time: immunoblot analysis (●), FdUMP binding assay (○) and dUMP catalytic activity (▲). The three graphs display results for NCI H630 (a), HT 29 (b) and MCF-7 (c) cells. Cells were plated at 2 × 10⁵ cells cm⁻² and grown for up to 288 h. After harvest, crude cellular extracts were made by sonication and aliquots were analysed in parallel by immunoblot, FdUMP binding and dUMP catalytic assays respectively. The results represent the mean ± s.d. of three separate experiments.

**Two-parameter flow cytometry with Ki-67 and PI**

This assay was based on a previously published method (Baisch and Gerdes, 1990). After harvest, one million cells were resuspended in 2 ml of 0.15 M sodium chloride at 4°C. The suspension was added dropwise to 8 ml of ice-cold pure acetone while gently shaking. Cells were stored for at least 24 h at −20°C. After centrifugation and decanting, 0.1% RNAse in PBS was added and the sample incubated for 20 min at 37°C. After pelleting, 100 μl of Ki-67 antibody (1:10 dilution in PBS containing 1% BSA) was added. Incubation lasted 30 min at room temperature with gentle shaking. Subsequently, 100 μl of FITC-conjugated goat anti-mouse antibody (1:40 in PBS–BSA) was added and incubated for 30 min at room temperature. After washing in PBS, 0.5 ml of PI (2 μg ml⁻¹ in PBS) was added for 20 min in the dark. Data were acquired and analysed as described above. The control populations in these experiments were incubated with non-specific mouse ascitic fluid rather than the Ki-67 antibody. The baseline fluorescence of the log-amplified Ki-67 FITC signal was set using the appropriate negative control for each sample. Peak fluorescence on FL1 of this population was used to determine the upper boundary for cells considered to be Ki-67 negative.

**Results**

**TS and cell cycle analysis during asynchronous growth**

NCI H630, HT 29 and MCF-7 cells were seeded at an equivalent density (10⁵ cells cm⁻²). The doubling time for NCI H630 cells was 25 h, for HT 29 cells 22 h and for MCF-7 cells 19 h. TS levels were measured at various time points during asynchronous growth (hours 0, 24, 48, 72, 96, 120, 144, 168, 192, 240 and 288). Immunoblot analysis revealed a 14- to 24-fold variation in TS protein levels from peak exponential growth phase to confluent growth phase in the three cell lines. TS levels were maximal after 48 h of growth and reached the lowest level after 120 h (Figure 1). The drop in TS from the maximal to the lowest basal level occurred over a brief period of time (48 h) between hours 72 and 120. Similar variations in TS protein levels and TS activity over time were also detected using the FdUMP binding and the dUMP catalytic assays respectively (Figure 1).

Comparing all three methods, the differences between the maximum and minimum TS level were 14- to 17-fold in the H630 colon cancer cell line, 15- to 23-fold in the HT 29 colon cancer cell line and 19- to 24-fold in the MCF-7 breast cancer cell line (Table 1). The change in TS levels was paralleled by similar variations in the distribution of cells through the cell cycle (Figure 2). The peak percentage of S-phase cells was reached after 24–48 h of growth and decreased 6- to 10-fold after 120 h. Thus, increased TS levels were associated with increased DNA synthesis.

**TS analysis by cell cycle phase**

**Two-parameter flow cytometry and cell sorting** Exponentially growing asynchronous NCI H630, HT 29 and MCF-7 cells were analysed by two-parameter flow cytometry for TS protein and DNA content. TS was measured as FITC fluorescence intensity and DNA content as PI fluorescence.

### Table 1 TS levels in confluent vs exponentially growing cells

| Cell line | Immuno blot relative density | FdUMP Assay | dUMP assay |
|-----------|-------------------------------|------------|------------|
|           | Variation² | Maximum² | Variation³ | Maximum³ | Variation³ |
| H630      | 14-fold  | 0.675 ± 0.04 | 17-fold  | 6.89 ± 0.2 | 15-fold  |
| HT 29     | 23-fold  | 0.269 ± 0.01 | 15-fold  | 4.84 ± 0.3 | 19-fold  |
| MCF-7     | 22-fold  | 0.809 ± 0.02 | 24-fold  | 13.7 ± 0.5 | 19-fold  |

*Variation between maximum level of mean TS (exponential growth phase) and mean basal level of TS (plateau phase, hours 120–288). *Maximum level of mean TS measured during exponential growth phase.
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Figure 2 Cell cycle analysis of asynchronous cell populations as a function of time in (a) NCI H630, (b) HT 29 and (c) MCF-7 cells. The graph shows the percentage of cells in G0/G1 (△), S (○) and G2/M (■) phase. After harvest, 2 million cells were fixed in ethanol and stained with propidium iodide for cell cycle analysis by flow cytometry. The results represent the mean ± s.d. of three separate experiments.

Table II Relative TS level at different cell cycle states measured by two-parameter flow cytometry

| Cell line | S vs G0/G1 | G2/M vs G0/G1 |
|-----------|------------|---------------|
| NCI H630  | 1.52 ± 0.20 | 1.68 ± 0.10   |
| HT 29     | 1.33 ± 0.09 | 1.53 ± 0.06   |
| MCF-7     | 1.47 ± 0.12 | 1.82 ± 0.22   |

TS levels were measured as mean FITC staining intensities in exponentially growing cells at different cell cycle stages. Relative TS levels are ratios of the staining intensity of S or G2/M phase cells compared with G0/G1 phase. Results are means ± s.d. of three experiments.

growing NCI H630 cells, densitometric scanning revealed a 1.5 ± 0.2-fold increase in TS from G0/G1 to G2/M (Figure 4, lanes 1–3). In HT 29 and MCF-7 breast cells, TS increased 1.3 ± 0.1-fold and 1.2 ± 0.3-fold between G0/G1 and S-phase and 1.8 ± 0.2-fold and 1.6 ± 0.4-fold between G0/G1 and G2/M phase respectively.

TS biochemical analysis We also measured TS catalytic activity and TS FdUMP binding activity in G0/G1 and S-phase cells sorted from exponentially growing NCI H630 and MCF-7 cells. In NCI H630 cells, a 1.1-fold increase in TS catalytic function and FdUMP binding activity were also noted between S and G0/G1 phase in MCF-7 cells (Table III). Thus, using biochemical analysis, two-parameter flow cytometry and Western immunoblot analysis, TS protein increased by approximately 1.1- to 1.5-fold between G0/G1 and G2/M phase in asynchronous, actively proliferating cell populations.

Measurement of thymidine kinase activity TK activity was measured in exponentially growing MCF-7 cells that had been sorted into G0/G1 and S-phases to determine if cell cycle...
H630, in TS is part tall Ki-67 Fugwe confluence (lane2) sorted NCI DNA to S-phase and 4-fold tubulin E peak). In OF H630 GD/GI, cells. 98% 5 Ki-67 27-36 50-110 102 units) 10% 26% Ki-67 is in these asynchronously growing cell populations. Maximum TS expression corresponds to the period when the highest percentage of cells are in S-phase (38-60%), while lowest TS protein levels are associated with the least percentage of cells in S-phase (5-10%). Thus, increased TS protein levels and TS activity are associated with cellular proliferation and DNA synthesis. This is in agreement with previous studies that have demonstrated a 17-fold variation in TS between resting and exponentially growing cells (Conrad, 1971; Conrad and Ruddle, 1972).

In contrast, two-parameter flow cytometry and immunoblot analysis of sorted cell populations demonstrates that TS is present in actively proliferating asynchronous cells in all (G0/G1, S and G2/M) phases of the cell cycle. In this population, S and G2/M cells have 1.1- to 1.8-fold more TS than cells in G0/G1. Conversely, in a confluent cell population, the TS level of the G0/G1 population in NCI H630 cells is undetectable. The G0/G1 population in proliferating NCI H630 cells is composed entirely of G1 cells (98%), while in confluent nonproliferating NCI H630 cells the G0/G1 popula-
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The exponential growth and release of TS is an association between TS and S-phase. Using elutriation and monoclonal antibody (Reddy et al., 1990), we have recently demonstrated that the in situ radiolabelled dUMP assay is subject to changes in apparent TS activity resulting from variations in dUMP activity by TK. Thus, differences between the in situ dUMP and radiolabelledFdUMP binding TS assays may be the result of an association of TK rather than TS with cell cycle phase.

Keyomarsi et al. (1991, 1993) synchronised MCF-7 cells using lovastatin and demonstrated large TS protein oscillations (10- to 20-fold) with cell cycle phase after release from synchrony using the FdUMP binding assay. This study suggested that in synchronised cells there is a specific association of TS with S-phase, since TS not only increases with entry into S-phase, but also decreases significantly when cells exit S-phase. The variations in TS noted in this study are in contrast with our data, but may be the result of the method of synchronisation using lovastatin.

We have previously reported that the TS protein half-life is 26 h in the NCI H630 cells (Chu et al., 1993). This is consistent with our data showing the persistence of TS throughout each cell cycle phase in asynchronously proliferating cells. In cells that are actively cycling, TS protein persists from one cell to the next. A major decrease in TS protein levels occurs only when a cell enters a resting phase and cell cycling is discontinued. Thus, the inhibiting conversion of dUMP to dTMP would be expected to have most activity in proliferating cells (G, S and G, M), in which the activity of TS protein is important for continued cellular proliferation.

In summary, we have shown that TS varies 14- to 24-fold between exponentially proliferating and confluent (quiescent) human cancer cells. TS is present in G, G, S and G, M in proliferating human cancer cells and is not detectable in G, G populations separated from confluent cells owing to the presence of increased numbers of G, cells. In exponentially growing asynchronous cells, the variations in TS between different cell cycle phases are less than 2-fold. In asynchronously growing tumour cells, TS protein levels are directly associated with cellular proliferation and, therefore, the percentage of cells in S-phase, but large increases in TS protein levels are not detected in the S-phase population of asynchronously growing cells.

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