Intracellular Location of Thymidylate Synthase and Its State of Phosphorylation*

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Thymidylate synthase (TS), an enzyme that is essential for DNA synthesis, was found to be associated mainly with the nucleolar region of H35 rat hepatoma cells, as determined both by immunogold electron microscopy and by autoradiography. In the latter case, the location of TS was established through the use of [6-3H]5-fluorodeoxyuridine, which forms a tight ternary complex of TS with 5-fluorodeoxyuridylate (FdUMP) and 5,10-methylenetetrahydrofolylpolyglutamate within the cell. However, with H35 cells containing 50–100-fold greater amounts of TS than unmodified H35 cells, the enzyme, although still in the nucleus, was located primarily in the cytoplasm as shown by autoradiography and immunohistochemistry. In addition, TS was also present in mitochondrial extracts of both cell lines, as determined by enzyme activity measurements and by ternary complex formation with [32P]FdUMP and 5,10-methylenetetrahydrofolate. Another unique observation is that the enzyme appears to be a phosphoprotein, similar to that found for other proteins associated with cell division and signal transduction. The significance of these findings relative to the role of TS in cell division remains to be determined, but suggest that this enzyme’s contribution to the cell cycle may be more complex than believed previously.

Thymidylate synthase (TS, EC 2.1.1.45) is a unique enzyme in nature by virtue of the fact that one of the substrates in the reaction, CH2PteGlu serves to reductively methylate the second substrate, dUMP, to yield dTMP and H2PteGlu. Because dTMP plays an essential role in the synthesis of DNA, the enzyme has been a chemotherapeutic target since its discovery about 40 years ago (1). The DNA sequences of some 30 different species of TS have been clarified (2) establishing it as one of the most phylogenetically conserved proteins known. X-ray crystal structures for the Lactobacillus casei (3), Escherichia coli (4, 5), and human TSs (6) have been utilized to define the mechanism by which the substrates interact with the enzyme to form product and the nature of the inhibition affected by substrate analogues, as well as to aid in the rational design of potential chemotherapeutic agents (7).

While much is known about the physical and enzymic properties of TS, less is known about how the enzyme is regulated within the cell, its structural location, and its potential interaction with other proteins. Recent studies indicate that this enzyme may be regulated at both the transcriptional (8) and translational levels of synthesis (9), while earlier studies suggested that TS forms multienzyme complexes involved in DNA synthesis both in T-even phage-infected E. coli (10, 11) and eukaryotic cells (12, 13). These findings are compounded further now by the enzyme’s apparent association with the nucleus of the cell and its state of phosphorylation, as will be described in this paper. In addition we will provide evidence that TS may be located also in the mitochondria of cells as has been described recently for a bifunctional dihydrofolate reductase-TS plant enzyme (14) and a dUTPase in HeLa cells (15).

Attempts to establish the intracellular location of TS have been inconclusive since depending on the technique and cells used, TS has been shown to be associated either with the nucleus (16, 17) or the cytoplasm (18, 19). Fluorescent antibody studies by us some 10 years ago using human TS antibody indicated that the enzyme was present mainly within the nucleus of the HeLa cell, but could not be confirmed with H35 hepatoma cells. More recently by using a similar technique with yeast the enzyme was shown to be localized to the nuclear periphery (20). To more clearly define the enzyme’s location within a eukaryote, we will demonstrate, using immunogold electron microscopy and autoradiography, the nuclear and nucleolar presence of TS in a rat hepatoma (H35) cell line.

MATERIALS AND METHODS

Immunogold Treatment of Cells—The fixation of cells and the labeling procedures used for the localization of the TS antigen after first treating the cells with a purified anti-TS rabbit antibody then with an IgG secondary antibody (goat anti-rabbit antibody conjugated with 5 nm colloidal gold, Janssen Life Sciences, Piscataway, NJ) were essentially as described in the instructions provided by Janssen and by Hechmy et al. (21). The TS antigen was purified to homogeneity (22) and 1 mg injected as an emulsion with Freund’s complete adjuvant several times along the midline region of a rabbit’s spine. After 2 weeks antibody production was boosted with another 0.5 mg of TS in saline that was injected into the rabbit’s hind legs intramuscularly and neck pad. After two more weeks 50 ml of blood was removed and allowed to clot. The TS antibody (IgG) was purified from the rabbit serum by

2 W. A. Samsonoff, G. Maley, and F. Maley, unpublished observations.

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1 The abbreviations used are: TS, thymidylate synthase; dUMP, 2'-deoxyuridine 5'-monophosphate; FdUMP, 5'-fluoro-2'-deoxyuridine 5'-monophosphate; dTMP, 2'-deoxythymidine 5'-monophosphate; FdUrd, 5-fluoro-2-deoxyuridine; CH2PteGlu, 5,10-methylene tetrahydrofolate; H2PteGlu, dihydrofolate; dUTPase, deoxyuridine triphosphatase; PAGE, polyacrylamide gel electrophoresis; DMEM, Dulbecco’s modified Eagle’s medium; RIPA, radioimmunoe precipitation buffer.

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CM-Affi-Gel blue chromatography (Bio-Rad) according to the manufacturer's instructions.

Quantitative Evaluation of Immunogold—Gold particles were counted on electron micrographs (final magnification \( \times 45,000 \)). Cell areas were determined by overlaying each micrograph with a calibrated lattice of known size, and underlying areas were determined for each cell compartment. The density of gold label is defined as the number of gold particles encountered per square micron of surface counted.

Electron Microscopy Autoradiography—H35S (FdUrd-resistant) and H35R (FdUrd-sensitive) cells (6 \( \times \) 10^6 in 12 ml of Swinn's medium containing 5% human serum 10% fetal bovine serum) were grown for 43 h in 75-mm plates in a CO2 incubator, after which 8 ml of medium was removed from each plate. Folinic acid was added to a final concentration of 100 \( \mu \)M to the remaining 4 ml, while [\( ^{32} \text{P} \)]FdUrd (22 Ci/mMol), DuPont NEN) was added to a final concentration of 0.26 \( \mu \)Ci. The plates were incubated for an additional 4 h, washed three times with media and incubated for 4 h with fresh Swinn's media. At the end of this period, the cells were fixed with 2 ml of Trump's fixative for 15 min and washed two additional times with 5 ml of fixative. The plates were then incubated overnight at 40 °C and the cells gently scrapped off the Petri dishes and centrifuged, followed by rinsing in 0.1 M sodium cacodylate buffer, pH 7.4. After a secondary fixation for 30 min at room temperature in a solution of 1% osmium tetroxide, 0.1 M cacodylate buffer, pH 7.4, the pellets were embedded in agar, dehydrated in a graded series of ethanol, and embedded in Epon-812. Sections of 0.1 \( \mu \)M were collected on carbon-coated grids, and autoradiographic emulsions were prepared essentially as described by Caro and van Tubergen (23). Grids were post-stained with uranyl acetate and Reynolds' lead citrate (24) and examined in a Zeiss 910 transmission electron microscope at 100 keV.

Mitochondrial Isolation and Ternary Complex Formation—H35S and H35R cells were grown in Swinn's medium to about 90% confluence in one to three roller bottles and harvested with 0.05% trypsin, 0.02% versene. The cells were added to an equal volume of Swinn's medium and centrifuged for 5 min at 1000 rpm followed by washing with 20 ml of phosphate-buffered saline. The centrifuged cells were resuspended in 4 ml of isolation buffer (230 mM mannitol, 70 mM sucrose, 0.5 mM EGTA, 0.5% bovine serum albumin, and 10 mM Hepes, pH 7.4). The cells were then homogenized and centrifuged in a 15-ml plastic centrifuge tube at 1000 \( \times g \) for 10 min at 4 °C using a Sorvall GLC-2B centrifuge. The supernatant fraction after transferring to another tube was recentrifuged at 20,000 \( \times g \) in a Sorvall RC-2B centrifuge for 15 min. The pellet was resuspended in 5 ml of isolation buffer and recentrifuged. Alternatively, mitochondria were purified by isopycnic centrifugation as described by Rickwood et al. (25). In either case, the final mitochondrial pellet was suspended in 1 ml of STE (100 mM NaCl, 10 mM EDTA, 50 mM Tris-HCl, pH 7.4) and centrifuged in an Eppendorf centrifuge at 14,000 rpm for 10 min at 4 °C. The pellet was resuspended in 50 \( \mu \)l of STE and sonicated for 7 s in an ethanol/ice bath and centrifuged as above. Alternatively, the mitochondria were suspended in cold water and after lysis the membranous fraction was centrifuged, as above, in an Eppendorf centrifuge. Ternary complexes containing [\( ^{32} \text{P} \)]dUMP were obtained by a slight modification of the procedure of Maley et al. (26) as follows: to 8 ml of H35R cell extract, mitochondrial supernatant fraction, or purified recombinant rat TS (27) was added 1 \( \mu \)l of 50 mM MgCl2, 3 \( \mu \)l of a 1.6 mM (R,S)-CH2H2PteGlu solution (26), 3 \( \mu \)l of 0.1 M cytidine 5'-monophosphate, and 5 \( \mu \)l of [\( ^{32} \text{P} \)]dUMP (1.1 \times 10^6 dpm). After incubating at room temperature for 30 min the reactions were stopped with an equal volume of SDS-loading buffer and boiled for 3 min. Half of the sample was subjected to 12.5% SDS-PAGE gel at 1 h at 180 volts. The gel was dried and exposed to Kodak X-omat x-ray film for 15–60 min at room temperature.

Labeling of H35S Cells with [\( ^{32} \text{P} \)] and TS Isolation—H35R rat hepatoma cells were grown in Swinn's medium as above on 60-mm tissue culture plates for 24 h prior to labeling. The cells, which were at 80% confluence at this time, were washed 3 \( \times \) with 2 ml of phosphate-free Dulbecco's modified Eagle's medium (DMEM) and then incubated with 2 ml of phosphate-free DMEM, at 37 °C in a CO2 incubator for 1 h. The media were replaced with 1.5 ml of phosphate-free DMEM containing 1% fetal bovine serum (1.5 mlplate) and 120 \( \mu \)Ci of [\( ^{32} \text{P} \)]dUMP, which was incubated in a 5% CO2 incubator for 4 h. The plates were washed three times with 5 ml of phosphate-buffered saline and the cells lysed in 0.5 ml of a modified RIPA (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 2 mM phenylmethylsulfonyl fluoride, 10 \( \mu \)g/ml aprotinin, 10 \( \mu \)g/ml leupeptin, 10 \( \mu \)g/ml leupeptin, 10 mM NaF, 0.1 mM Na3VO4) on ice with gentle rocking for 30 min. The lysates were transferred to 1.5-ml Eppendorf tubes, which were centrifuged at 14,000 rpm for 10 min at 4 °C in an Eppendorf 5415 centrifuge. The supernatant fractions were transferred to fresh Eppendorf tubes, to which were added 25 \( \mu \)l of protein A-Sepharose (10% slurry in modified RIPA), incubated for 1 h, and centrifuged for 10 min at 14,000 rpm at 4 °C. To the supernatant fractions transferred to fresh tubes was added 10 \( \mu \)l of rabbit anti-rat TS serum, and after incubation on ice for 1 h, 25 \( \mu \)l of protein A-Sepharose beads was added to each tube, which were shaken for 1 h. The beads were centrifuged and washed four times with 200 \( \mu \)l of modified RIPA, then resuspended in 20 \( \mu \)l of SDS-loading buffer containing 2-mercaptoethanol and boiled for 5 min. Each supernatant fraction (10 \( \mu \)l) was loaded onto a 12.5% SDS-PAGE gel, and after 1 h at 175 volts, the gel was stained with Coomassie Blue, followed by destaining and drying onto Whatman No. 3MM paper. The dried gel was exposed to Kodak X-Omat film for 1–3 days at ~70 °C using an enhancing screen.

In Vitro Phosphorylation of Thymidylate Synthase—H35R cells were grown for 24 h as described above. The medium was removed, and the plates were rinsed with a buffer solution containing 0.25 m sucrose, 50 mM Tris-Cl, pH 7.4, and 5 mM MgSO4. The cells from six plates were scraped off with the above buffer, but containing 2 mM phenylmethylsulfonyl fluoride, 10 \( \mu \)g/ml aprotinin, 10 \( \mu \)g/ml leupeptin, 10 mM NaF, and 0.1 mM sodium vanadate, and sonicated for 10 s in an ethanol/ice bath. Aliquots of the sonicates (0.28 ml) were added to each Eppendorf tube with 20 \( \mu \)Ci of [\( ^{32} \text{P} \)]ATP. The incubations, conducted at room temperature, were terminated at various times by the addition of 1 \( \mu \)mol of cold ATP, which so diluted the [\( ^{32} \text{P} \)]ATP to effectively end the measurable incorporation of labeled phosphate into the protein acceptors. The cellular debris was centrifuged for 10 min at 14,000 rpm in an Eppendorf centrifuge at 4 °C. The supernatant fractions were transferred to fresh Eppendorf tubes, where they were treated with 7 \( \mu \)l of rabbit anti-rat TS serum for 1 h on ice, followed by 15 \( \mu \)l of protein A-Sepharose (Pharmacia Biotech Inc.) for 1 h on ice.

RESULTS

Evidence for the Nuclear Presence of TS—Earlier studies by us using fluorescent guinea pig antibody to HeLa cell TS, as well as immunogold electron microscopy, strongly implicated the association of TS with the nucleus.2 However, attempts to repeat these studies by exposing H35S cells to rat TS fluorescent antibody were inconclusive, mainly due to the difference in membrane permeability of the two cell lines. Since we had available to us much larger quantities of rat TS (22) than HeLa TS for the preparation of antibody, we explored the use of immunogold secondary antibody (goat anti-rabbit IgG) fixation

![Image](https://example.com/image.png)
to rabbit anti-rat TS antibody following treatment of H35S cells with the latter.

Fig. 1A reveals that little or no gold particles could be detected when the cells were exposed to nonspecific IgG. In contrast, when the H35S cells were treated with purified anti-TS IgG, a major share of the immunogold particles were found to be localized to the nucleus (Fig. 1B) with most of the particles being associated with the nucleolus (Fig. 1C). The nuclear distribution of the particles could be partially blocked if the antibody was first treated with H35 TS, verifying the specificity of the reaction (Fig. 2). That the nuclear (nucleolar) association of the TS antibody was not a random event was verified by measuring a statistically significant number of immunogold particles in a large number of areas inside, as well as outside of the cell (Fig. 2). However, because of the uniqueness of these findings and the possibility that the results of Fig. 1 were a consequence of the technology employed and did not truly represent what was observed, a more direct approach was sought, that of autoradiography.

Nuclear-Nucleolar Location of TS via Autoradiography—To unambiguously confirm the immunogold results a biochemical approach was taken, that of exposing the cells to \([6-^3H]FdUrd\) and leucovorin, which in the case of the former compound will be converted to \([6-^3H]FdUMP\) once in the cell, while the latter is metabolized to 5,10-methylenetetrahydrofolatepolyglutamate. Both compounds then combine with TS to form a tight ternary complex. To demonstrate that the enzyme was not associated with the mitochondrial membranes, lysis of the mitochondria in water followed by centrifugation to remove the membranous fraction revealed TS to be present mainly in the supernatant fraction. As in the case of the dUTPase (15), TS appears to be imported, since evidence for a sequence in mitochondrial DNA, which hybridizes to the rat TS cDNA could not be detected, although it is possible that the mitochondrial TS sequence, if it does exist, is sufficiently different from the genomic DNA that it does not hybridize to mitochondrial TS DNA. However, based on the high degree of evolutionary homology of the various TSs described to date (2), this would appear to be unlikely.

Evidence That Thymidylate Synthase Is Phosphorylated—In
view of the fact that many proteins, in particularly those associated with the regulation of cell division are phosphorylated (30), we examined this possibility for TS. H35R cells were incubated with $^{32}$P$_{i}$, and the TS present within the cells was extracted with antibody specific for rat TS using protein A. As shown in the SDS-PAGE autoradiogram (Fig. 5), the TS extracted from the cells was labeled with $^{32}$P$_{i}$ (Fig. 5, lane 2), but no label was present in this region when nonspecific serum was used (Fig. 5, lane 1). In the presence of increasing amounts of okadaic acid, an inhibitor of phosphoserine/phosphothreonine phosphatases, the amount of label associated with TS appeared to increase (Fig. 5, lanes 3 and 4), while the addition of staurosporine, a protein kinase inhibitor, blocked TS labeling completely (Fig. 5, lane 5).

Phosphorylation of Thymidylate Synthase in Vitro—To verify the results in Fig. 5, we determined if the apparent phosphorylation of TS that was occurring in situ could be measured in cellular extracts. It is apparent from the results in Fig. 6 that a kinase is present in the extracts that transferred $^{32}$P from $\gamma$-$^{32}$P]ATP to a specific site on the TS present in the extracts. For the purpose of measuring potential kinase activity, we used H35R cells rather than H35S cells, since a much greater quantity of TS is present in extracts from these cells, which did not require the addition of TS as a substrate. To reduce the background and to basically terminate the transfer of $^{32}$P, a large excess of cold ATP was added at the indicated times in Fig. 6, which appeared to have the desired effect of ending measurable incorporation of radioactivity into protein. It is clear from the results in Fig. 6 that the transfer is time-dependent. Preliminary results indicate that at least one serine in the enzyme is phosphorylated.

**DISCUSSION**

Numerous proteins, particularly those associated with cell division, have been found in the past decade to be associated with the nucleus. Even before these more recent developments, TS was reported to be present in the nucleus of eukaryotic cells as part of a “replisome” complex consisting of enzymes involved mainly in providing substrates for DNA synthesis (17), although the cytoplasmic location of TS has also been reported (18, 19). We have shown in this paper by both immunogold labeling and autoradiography that not only is TS associated with the nucleus of H35S cells, it is located predominantly in the nucleus. It was hoped that cells selected for their resistance to FdUrd (H35R), as a result of a 50–100-fold increase in TS, would show an even greater nuclear content of TS. However, much to our surprise, when these cells were examined by autoradiography to locate TS by means of ternary complex formation, most of the TS was found in the cytoplasm, although some was still in the nucleus. It should be emphasized that the presence of such high concentrations of TS in H35R cells is not encountered normally, since TS, even in actively dividing cells, can be detected only by a sensitive tritium release assay (29), whereas the enzyme can be measured easily in H35R cell extracts using a spectrophotometric assay (26). This massive level of TS in the cytoplasm of H35R cells is also associated with an altered morphology in that these cells appear to be considerably larger than the H35S cells. It is not known at this time whether the morphologic differences are associated with an apparent impaired transport of TS to the nucleus. Even so, it is not obvious how TS is directed to the nucleus, since it does not possess a typical nuclear localization signal (31), although there are three sites in the protein sequence of human, mouse, and rat TS (27) with three to four basic residues within a seven- to eight-amino acid peptide sequence that could serve this purpose. Alternatively, the enzyme might be chaperoned into the nucleus by a protein similar to that described recently for the Id family of helix-loop-helix proteins (32). It is of interest to note that dUTPase, like TS, although associated with the nucleolus, does not contain a characteristic nuclear localization signal either (15, 33).

Other properties that dUTPase and TS share relate to their presence in mitochondria and their state of phosphorylation. It is possible that the mitochondrial location of TS is due to the nonspecific adherence of TS to the surface of the mitochondria, but the extensive washing procedure employed would appear to mitigate against this possibility, as well as our recent finding that the enzyme can be released on hypotonic lysis of the mitochondria. Preliminary results reveal that the H35 TS is labeled on a serine, as has been found also for dUTPase (33). Because of the common properties of TS and dUTPase, it would not be unreasonable to assume that the two enzymes reside in the same regions of the cell, possibly in the proposed replisome complex (12). As shown in Fig. 6, extracts of H35S cells contain a kinase (5) that phosphorylates TS. More recently, to test TS’s susceptibility to phosphorylation, we have found such kinases as protein kinase C and calmodulin kinase to phosphorylate recombinant rat TS quite well, and with a fair degree of
specificity, as several other proteins including E. coli TS, were not phosphorylated.3

A function for the phosphorylation of TS, if any, is not known, although based on similar studies, it has been shown that phosphorylation can affect the stability, the activity, and even the location of a protein (34). As shown recently in the case of MAD2R, its nuclear accumulation and signaling depend on it being phosphorylated (35). This does not appear to be an isolated case as more and more instances are being reported on the influence of protein phosphorylation on the cellular location of various proteins (34, 36, 37), particularly those associated with cell division. It is interesting to note that only the nuclear form of dUTPase is also phosphorylated (33). Whether a similar involvement in providing a substrate for the synthesis and repair of DNA, this could very well be its function. In any event it would appear from the data presented here that the functional role of TS may be more complex than merely serving as a provider of dTMP. A preliminary account of these findings has been presented (39).

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