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# Isolation and Characterization of a Thymidylate Synthase-Deficient Human Colon Tumor Cell Line

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**ABSTRACT.** Following mutagenesis of the human colorectal tumor cell line HCT C with ethyl methanesulfonate, clonal sublines were isolated that survived on medium toxic to cells expressing thymidylate synthase (TS). The subline exhibiting the lowest TS activity, designated as C18, was characterized. Extracts from C18 cells were mixed with extracts from parental C cells to determine whether the TS-deficient phenotype is *trans*-acting. No effect was observed on the activity of TS in parental extracts. The levels of functional TS in C18 cells were analyzed by the binding of the mechanism-based inhibitor 5-fluoro-2′-deoxyuridylate (FdUMP) under conditions that allowed for the detection of 10 fmol of TS. Only a low level of FdUMP–TS complexes was detected in C18 extracts. The level of TS expression in C18 cells was similar to that in parental C cells, as indicated by immunoblot and RNA analyses. DNA sequence analysis of TS cDNA from C18 cells revealed the existence of a point mutation (C→T) at nucleotide 647 that predicts the replacement of Ser216 by a leucine residue. That the C18 cell line was homozygous for this mutation was indicated by restriction fragment-length polymorphism analysis and by primer extension analysis. To provide additional evidence that substitution of Ser216 by a leucine residue created a defective protein, a TS-deficient bacterial strain was transformed with an expression vector containing the mutated human TS cDNA. The transformed strain exhibited thymidine auxotrophy, indicating that the mutant TS (Leu216) is nonfunctional. BIOCHEM PHARMACOL **58**;10:1529–1537, 1999. © 1999 Elsevier Science Inc.

**KEY WORDS.** thymidylate synthase; thymidine; protein deficiency; mutagenesis; point mutation; genetic complementation

The TS**-catalyzed reaction is the sole *de novo* source of dTMP, which is required for DNA biosynthesis and cell growth. Because of its role in cell growth, TS has been a target of antineoplastic chemotherapy (reviewed in Ref. 1). Of the drugs that inhibit TS, the most well-studied are the 5-fluoropyrimidines, 5-fluorouracil, and FdUrd. Fluoropyrimidine drugs are metabolized intracellularly to FdUMP, which is an analog of the TS substrate dUMP. FdUMP is a mechanism-based inhibitor of TS, which, in the presence of the TS co-substrate $CH_2H_4$PteGlu, binds to TS in an essentially irreversible manner [1]. The resulting ternary complex is devoid of catalytic activity. The cytotoxicity that is associated with the TS-directed actions of the 5-fluoropyrimidines is reversed by the addition of dThd. It is postulated that depletion of dTMP results in DNA fragmentation due to misincorporation into DNA of dUTP (and FdUTP after exposure to fluoropyrimidines) and/or to the induction of a DNA endonuclease (reviewed in Ref. 2).

Because of its TS-directed actions, FdUrd has been utilized to investigate the biological effects of dTMP deprivation and to isolate cell lines expressing TSs that confer a drug resistance phenotype [3–6]. A more direct approach for conducting studies of TS function is the use of cells that are deficient in TS. TS-deficient mammalian cells have been isolated by several investigators [7–10]. A TS-deficient human cell line has been utilized to examine the cellular events resulting from dTMP deprivation [11, 12]. Withdrawal of dThd from the growth medium of this cell line was associated with internucleosomal cleavage, which is a hallmark of apoptosis [11]. Recent studies have indicated that dTMP deprivation induces apoptosis in this cell line by activating a death receptor that plays a central role in physiological apoptosis [12]. TS-deficient hamster and murine cells have been utilized to investigate the relationship between structural alterations in human TS and its

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** *Abbreviations*: TS, thymidylate synthase; $CH_2H_4$PteGlu, 5,10- methylenetetrahydrofolate; FdUMP, 5-fluoro-2′-deoxyuridylate; FdUTP, 5-fluoro-2′-deoxyuridine 5′-triphosphate; dThd, thymidine; FdUrd, 5-fluoro-2′-deoxyuridine; EMS, ethyl methanesulfonate; HTG, hypoxanthine, thymidine, and glycine; FACT, folinic acid, aminopterin, cyanocobalamin, and thymidine; FBS, fetal bovine serum; RT, reverse transcriptase; and PCR, polymerase chain reaction.

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function as a target for TS inhibitors [13, 14]. In these studies, TS-deficient cells were transfected with expression vectors containing human TS cDNAs encoding mutant proteins. The response of cell transfectants to TS inhibitors was utilized to identify those substitutions in the protein that confer drug resistance.

Whereas several mammalian TS-deficient cell lines have been isolated, only one cell line of human origin has been reported [10]. This cell line, derived from a human colonic adenocarcinoma cell line, exhibited dThd auxotrophy, which was dependent upon the extracellular folate concentration [15]. The molecular basis for the TS deficiency in this cell line was examined by partial DNA sequence analysis. Two mutations were observed in the region of analysis. No information was provided regarding the relative contributions of the mutations to the TS-deficient phenotype.

In the present studies, the molecular basis for the dThd auxotrophy of a human cell line expressing a highly defective TS is reported. The gene encoding this enzyme contains a missense mutation that alters a residue essential for catalytic activity [16]. Based on biological characteristics, the TS-deficient human tumor cell line isolated in this study will be useful for studies of the role of TS in cell metabolism and growth and for analyses of structure–function relationships among human TSs.

## MATERIALS AND METHODS

### *Materials*

[α-$^{32}$P]dCTP (3000 Ci/mmol), [α-$^{35}$S]dATP (1000 Ci/mmol), and [γ-$^{32}$P]ATP (3000 Ci/mmol) were purchased from New England Nuclear. [6-$^{3}$H]FdUMP (18 Ci/mmol) and [5-$^{3}$H]dUMP (25 Ci/mmol) were purchased from Moravek Biochemicals. [$^{32}$P]FdUMP (620 Ci/mmol) and (6*RS*)$CH_2H_4$PteGlu were prepared as described previously [17]. RPMI 1640 medium, folate-free RPMI 1640 medium, and FBS were purchased from GIBCO. Hypoxanthine, dThd, glycine, folinic acid, aminopterin, cyanocobalamin, and EMS were purchased from the Sigma Chemical Co. Bradford protein dye-binding reagent was purchased from Bio-Rad Laboratories. Restriction endonucleases were obtained from New England Biolabs and utilized as directed by the manufacturer. Ribonuclease inhibitor (RNasin) was supplied by Promega. Oligonucleotides utilized as primers for polymerase-directed reactions are listed in Table 1. Oligonucleotides were prepared using an Applied Biosystems 380B synthesizer in the Oligonucleotide Synthesis Facility of the Institute for Biological Research and Technology, University of South Carolina.

### *Cell Culture*

The human colonic tumor cell line HCT C was obtained from M. Brattain, Medical College of Ohio [19] and was maintained as a monolayer in RPMI 1640 medium supplemented with 5% FBS. Cells were depleted of folates by growth in folate-free RPMI 1640 medium supplemented with HTG (100 μM hypoxanthine, 30 μM dThd, 30 μM glycine) and 5% charcoal-stripped FBS [17]. The TS-deficient subline of HCT C, designated C18, was maintained in FACT medium (folate-free RPMI 1640 medium supplemented with 5% charcoal-stripped FBS and $10^{-8}$ M folinic acid, 1 μM aminopterin, 0.15 μM cyanocobalamin, and 10 μM dThd). This selection medium is a modification of a previously described medium [9]. Cells were monitored for the absence of Mycoplasma by the Mycoplasma T. C. Rapid Detection System (Gene-Probe).

**TABLE 1. Oligonucleotides complementary to human TS cDNA sequences**

| Sense oligonucleotides | | Antisense oligonucleotides | |
|---|---|---|---|
| 1S | −63 to −44* | 1AS | 99 to 118 |
| 2S | −33 to −13 | 2AS | 181 to 205 |
| 3S | 88 to 107 | 3AS | 519 to 542 |
| 4S | 108 to 127 | 4AS | 636 to 657† |
| 5S | 111 to 129 | 5AS | 654 to 673 |
| 6S | 453 to 475 | 6AS | 689 to 708 |
| 7S | 457 to 477 | 7AS | 968 to 987 |
| 8S | 478 to 498 | 8AS | 979 to 998 |
| 9S | 807 to 826 | 9AS | 1040 to 1059 |

Oligonucleotides were utilized as primers for reverse transcription-polymerase chain reactions, DNA sequence analysis, primer extension analysis, and site-directed mutagenesis.

*Numbering of nucleotides is based on the sequences of Takeishi *et al.* [18].

†The oligonucleotide contains an A at position 647.

### *Isolation of TS-Deficient Cells*

HCT C cells ($2.5 \times 10^6$), grown in monolayer in maintenance medium, were exposed for 16 hr to 1.8 mM EMS. After treatment, $1 \times 10^7$ cells were plated in medium supplemented with HTG for 7 days, after which the medium was replaced with FACT medium. After 15 days in FACT medium, clones were isolated by cloning cylinders. To identify TS-deficient clones, cells were grown for 5 days in RPMI 1640 containing 5% dialyzed FBS, with or without 10 μM dThd.

### *TS Activity Determination*

TS activity in intact cells was determined by measuring the release of tritium from [5-$^{3}$H]dUMP formed intracellularly from [5-$^{3}$H]dUrd. Cells ($1 \times 10^5$) were grown for 24 hr in RPMI 1640 medium supplemented with 10 μM dThd and 5% FBS, after which the medium was replaced with medium containing 1 μM [5-$^{3}$H]dUrd (2.5 Ci/mmol). Tritium release into extracellular water was analyzed as described previously [20]. TS activity in cell extracts was determined by measuring the formation of [$^{3}$H]$H_2O$ from [5-$^{3}$H]dUMP as described previously [20]. In studies of TS activity in mixed extracts, extracts were prepared in the presence or absence of protease inhibitors (50 μg/mL of aprotinin, 0.1 mM phenylmethylsulfonyl fluoride, and 20

μg/mL of leupeptin). TS activity was determined in reactions containing 1:2 (v/v) of parental and clonal extracts, and the specific activity was based on the amount of parental extract protein in the assay. One unit of TS is defined as the amount of enzyme required to release 1 picomole of $^3H$ per minute per milligram of soluble protein.

### *Isoelectric Focusing of TS–FdUMP–$CH_2H_4$PteGlu Ternary Complexes*

TS in cell extracts was labeled by ternary complex formation by utilizing (6*RS*)$CH_2H_4$PteGlu and [$^{32}$P]FdUMP, and the resulting complexes were separated by isoelectric focusing gel electrophoresis as described previously [21].

### *Extraction and Analysis of Nucleic Acids*

DNA and RNA were isolated from cells according to standard procedures. The procedures utilized for Northern and Southern blot analyses have been described previously [21]. TS sequences were identified by hybridization with a single-stranded $^{32}$P-labeled 1.4-kb *Acc*I/*Hpa*I fragment of pKB169 (provided by F. G. Berger, University of South Carolina). pKB169 contains a full-length human TS cDNA [13].

### *Immunoreactivity Analyses*

For immunoblot analysis, cell-free extracts (100 μg of total protein per lane) were separated on 10% SDS–polyacrylamide gels by the method of Laemmli [22]. The protein was transferred electrophoretically to Immobilon-PVDF membranes (Millipore) by standard procedures. TS polypeptide was detected by successive incubations with D3B31, a murine anti-human TS monoclonal antibody (IBRT Monoclonal Antibody Facility), goat anti-murine IgG conjugated with horseradish peroxidase (Boehringer Mannheim Biochemicals), and diaminobenzidine/$H_2O_2$ by standard procedures. Immunocomplexes were detected with the ECL Western blotting kit detection system (Amersham). Recombinant human TSs expressed in a mutant strain of *Escherichia coli* were analyzed by ELISA, as described previously [16].

### *RT–PCR*

Total RNA (1 μg) was reverse transcribed utilizing Moloney murine leukemia virus reverse transcriptase (New England Biolabs) and oligonucleotide 9AS by standard procedures. Single-stranded cDNA was amplified in a Perkin Elmer Cetus model 480 thermal cycler utilizing *Taq* DNA polymerase in 30–35 cycles of denaturation (95°, 1 min), annealing (55°, 1 min), and extension (72°, 1 min) as described previously [13]. Amplification of the 5′ region of TS cDNA required modification of the PCR procedure. The reaction was conducted in 5% deionized formamide. Denaturation was carried out for 1.5 min at 94°. *Taq* DNA polymerase was added during the annealing step; annealing was carried out for 40 sec at 60° and extension for 40 sec at 72°.

### *Sequence Analysis of PCR Products*

For sequence analysis between nucleotides 108 and 1059, single-stranded DNA was obtained by asymmetric PCR utilizing the oligonucleotide primers 1S, 3S, 7S, 3AS, 6AS, and 9AS at a primer ratio of 50:1. The primers utilized for sequence analysis of asymmetric PCR products were 4S, 8S, 9S, 2AS, 5AS, and 7AS. For sequence analysis of the 5′ region, PCR products obtained with primers 1S and 3AS were cloned into pBluescript KS+ (Stratagene) by standard procedures. Primers utilized for sequence analysis of the 5′-fragment were 2S and 1AS. DNAs were sequenced by the dideoxy chain termination method utilizing Sequenase Version 2.0 modified T7 DNA polymerase (U.S. Biochemical). The sequence reactions were separated on 8 M urea–8% acrylamide gels. After drying, the gels were subjected to autoradiography.

### *Digestion with Restriction Endonucleases*

TS cDNAs were generated by RT–PCR, using as primers 5S, 6S, 8AS, and 9AS. After the removal of primers and nucleotides, the DNAs were digested with the restriction endonuclease *Mbo*I, using conditions recommended by the manufacturer. The resulting DNA fragments were separated by electrophoresis using 3% agarose gels and detected by UV light after staining with ethidium bromide.

### *Primer Extension Analysis*

TS cDNA fragments were obtained by RT–PCR utilizing the primers 8S and 6AS. For primer extension, the fragments were incubated in a reaction mixture containing 200 μM dATP, dCTP, dTTP, and ddGTP, $^{32}$P-end-labeled 5AS, and 5 U *Taq* DNA polymerase. After denaturation, the DNA was subjected to 20 cycles of extension (60°, 1 min) and denaturation (94°, 2 min). After denaturation, the DNA was separated on 7 M urea–12% acrylamide gels. Extension products were detected by autoradiography at −70°.

### *Site-Directed Mutagenesis of Human TS*

Plasmid pTS080, which contains the human TS cDNA under the transcriptional control of the T7 RNA polymerase promoter, was provided by W. S. Dallas (Glaxo-Wellcome Research Laboratories) [23]. A C to T mutation was produced at position 647 in the TS cDNA sequence by site-specific mutagenesis as described previously [16]. Oligonucleotide 4AS was utilized as the mutator primer. The TS cDNA insert of the mutated plasmid was sequenced in its entirety by dideoxy chain termination using modified T7 DNA polymerase.

### *Complementation of dThd Auxotrophy*

A TS-negative strain of *E. coli*, TX61 (provided by W. S. Dallas, Glaxo Wellcome Research Laboratories), was transformed with pTS080 containing either wild-type or mutated TS cDNA. The growth rates of transformed bacteria were determined in the presence or absence of dThd as described previously [16].

## RESULTS

To determine an appropriate concentration of mutagen to achieve maximal recovery of mutants, HCT C cells were exposed to various concentrations of EMS. Previous studies in this laboratory indicated that concentrations of EMS resulting in 40% cell survival yielded the highest mutation frequency per exposed population, as determined by loss of hypoxanthine-guanine phosphoribosyltransferase. For HCT C, treatment with EMS at a concentration of 225 μg/mL (1.8 mM) for 16 hr resulted in 40% survival (data not shown).

For the isolation of TS-deficient sublines, HCT C cells were depleted of intracellular folates prior to exposure to EMS. Intracellular folates play a critical role in the selection of TS-deficient cells. FACT medium, which is utilized to isolate mammalian cells deficient in TS, contains a reduced folate, such as 5-methyltetrahydrofolate or 5-formyltetrahydrofolate (folinic acid), a dihydrofolate reductase inhibitor, such as methotrexate or aminopterin, cyanocobalamin, and dThd [7–10]. This medium selects for cells lacking TS because it creates an environment in which intracellular tetrahydrofolate is limiting for cell growth. TS catalyzes the sole reaction in which a tetrahydrofolate derivative is converted to dihydrofolate. Regeneration of tetrahydrofolate from dihydrofolate requires the action of dihydrofolate reductase. If TS is active under conditions in which dihydrofolate reductase is inhibited, dihydrofolate accumulates and tetrahydrofolate is depleted. Cells that lack TS survive under these conditions as long as dThd is supplied.

Since HCT C cells were depleted of intracellular folates, it was necessary to establish the optimal concentration of folinic acid to utilize in FACT medium for the selection of TS-deficient cells. Cells were incubated with medium supplemented with aminopterin, dThd, cyanocobalamin, 10 μM FdUrd (to inhibit TS), and various concentrations of folinic acid. In these studies, it was observed that $10^{-8}$ M folinic acid was the minimal concentration that supported cell growth (data not shown). Growth studies also were carried out utilizing FACT medium without FdUrd to establish that these conditions were selective against cells that express TS. Folate-depleted HCT C cells were grown in either FACT medium or folate-free medium supplemented with 5% charcoal-stripped FBS and HTG. After 5 days, cells grown in FACT medium exhibited 6.8% growth relative to the growth of cells grown in HTG-supplemented medium (data not shown).

After mutagenesis and selection in FACT medium, resistant clones were isolated, and the requirement of dThd for growth was determined by cultivation in medium lacking dThd. Approximately 50% of FACT-resistant clones were dThd auxotrophs. The frequency of FACT-resistant clones after mutagenesis was $10^{-6}$ to $10^{-7}$. This is comparable to a frequency of $8.5 \times 10^{-7}$ for selection of FAT-resistant human colon tumor cells reported by Houghton *et al.* [10].

TS activity was determined in 18 dThd auxotrophs that were isolated after mutagenesis and FACT selection. TS activity was determined *in situ* in cells exposed to [5-$^3$H]dUrd. The dUrd is converted intracellularly to [5-$^3$H]dUMP, and the amount of [$^3$H]$H_20$ released into the medium is a measure of TS activity. Greater than 90% of dThd auxotrophs exhibited less than 8% TS activity, relative to parental activity. The subline, designated as C18, exhibited the lowest *in situ* TS activity. TS activities, determined in cell extracts of C and C18, were 97.0 and 0.96 units, respectively. In one approach to determining whether the TS deficiency observed in C18 cell extracts was due to mutations that were *trans*-acting, extracts from parental C cells were mixed with extracts from C18 cells. The TS activity of the combined extracts was 91.3 units. The specific activities were based on the amount of parental extract protein. The omission of protease inhibitors from the extracts did not alter the data.

As an independent method of assessing TS deficiency, the formation of a ternary complex composed of FdUMP, $CH_2H_4$PteGlu, and TS in extracts of C18 cells was examined. FdUMP is a mechanism-based inhibitor of TS that is an analog of dUMP. FdUMP binds tightly to TS in the presence of the folate co-substrate, $CH_2H_4$PteGlu, forming a covalent complex that is irreversible upon denaturation of the enzyme [1]. If [$^{32}$P]FdUMP is utilized in the assay, as little as 10 fmol TS/mg protein can be detected. To determine if C18 contained TS polypeptide capable of forming ternary complexes, cell extracts were incubated with $^{32}$P-labeled FdUMP and $CH_2H_4$PteGlu, and the resulting complexes were denatured and separated by isoelectric focusing gels. Even after extended exposure, ternary complex formation was minimal in extracts from C18 (Fig. 1).

To determine if TS protein was present in C18, Western blot analysis utilizing a murine anti-human TS monoclonal antibody was carried out. A cross-reacting protein was observed in C18 extracts that is similar in size (36 kDa) and abundance to TS protein in parental cells (Fig. 2A). Northern analysis was carried out on total RNA isolated from parental C cells and C18 cells (Fig. 2B). TS-specific RNA sequences were similar in size (~1.6 kb) and abundance in the HCT C and C18 cells.

Since EMS causes base pair changes, which could result in the loss of restriction endonuclease digestion sites, Southern analysis was performed to determine whether such alterations could be detected in the TS gene of C18. Genomic DNA was digested with *Taq*I restriction endonuclease, which cleaves DNA at a 4-bp recognition site,

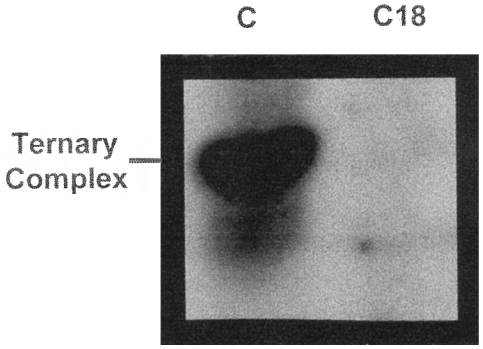


**FIG. 1. Ternary complex formation in extracts from HCT C and C18 cell lines. Cell extracts (28 μg of soluble protein; 85 pmol of TS in HCT C) were incubated with 340 nM [$^{32}$P]FdUMP and 94 μM (6*RS*)$CH_2H_4$PteGlu as described in Materials and Methods. The resulting ternary complexes were denatured and separated by isoelectric focusing gel electrophoresis in 9 M urea–4% acrylamide gels containing 0.67% (v/v) each of ampholines pH 4–6, pH 6–8, and pH 3.5–10. The labeled complexes were detected by autoradiography.**

T/CGA. This site is highly prone to mutation in mammalian cells [24]. No difference was observed in the restriction fragment patterns between DNA isolated from parental and mutant cells (data not shown). This indicated that point mutations did not occur at *Taq*I recognition sequences.

DNA sequence analysis was carried out by utilizing

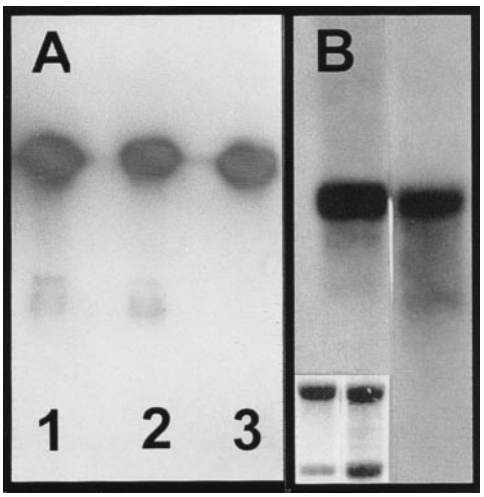


**FIG. 2. Analysis of TS expression in HCT C and C18 cell lines. (A) Western blot analysis of TS expression. Cell protein (100 μg) was denatured, separated on 10% SDS–polyacrylamide gels, and transferred to Immobilon-PVDF membranes as described in Materials and Methods. TS polypeptide was detected as described in Materials and Methods. The levels of cross-reacting protein are shown in HCT C (lane 2) and C18 (lane 3). The mobility of purified recombinant human TS is shown in lane 1. (B) Northern blot analysis of TS expression. Total RNA (20 μg) was fractionated on 1.5% formaldehyde–agarose gels and transferred to nylon membranes. RNA loading was analyzed by methylene blue staining of the membranes (inset). The RNA was hybridized to a $^{32}$P-labeled single-stranded human TS cDNA probe as described in Materials and Methods. TS-specific sequences in HCT C (left lane) and C18 (right lane) were detected by autoradiography.**

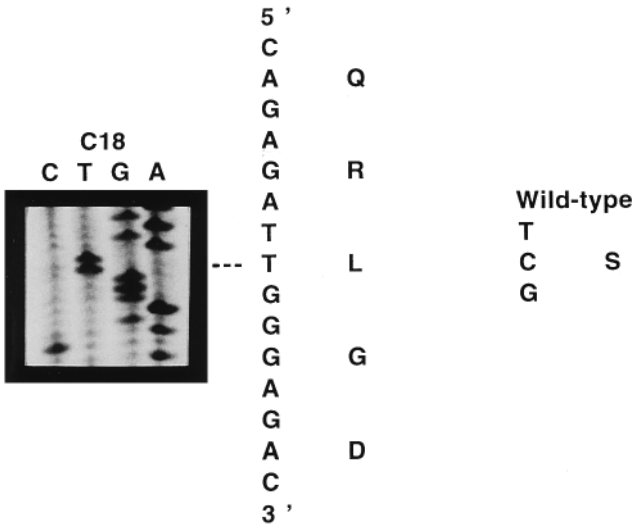


**FIG. 3. Region of mutation in TS cDNA from C18. TS cDNA was prepared by RT–PCR utilizing RNA isolated from C18 as described in Materials and Methods. Single-stranded cDNA was prepared by asymmetric PCR and sequenced by the dideoxy chain termination method as described in Materials and Methods. The region of an autoradiogram of a sequencing gel in which a mutation occurs in TS cDNA from C18 is shown. The site of the point mutation is designated by the dotted line.**

cDNA generated with RNA isolated from C18 cells. The sequence from nucleotide 108 to the 3′ end of the coding region was determined by utilizing single-stranded cDNA generated by RT–PCR and asymmetric PCR. The first 170 bp of the TS cDNA are high in G-C content, in some regions as high as 88%. Furthermore, three tandem repeat sequences are present that are predicted to form interconvertible stem–loop structures [18]. For this reason, sequence analysis is difficult in the 5′-region. To overcome the problem, the 5′ portion of the cDNA was cloned, and the sequence of the TS insert was determined by analysis of four clones. Base pair changes were observed at nucleotides 647 (C→T) and 961 (T→C). The mutation at position 961 lies 19 bp 3′ of the stop codon. The region in the proximity of the mutation at position 647 is shown in Fig. 3. There was no evidence for heterozygosity at this position in the sequence. This change predicts the substitution of Ser216 by a leucine residue in the TS polypeptide.

To provide additional evidence for the presence of a mutation at nucleotide 647 in TS cDNA from C18 cells, restriction fragment-length polymorphism analysis was conducted utilizing cDNA fragments extending from nucleotides 111 to 998 and 453 to 1059. *Mbo*I digestion sites occur at positions 281, 338, 556, and 644 in the wild-type TS cDNA. Mutation at nucleotide 647 predicts the loss of the restriction enzyme cleavage site at nucleotide 644, resulting in the loss of an 88-bp digestion fragment. The *Mbo*I digestion pattern of TS cDNAs generated from RNA isolated from parental C cells and C18 cells is shown in Fig. 4. Digestion of the 5S/8AS PCR product from parental C cells resulted in fragments with mobilities corresponding to the expected lengths of 354, 218, 170, and 88 bp. In digests

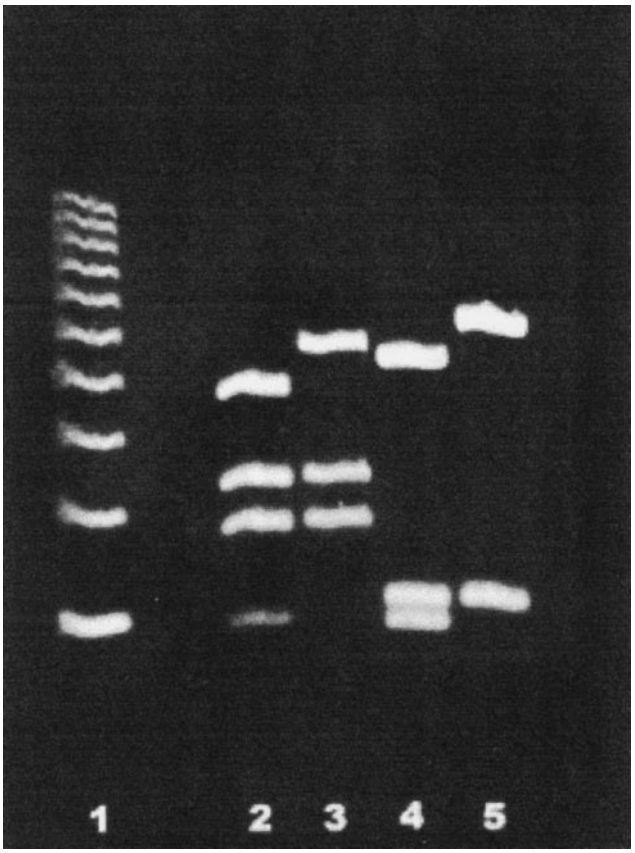


**FIG. 4. MboI digestion pattern of TS cDNA fragments. TS cDNA fragments were prepared as described in Materials and Methods. The fragments were digested with *Mbo*I, and the resulting fragments were separated on 3% agarose gels. The restriction fragments were stained with ethidium bromide and visualized by exposure to UV light. The digestion patterns of 888-bp (lanes 2 and 3) and 607-bp (lanes 4 and 5) fragments from HCT C (lanes 2 and 4) and C18 (lanes 3 and 5) are shown. A 100-bp DNA ladder was utilized as a DNA size marker (lane 1).**

of the corresponding PCR product from C18 cells, a novel fragment was observed of approximately 440 bp, a length that is consistent with the loss of an *Mbo*I digestion site at nucleotide 644. Similar results were obtained after digestion of the 6S/9AS PCR products from HCT C and C18 (Fig. 4). The data supported the results of DNA sequence analysis and provided additional evidence that the C18 cell line is homozygous for the mutant allele.

To address the issue of the homozygosity of the C18 mutation more directly, primer extension analysis of cDNA generated from parental C cells or C18 cells was carried out. The analysis was based on differential extension of a primer in the presence of ddGTP, utilizing as templates DNA fragments containing either a C or a T at nucleotide 647. TS cDNA containing the C18 mutation (T at nucleotide 647) was expected to produce a longer (34-bp) extension product than DNA containing a C at nucleotide 647 (27-bp extension product). RNA was isolated either from parental C cells or from C18 cells maintained in either FACT or folate-free medium supplemented with HTG. A cDNA fragment extending from nucleotides 478 to 708 was hybridized to oligonucleotide 5AS, and the primer was extended in the presence of dATP, dCTP, dTTP, and ddGTP. TS cDNA prepared from C18 maintained in either medium resulted in the formation of a 34-bp product, whereas TS cDNA prepared from parental C cells resulted in the expected 27-bp product (Fig. 5). This result indicated that the *ts* locus in C18 is variant and is homozygous for the variation. In addition, the experiment suggested that the genotype was unaltered, regardless of growth under conditions that were selective (FACT medium) or nonselective (HTG medium) for dThd auxotrophy.

The relationship between the presence of a C→T mutation at 647 and the loss of TS function was investigated by site-directed mutagenesis. A C→T mutation was created at nucleotide 647 of a human TS cDNA insert in a bacterial expression vector. A TS-deficient bacterial strain was transformed with vectors containing either a C or a T at position 647 of the TS cDNA. The growth of the transformants in the presence or absence of dThd was determined (Fig. 6). The transformants exhibited similar rates of growth in the presence of dThd. Transformants containing TS cDNA with a C at position 647 grew in the absence of dThd. Bacteria transformed either with vector DNA alone or with vector DNA containing a human TS insert with a T at position 647 exhibited no growth in the absence of dThd. The lack of growth of bacteria transformed with TS cDNA containing a T at position 647 was not due to lack of expression or pronounced instability of the mutant polypeptide. This was demonstrated by ELISA

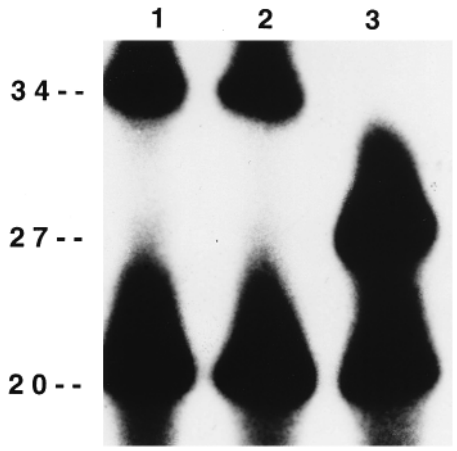


**FIG. 5. Primer extension analysis of TS cDNA fragments. TS cDNA fragments were prepared as described in Materials and Methods. The fragments were utilized as templates for extension of a $^{32}$P-end-labeled primer in the presence of dATP, dCTP, dTTP, and ddGTP as described in Materials and Methods. The extension products were separated by electrophoresis on 7 M urea–12% acrylamide gels. The labeled bands were detected by autoradiography. Shown in the figure are the extension products obtained from TS cDNA from C18 maintained in HGT medium (lane 1), C18 maintained in FACT medium (lane 2), and HCT C (lane 3).**

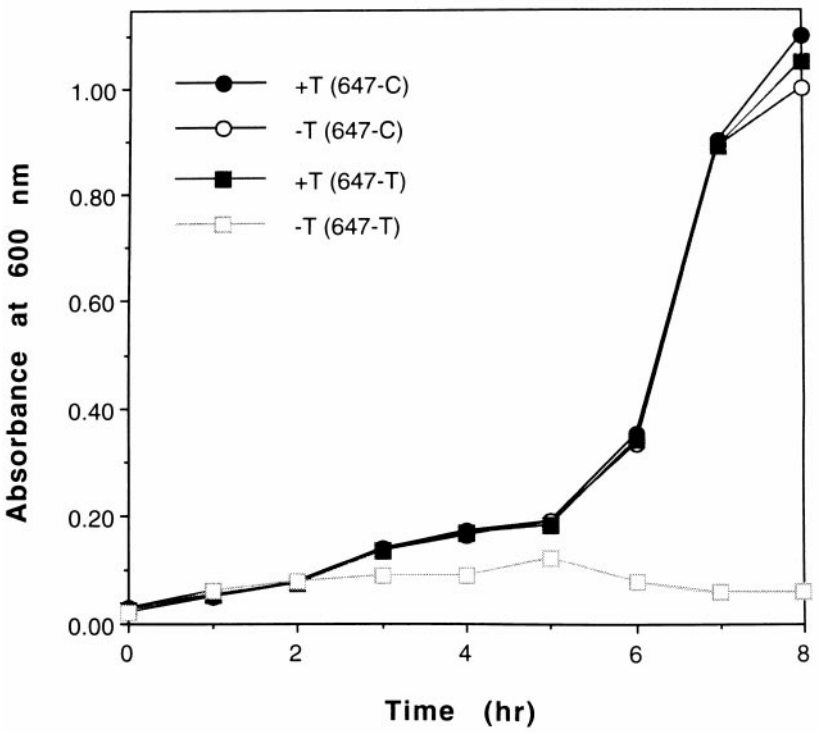


**FIG. 6. Growth of TS-deficient bacteria transformed with vectors containing wild-type or mutated human TS cDNA. A TS-deficient strain of *E. coli*, TX61, was transformed with the expression vector, pTS080, containing either a C (wild-type) or a T (mutated) at nucleotide 647 in the human TS cDNA insert. The transformed bacteria were grown in the presence (+T) or absence (−T) of dThd, as described in Materials and Methods. The growth of the transformants was determined by measuring the absorbance at 600 nm.**

using a human TS monoclonal antibody to detect cross-reacting protein. Similar levels of cross-reacting protein were detected in extracts of transformants expressing either the wild-type (Ser216) or the mutant (Leu216) TS; no cross-reacting protein was detected in extracts of untransformed bacteria (data not shown).

## DISCUSSION

A TS-deficient human colon tumor cell line, designated as C18, has been isolated that expresses TS mRNA and TS cross-reacting protein at levels similar to those in parental cells. The TS-deficient cell line is homozygous for a C→T mutation in TS cDNA, which predicts the substitution of serine by leucine at position 216 of the TS polypeptide. TS-deficient bacteria expressing a human TS polypeptide with leucine at position 216 are dThd auxotrophs, even though the protein is expressed at a level of 5–6% of the total soluble protein. Recent studies of mutant proteins with substitutions at position 216 of human TS revealed that the presence of a hydroxyl group at position 216 is essential for catalysis by human TS [16]. The mutant protein with a leucine residue at position 216 exhibited $K_m$ values for the substrates dUMP and $CH_2H_4$PteGlu that are increased by 82- and 56-fold, respectively, relative to the wild-type enzyme [16]. The $k_{cat}$ for the mutant enzyme is 78-fold lower than that of wild-type human TS [16]. The steady-state kinetic data are consistent with the observation that the activity of the defective enzyme expressed in C18 extracts is ~1% of the activity of the enzyme expressed in parental C cells. In studies of the binding of [$^{32}$P]FdUMP and $CH_2H_4$PteGlu to the enzyme in C18 extracts, only a faint band corresponding to the inhibitory ternary complex was observed. In recent studies, the rate of inhibitory ternary complex formation was observed to be approximately $10^5$ slower for a mutant protein with leucine at position 216 than for the wild-type protein (data not shown). The data are consistent with the hypothesis that the basis for the dThd auxotrophy of C18 is the expression of an enzyme in which Ser216 is replaced by a leucine residue.

Though TS-deficient cell lines have been isolated previously, the molecular basis for the TS deficiency had not been established [7–10]. A TS-deficient human tumor cell line has been isolated that exhibits dThd prototrophy in the presence of high exogenous folate concentrations [15]. Sequence analysis of TS cDNA isolated from the cell line revealed the presence of two mutations: Asp218→Asn and His256→Tyr. The investigators presented a logical argument to support their hypothesis that the cell line is heterozygous for two mutated alleles; however, no evidence was presented that establishes that these mutations create loss of TS function. This is of particular concern since the TS cDNA isolated from the TS-deficient cell line was not sequenced in its entirety.

In the present investigation, three independent approaches indicated that the C18 subline is homozygous for the C→T transition at nucleotide 647. The basis for the apparent homozygosity of the mutation at nucleotide 647 is unknown. In parental C cells, the modal number of chromosomes has been reported to be 46 [19]. Whereas loss of either chromosome 18 or a region of the p arm, which is

the site of the *ts* locus, could have occurred subsequent to exposure to EMS, no decrease in gene dosage was detected by analysis of genomic DNA isolated from C18. Parental C cells are reported to exhibit microsatellite instability, and studies of other colon tumor cell lines with this phenotype have revealed that genetic defects in mismatch repair frequently underlie the microsatellite instability [25–27]. Genetic defects in mismatch repair are associated with a higher rate of endogenous mutation, suggested to be increased by 2–3 orders of magnitude [28, 29]. Chemical mutagens such as EMS frequently alkylate N-7 of guanine in DNA, resulting in a G:C→A:T transition, which is consistent with the base change observed at 647 in TS cDNA from C18 [30]. The combined effect of a higher intrinsic mutation rate and the use of a potent chemical mutagen is expected to increase significantly the probability of isolating cell mutants. Interestingly, the frequency of isolation of TS-deficient cells from HCT C was not significantly higher than that reported previously for isolation of human TS-deficient cells [10]. Perhaps the mechanism that underlies the apparent homozygosity of C18 is an increase in the frequency of homologous recombination. In studies of human somatic cell lines, evidence has been obtained for the production of homozygous from heterozygous loci by homologous recombination [31]. Interestingly, defects in mismatch repair are associated with an increase in the frequency of homologous recombination [32].

The availability of a human cell line deficient in TS is of importance for several areas of research. A current area of interest is the relationship between chemotherapy-induced cell damage and the induction of apoptosis in tumor cells. TS is a direct or indirect target of several classes of antimetabolite drugs; thus, elucidation of the downstream effects of dTMP deprivation may provide insight into the pathways by which drug-mediated cell damage and apoptosis intersect. Relative to TS inhibitors, TS-deficient cell lines offer the advantage that the effects of dTMP deprivation can be examined without the concern that secondary actions unrelated to TS inhibition are occurring. Seminal studies of dTMP deprivation and apoptosis have been conducted in a TS-deficient human colon tumor cell line [11, 12]. These studies have indicated that dTMP deprivation induces apoptosis by autocrine signaling via Fas–FasL (CD95–CD95L) interactions. The Fas pathway is involved in activation of physiologic apoptosis in the immune system. The binding of FasL to Fas at the cell surface results in protein–protein interactions that eventually activate downstream caspases, committing a cell to apoptosis [33]. It will be important to determine whether this mechanism is a general phenomenon in colon tumor cells by utilizing cell models such as C18.

TS-deficient human cells will also be useful for studies of TS structure–function relationships among human TSs. Such studies have been essential for the identification of residues and structural regions that play a role in the function of TSs from prokaryotes [34–36]. Since the human enzyme is a target for chemotherapy aimed at cancers of the breast, ovary, head and neck, and gastrointestinal tract, there is considerable interest in studies of structure–function relationships among human TSs. Within the last year, several investigations have been published that focus on mutant TSs of human origin [14, 16, 37, 38]. This interest is derived, in part, from gene therapy approaches that are aimed at the protection of normal tissues from the cytotoxic effects of high-dose chemotherapy via heterologous expression of mutant TSs. Such proteins confer resistance to TS-directed inhibitors but retain sufficient catalytic activity for normal cell growth. Studies of the effect of a structural change(s) in human TS on the cytotoxicity of TS-directed inhibitors would be facilitated by the availability of a functional assay of human origin. Although rodent cell lines deficient in TS activity have been utilized to examine structure–function relationships among human TSs, differences exist between rodent and human cells in the levels of folate interconverting enzymes and in folate pools [39]. As TS function is intricately linked to folate pools, a human TS-deficient cell line is preferable for studies utilizing human TSs.

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