Thymidylate synthase plays an essential role in the synthesis of DNA. Recently, several new and specific thymidylate synthase inhibitors that occupy the folate binding site, including Tomudex®, BW1843U89, and Thymitaq, have demonstrated therapeutic activity in patients with advanced cancer. In order to find drug-resistant forms of human thymidylate synthase for gene therapy applications, human sarcoma HT1080 cells were exposed to ethyl methanesulfonate and Thymitaq selection. Thymitaq-resistant clonal derived sublines were established, and analysis indicated that both gene amplification and point mutations contributed to drug resistance. Eight mutant cDNAs that were identified from Thymitaq-resistant sublines were generated by site-directed mutagenesis and transfected into thymidylate synthase-negative cells. Only K47E, D49G, or G52S mutants retain enzyme activity. Moreover, cytotoxicity studies demonstrated that D49G and G52S transfected cells, besides displaying resistance to Thymitaq with IC50 values 40- and 12-fold greater than wild-type enzyme transfected cells, respectively, also lead to fluorodeoxyuridine resistance (26- and 97-fold in IC50 values, respectively) but not to Tomudex or BW1843U89. Characterization of the purified altered enzymes obtained from expression in Escherichia coli is consistent with the cell growth inhibition results. We postulate that the D49G or G52S mutation leads to the structural perturbation of the highly conserved Arg 106 loop, decreasing the binding of thymidylate synthase to the inhibitors, Thymitaq and fluorodeoxyuridine.

Thymidylate synthase (TS, EC 2.1.1.45) catalyzes the de novo biosynthesis of thymidylate, which is necessary for DNA synthesis and repair (1). The mechanism of TS activity involves the reductive methylation of the substrate, 2'-deoxyuridine 5'-monophosphate (dUMP) by transfer of a methylene group from the cofactor, 5,10-methylene-5,6,7,8-tetrahydrofolate (CH2H4folate), to generate 2'-deoxythymidine 5'-monophosphate (dTMP) and 7,8-dihydrofolate. Human TS has been sequenced (2), purified (3,4), and crystallized (5). As an attractive target for anti-cancer drug design, since the 1950s, many TS analogues of both the substrate, dUMP, and the cofactor, CH2H4folate, have been synthesized and tested as potential anti-cancer therapeutics. Until recently, 5-fluorouracil and fluorodeoxyuridine (FdUrd) were the sole TS-targeted drugs approved for clinical application. In vivo, 5-fluorouracil and FdUrd are metabolized to 5-fluoro-2-deoxyuridylate (FdUMP), a compound that subsequently occupies the pyrimidine binding site forming a ternary complex with TS and the folate cofactor, resulting in inhibition of enzyme function. The recent determination of the three-dimensional structure of human TS has allowed the design of highly specific inhibitors, leading to the emergence of novel folate analogues, such as Tomudex (ZD1694), BW1843U89, and Thymitaq (AG337) (Fig. 1) (6). These promising compounds have entered clinical trials in recent years (7).

Previous studies have attempted to correlate enzyme structure and function using mutagenesis. To date, a large number of mutations have been made in Lactobacillus casei and Escherichia coli TS (1, 8, 9). In most reports, the procedures included the following two steps: generated mutants were first screened for their enzyme activity by genetic complementation in a TS-deficient E. coli host in the absence of thymine, and kinetic characterizations in vitro were subsequently performed for functional mutants. A few mutants of human TS and their expressed enzymes in mammalian cells have been studied (10–14). The Y33H human TS mutant, the only mutation in TS reported to be related to TS-directed drug resistance, was discovered in a human colon tumor cell line and conferred approximately a 3–4-fold resistance to FdUrd (10–12). This mutation was reported to affect the catalytic properties of human TS enzyme, showing an 8-fold decrease in Kcat but without significant change in the Km values for both dUMP and CH2H4folate between the mutant and wild-type TS.

As point mutations in human TS leading to the generation of antifolate-resistant genes have not yet been reported, an important goal of this work was to obtain human TS mutants conferring resistance to novel antifolates with minimal changes.
in the catalytic activity of the altered enzymes. Such mutants would be of much interest both for understanding structure-function for human TS and for their potential applications in gene therapy by protecting hematopoietic progenitors from TS inhibitor toxicity.

In the present study, we generated TS mutants from human sarcoma HT1080 cells following ethyl methanesulfonate (EMS) exposure and Thymitaq selection. Clonal sublines containing these mutations were identified by DNA-SSCP and sequencing. These mutant cDNAs were cloned into mammalian expression vectors and transfected into mouse TS-negative cells for cytotoxicity assays. The relevant enzymes were expressed in E. coli purified and characterized by kinetic studies in vitro.

**EXPERIMENTAL PROCEDURES**

**Materials—**Ethyl methanesulfonate (EMS) was supplied by Sigma. The Transformer™ site-directed mutagenesis kit was purchased from CLONTECH Laboratories, Inc. (Palo Alto, CA). The bacterial expression plasmid pET-17b and competent E. coli BL21(DE3) cells were from Novagen, Inc. (Madison, WI). The mammalian expression vector pcDNA3 was from Invitrogen (San Diego, CA). DEAE-cellulose (DE52) was from Whatman, and phenyl-Sepharose CL-4B was from Amersham Pharmacia Biotech. Oligonucleotide primers were synthesized by either IDT, Inc. (Coralville, IA), or Operon Technologies, Inc. (Alameda, CA). Human recombinant TS cDNA modified to enhance expression (15) was kindly provided by Dr. Frank Maley (New York Health Department, Albany, NY). Thymitaq (AG337) was a generous gift of Agouron Pharmaceuticals, Inc. (Sacramento, CA). Mudex (ZD1694) was a kind gift from Zeneca (Macclesfield, UK). Alamar Blue was from Alamar Biosciences, Inc. (Sacramento, CA). Thymitaq without EMS exposure (16, 17).

**DNA Sequence Analysis for Mutant Detection—**The reverse transcriptase-PCR fragments exhibiting abnormal mobility on SSLCP gels were subsequently subcloned into pCR-Script™SK (+) vector, which permits the efficient cloning of PCR fragments with a high yield and sequencing by two commercially designed primers (M3 (−20) and M13 reverse). Sequence analysis was performed by the dyeoxy chain termination method with α-35S-dATP using modified T7 DNA polymerase according to the manufacturer’s instructions (Amersham Pharmacia Biotech, Uppsala, Sweden).

**Cell Lines and Culture Conditions—**Human fibrosarcoma HT1080 cells were obtained from the American Type Culture Collection (Rockville, MD). Stock cultures of the parental cell line HT1080 and resistant sublines were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM l-glutamine, 100 µg/ml streptomycin, and 100 units/ml penicillin. The TS-negative cell line FSthy21, a kind gift of Dr. T. Seno (9), was originally established from mouse FM3A cells. FSthy21 cells were grown in Eagle’s minimum essential medium supplemented with 10% dialyzed fetal bovine serum, 1 µM 5,10-CH2tetrahydrofolate, and 10 µM thymidine. Prototrophic transformant clones, derived from FSthy21 cells by transfection of wild-type or mutant human TS cDNAs, were cultured in the same medium as mentioned above without thymidine and reduced folate supplementation.

**Chemical Mutagenesis and Drug Selection—**In order to determine the optimum concentration of EMS and Thymitaq for random mutagenesis experiments, we first carried out the cytotoxicity assay of human fibrosarcoma HT1080 cells with EMS or Thymitaq. IC50 values and the minimal concentration of EMS or Thymitaq that resulted in no colony formation in HT1080 cells were obtained by plots of colonies surviving versus various EMS or Thymitaq concentrations.

HT1080 cells (50 ml, total 4 × 106 cells) growing in logrhythm phase were exposed to EMS (400 µg/ml) for 18 h. The cells were washed and then incubated for an additional 3 days in EMS-free medium to allow phenotypic expression. EMS-treated cells were subcultured at 6 × 107 cells/100-mm dishes in 15 ml of medium and then grown in the presence of 40 µg Thymitaq for 14 days. Surviving clones obtained from EMS exposure and Thymitaq selection were isolated with a ring cylinder and expanded into stable resistant sublines. Control cells were treated with Thymitaq without EMS exposure (16, 17).

**Single-stranded Conformation Polymorphism (SSCP) Analysis—**RNA from the HT1080 cells and resistant sublines was isolated, and first-strand cDNAs were synthesized by reverse transcriptase-PCR. For DNA-SSCP analysis, TS fragments (150–260 bp) were obtained by PCR amplification using the cDNAs as the template and 6 pairs of TS-specific primers (the sequences of oligonucleotide primers used in various experiments such as PCR amplification, DNA-SSCP, and sequence analysis are described in Table 1). The reaction mixture containing 1 µCi of [α-32P]dCTP and a small volume (3 µl) of final PCR products was subsequently mixed with 10 µl of loading buffer containing 36% formamide. Samples were denatured at 94 °C for 3 min and chilled on ice for at least 5 min, and 2 µl was loaded onto a 6–8% non-denaturing polyacrylamide gel with 10% glycerol. Gels were electrophoresed at 10 watts for 6–8 h at 4 °C, using 0.5× Tris borate/EDTA buffer. The separated single strand DNA fragments were visualized by autoradiography.

An alternative method (nonisotopic SSCP) was also utilized in which the single strand DNA bands are detected by ethidium bromide staining instead of autoradiography. At least 40 ng (20 µl) of amplified DNA was denatured by addition of 1 µg of 0.5 M NaOH, 10 mM EDTA at 42 °C for 5 min. Before loading, 1 µl of formamide containing 0.5% bromphenol blue and 0.5% xylene cyanol were added. Non-denaturing gels (1.5 mm thick, 6–8% polyacrylamide) with 5% glycerol were made in a standard vertical gel apparatus. Gels, using 0.5× TBE as running buffer, were electrophoresed at 15 V/cm for 4 h, and the temperature was maintained at 4 °C by circulating cold water. Finally, SSCP gels were neutralized and stained in 0.5× TBE containing 0.5 µg/ml ethidium bromide for visualization of bands.

**DNA Sequence Analysis for Mutant Detection—**The reverse transcriptase-PCR fragments exhibiting abnormal mobility on SSLCP gels were subsequently subcloned into pCR-Script™SK (+) vector, which permits the efficient cloning of PCR fragments with a high yield and sequencing by two commercially designed primers (M3 (−20) and M13 reverse). Sequence analysis was performed by the dyeoxy chain termination method with α-32S-dATP using modified T7 DNA polymerase according to the manufacturer’s instructions (Amersham Pharmacia Biotech, Uppsala, Sweden).

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(6.4 kilobase pairs) and orientation was confirmed by restriction mapping and sequencing. The Transformer™ site-directed mutagenesis kit was used to obtain point mutations in human TS. The mutagenic and selective primers used for site-directed mutagenesis experiments are described in Table II. A selection primer that contains a unique NdeI restriction site, and the 3'KspI restriction site on the pcDNA3 vector is shown last, and the other 8 primers were designed to generate mutants of the human TS gene at the targeted site with 1 to 3 nucleotide changes. Mutant human TS cDNA was obtained by annealing one mutagenic primer and one selection primer to the single strand pcDNA3-hTS plasmid. The experimental procedures followed the manufacturer’s instructions. The resulting colonies were initially screened using NdeI digestion that only cuts newly synthesized plasmids. Plasmids with correct point mutations in human TS were examined further by restriction mapping and DNA sequencing. Transfection of Mouse TS-negative Cells with Wild-type and Mutant Human TS cDNAs—The mouse TS-negative F9hy21 cells were used as host for DNA transfections that were performed using a DOTAP™ transfection kit (Boehringer Mannheim). Three days after transfection, cells were placed in selective media, which lacked thymidine and reduced folate. Surviving cells having the ability to grow in the absence of thymidine were cloned in soft agarose. Growth Inhibition Assay for Transfected Cells—Cloned logarithmically growing suspension mouse TS-negative F9hy21 cells transfected with either wild-type or variant human TS cDNA were seeded in 96-well plates at 2,000 cells/well in 180 μl of complete medium. Two hours later, drug (Tomudex, Thymitaq, BW1843U89, or FdUrd) was added, and the cells were grown in drug-containing medium for an additional 7 days. Cell viability was measured by the Alamar Blue™ assay. To the above 96-well cultured cells, 25 μl of Alamar Blue (10% of incubation volume) was added according to the manufacturer’s instructions. The 96-well plates were then incubated at 37 °C for 4 h. Viability of cells increases in chemical reduction of the media which results in a change in color from blue to red. The intensity of red color (and fluorescence) is proportional to the number of viable cells. After incubation, fluorescence was read at 530–560 nm excitation wavelength and 590 nm emission wavelength by an automated plate reader (model EL340; Bio-Tek). Drug concentrations needed to reduce cell growth by 50% (IC50 values) were determined graphically by plotting cell growth versus inhibitor concentrations.

**TS Protein Expression Vector Construction and Purification—**Human TS variants (G528 and D49G) selected for enzyme kinetic characterization were recloned into the protein expression vector pET-17b. DNA fragments carrying the entire mutant human TS cDNA were amplified using the corresponding pcDNA3-STS vector as the template and two primers designed to contain appropriate restriction site for cloning. The 5’-primer (5’ GGAATTCGACAGCATATGCTTGTTGC 3’) contains a created NdeI restriction site, and the 3’-primer (5’ CTAGATGCATGCTGACGCCGCCC 3’) has an XhoI site. After PCR amplification, the reaction mixture was digested with NdeI and XhoI enzymes, and the restricted DNA fragment (950 bp) was inserted into the corresponding site of pET-17b vector. The correct construction of mutant pET-17b(hTS) was verified by restriction mapping and sequence analysis.

The protein expression pET-17b(hTS) plasmid (wild-type or mutant TS) was used to transform E. coli strain BL21(DE3). Bacterial cells were grown at 30 °C in 1 liter of tryptone phosphate medium (2% bacto-tryptone, 1.5% yeast extract, 0.2% sodium phosphate (dibasic), 0.1% potassium phosphate (monobasic), 0.8% sodium chloride, and 0.2% glucose) supplemented with 100 μg/ml ampicillin (18). The wild-type and the two mutant TSs were induced with 1 mM isopropyl-β-D-thiogalactose for 5 h at which point the enzyme was induced to about 10–15% of the soluble protein. The purification procedures were basically carried out as described by Ciesla et al. (19) for rat TS purification, which included streptomycin treatment of crude extract, followed by ammonium sulfate precipitation, ion-exchange cellulose DE-52, and phenyl-Sepharose CL-4B chromatography. A minor modification was made by eluting TS protein from a phenyl-Sepharose CL-4B column with a decreasing linear gradient of ammonium sulfate from 0.8 m to 0 instead of 0.8 to 0.4 m. Finally, TS fractions were precipitated with solid ammonium sulfate and stored at −80 °C until use. TS purity was demonstrated by 12% SDS-PAGE.

**TS Characterization—**TS activity was monitored spectrophotometrically at 340 nm as described previously (20, 21). The assay mixture contained 50 mM Tris-HCl, pH 7.4, 25 mM MgCl2, 6.5 mM formaldehyde, 1 mM EDTA, 75 mM β-mercaptoethanol, and 100 μM dUMP. CH2H4folate was present at a concentration of 600 μM, and dUMP was varied between 1 and 300 μM. Steady-state kinetic parameters were determined from initial velocity measurements, which were obtained by measuring the change in A340 with a Shimadzu UV-2101PC spectrophotometer. For determination of Km of CH2H4folate, the concentration of dUMP was fixed at 500 μM, whereas CH2H4folate concentrations were varied between 5 and 600 μM. For determination of Km of dUMP, CH2H4folate was present at a concentration of 600 μM, and dUMP was varied between 1 and 300 μM. Steady-state kinetic parameters were subsequently obtained by a nonlinear least squares fit of the data to the Michaelis-Menten equation using a computer program. hKm (n5) values were obtained by dividing Vmax (μmol/min/mg protein) by the estimated concentration of enzyme (μmol) used in the reaction.

Inhibition constants (Ki) were determined from steady-state inhibition reaction rates for mixtures of enzyme, dUMP, CH2H4folate, and inhibitor. A high fixed CH2H4folate concentration (600–800 μM) and variable antifolate concentrations were used to measure the inhibition produced by Tomudex, Thymitaq, and BW1843U89. A constant dUMP concentration (500 μM) was used, and FiDUMP concentrations were varied to measure inhibition by FiDUMP.

**RESULTS**

**Random Mutagenesis—**Based on EMS and Thymitaq cytotoxicity assays, a concentration of 400 μg/ml EMS that resulted in 80% inhibition of colony formation and 40 μM Thymitaq, approximately 20-fold higher than IC50 value for this compound, were chosen for selection of resistant clones. Following EMS exposure and Thymitaq selection, a relatively large number of resistant clones (41 per 108 cells) were generated in comparison with EMS pretreatment not selected in Thymitaq (1 per 106 cells), indicating that EMS increased the frequency of surviving Thymitaq-resistant clones by approximately 10-fold. All 41 of these clones from EMS and Thymitaq treatment and 1 clone from a control experiment without EMS were expanded to stable Thymitaq-resistant cell lines, which were grown in the presence of 40 μM Thymitaq.

**DNA-SSCP Analysis—**To detect putative point mutations leading to drug resistance, all 41 EMS-exposed Thymitaq-resistant clones were analyzed by DNA-SSCP analysis. The sequences of oligonucleotides used for PCR amplification, DNA-SSCP screen, and sequence analysis are provided in Table I. Results of DNA-SSCP screening indicated the presence of multiple resistant clones that showed altered banding patterns in the DNA-SSCP gels. DNA sequencing confirmed the presence of point mutations in the TS genes of these resistant clones. The point mutations in human TS were classified into two types: missense and silent mutations. Missense mutations were further classified into two categories: single-base substitutions and small insertions or deletions. Silent mutations were classified into two categories: single-base substitutions and small insertions or deletions.

**Table I—**The sequences of oligonucleotides used for PCR amplification, DNA-SSCP screen, and sequence analysis

| Oligonucleotide primers | Sequence of oligonucleotides | Annealing codon to human TS |
|-------------------------|-----------------------------|---------------------------|
| hTS-1A*                 | 5’ CACAGGAGCGGACCGGAG 3’    | nt 50–60                   |
| hTS-1B                  | 5’ CATGAGATCCGTGAG 3’       | nt 354–334                 |
| hTS-2A                  | 5’ GGCTGTCCTTCAGAAGGTA 3’   | nt 298–319                 |
| hTS-2B                  | 5’ TCTCTGACAGCGAGGACAC 3’   | nt 645–622                 |
| hTS-3A                  | 5’ CGCCACGTTCATAGGGACACG 3’ | nt 905–915                 |
| hTS-4A                  | 5’ TACCTGCTGGGCAGTACCAAC 3’ | nt 97–117                  |
| hTS-4B                  | 5’ TATCTTCGTACCGTGTCAGGGC 3’ | nt 210–188                 |
| hTS-5A                  | 5’ TACTAGTATCTCGAGAACGATG 3’ | nt 451–475                 |
| hTS-5B                  | 5’ ATGGGTCATACCTCTTCCGAC 3’ | nt 503–481                 |
| hTS-6A                  | 5’ GGAGATCCACATATATCTCTGGA 3’ | nt 756–779                 |
| hTS-6B                  | 5’ TCGGTCGCTTCGCTGANGCT 3’  | nt 822–803                 |

* A represents sense, and B indicates antisense.

† nt, nucleotides.
Drug-resistant Mutants of Human Thymidylate Synthase

The Expressed Levels of Human TS in Resistant Cells—Western blotting revealed that some resistant sublines (HT1080-A, -1b, -2b, -2c, -1d, -2d, -1e, -2e, and -6e) demonstrated elevated expression levels, and modest increases in TS genomic DNA were also seen in these sublines by Southern blotting. The corresponding mRNA levels were observed to increase in only HT1080–2c, -2d, and -2e sublines as determined by Northern blotting, using ribosomal phosphoprotein 36B4 mRNA as controls. In contrast, some sublines (HT1080–1c, -1d, and -1e) did not amplify the TS gene and nor did they overexpress TS mRNA and protein (Fig. 3).

Mutations Identified by Sequencing Analysis—The fragments produced by PCR amplifications with abnormal migration on SSCP gels were cloned into pCR-Script vector for sequencing. More than 20 mutations in the 9 Thymitaq-resistant cell lines were identified, which are K214R, A228T, and K266I mutations from HT1080-1b; I40T and D49N from HT1080-2b; T51A from HT1080-1d; R25H and F59L from HT1080-2d; and G52S from HT1080-1e; D49G from HT1080-2e; and T234M from HT1080-2e; and S572 as an example is shown in Fig. 4). The results combining Western and SSCP analyses indicated that human TS protein overexpression and mutations in the TS coding region were both present in the same cell lines. No mutations were detected in the untreated HT1080 cell line or in the HT1080-A line that was obtained by Thymitaq selection without EMS exposure.

Construction of Mutations in Human TS by Site-directed Mutagenesis—To determine whether only single point mutations result in changes in Thymitaq binding to TS, we expressed mutant TS genes with only single point mutations. The human TS expression vector pcDNA3-hTS, which contains the entire coding sequence of the human TS gene with minor modifications of the N-terminal nucleotide codon, was utilized for these studies (15). Eight TS mutants, including K47E, D49N, F59L, R50C, T51A, G52S, Q214R, and G52S, were detected in the resistant subline analysis, were generated by site-directed mutagenesis using pcDNA3-hTS as a template. These mutations were selected from the 20 or more mutations found, based on their occurrence in highly conserved regions of this enzyme (Table III). Sequencing the coding regions demonstrated that the expected substitution had been introduced and that no other alteration had occurred (data not shown).

Rescue of TS-negative Cells by Transfection of Various Human TS cDNAs—Transfection of the eight human TS variants

Table II

| Oligonucleotide primers | Sequences | Amino acid changed |
|-------------------------|-----------|-------------------|
| K47E                    | 5'-GGCCCGTCAGGAGGACGACC 3' | Lys47 → Glu |
| D49G                    | 5'-TCAAGAAGGACGCCCACAG 3' | Asp49 → Gly |
| D49N                    | 5'-GCTAGAAGGACGCCCACAG 3' | Asp49 → Asn |
| R50C                    | 5'-AGGAGGAGGACGCCCACAG 3' | Arg30 → Cys |
| T51A                    | 5'-AAGGAGGACGCCCACAG 3' | Thr51 → Ala |
| G52S                    | 5'-GAGGACGCCCACGCCCAC 3' | Gly52 → Ser |
| F59L                    | 5'-ACCCGTGCGATCTGGCATGAG 3' | Phe59 → Leu |
| Q214R                   | 5'-TGCCAGCTGACTGGGAGTCGAG 3' | Gln214 → Arg |
| pcDNA3*                 | 5'-CAAAAAGTCTCGGGGAGGATTGATA 3' | Sma1 → KspI |

a The mismatches to wild type directing the codon changes are underlined.

b Selection primer for destroying the unique SmaI restriction site to generate another unique KspI site on pcDNA3 vector.

FIG. 2. Autoradiogram of SSCP analysis of human TS gene mutations in AG337-resistant cells. Amplified DNA fragments by reverse transcriptase-PCR corresponding to region A were denatured by heating, and electrophoresis was performed in 8% polyacrylamide gel containing 5% glycerol at constant 30 watts at 4 °C. Lane 1, control HT1080 cells; lanes 2–5, AG337-resistant cells. Fragments with a mobility shift in addition to wild-type bands are observed in lanes 4 and 5, suggesting mutations in human TS gene from nucleotides 70 to 187 for HT1080-1b and HT1080-2b.

FIG. 3. Southern, Northern, and Western blot analysis of 10 resistant sublines and parental HT1080 cell line. A, Southern blot analysis of DNA restricted with EcoRI. B, Northern blot analysis of total RNA from drug-resistant and parental cells. Both were hybridized with a [32P]dCTP-labeled human TS cDNA. C, a control for RNA loading, using ribosomal phosphoprotein 36B4 mRNA. Northern blot membrane was stripped and rehybridized with a [32P]dCTP-labeled cDNA probe for the 36B4 mRNA. D, Western blot analysis of human TS proteins, which were probed by a rabbit polyclonal antibody against human TS. Lanes 1, HT1080 cells. Lane 2, resistant cell line HT1080-A, which was selected by AG337 without EMS pretreatment. Lanes 3–11 represent nine different resistant sublines from EMS treatment, followed by AG337 selection.
Drug-resistant Mutants of Human Thymidylate Synthase

Based on the drug sensitivity results presented above, TS mutants D49G and G52S were selected for enzyme kinetic studies. The D49G and G52S human TS cDNA were inserted into protein expression vectors pET-17×b. The pET system provided high yields of soluble protein in a derivative of the E. coli strain BL21(DE3). The activity of the enzyme was monitored and found to be highest 5 h after the addition of isopropyl-β-thiogalactoside. E. coli extracts were analyzed by SDS-PAGE and revealed an intensely staining band at a molecular mass of about 36 kDa, absent from extracts of the host E. coli cells. This new protein band was estimated to represent about 10–20% of the total soluble protein in the extract. The crude extracts from bacterial cells transformed by mutant TS vectors had similar high levels of the altered proteins, comparable to wild-type enzyme.

Purification of Mutant TS Proteins—A procedure previously used to purify rat TS, using sequential ion-exchange/phenyl-Sepharose chromatography, was adopted for purification of wild-type and mutant human TS proteins (19), modified in that the human TS was eluted from phenyl-Sepharose using a linear gradient of ammonium sulfate of 0.8 M to 0 instead of 0.8 to 0.4 M employed for rat proteins, which is similar to that described in a recently published paper (15). After purification, a single major component on SDS-PAGE gel migrating with an apparent molecular weight of human TS protein was observed for the wild-type and mutant enzymes. Purity was estimated to be greater than 80% as determined by densitometric scanning.

Kinetic Properties of Mutant Enzymes—To obtain information about the catalytic and ligand-binding properties of these TS variants, the kinetic parameters 

\[ V_{max} \] and \[ K_m \]

values for substrate and cofactor and \[ K_f \] values for inhibitors were evaluated. The \[ K_m \] values for CH₂H₄folate and dUMP were not significantly different between the wild-type and G52S mutant forms, whereas the catalytic efficiency \( (k_{cat}/K_m) \) of G52S was even lower than the \( k_{cat} \) of wild-type TS (Table V). The \[ K_m \] for dUMP and CH₂H₄folate of the D49G variant was increased 3-fold over wild-type TS, and D49G mutants showed diminished catalytic activity with \( k_{cat} \) values 3-fold lower than wild-type TS.

The \( K_f \) values of wild-type and mutant TS proteins for Tomudex, BW1843U89, and Thymitaq were determined at a high concentration (over 8-fold \( K_m \) of TS variants) of CH₂H₄folate. The \[ K_f \] values for FdUMP were determined at a fixed concentration (500 μM) of dUMP. As expected, the \[ K_f \] values for FdUMP of the G52S mutant was 20-fold greater than the \[ K_f \] of wild-type TS, consistent with cytotoxicity results. The \[ K_f \] values of the D49G mutant for FdUMP was only 5.4-fold higher than the wild-type. By comparison, inhibition of G52S and D49G mutants by the folate inhibitors of TS was 3–6-fold less effective than against wild-type TS (see Table V).

In addition, to address the question about possible inhibitory effects of the unnatural stereoisomer of CH₂H₄folate upon the kinetic analyses of the enzyme, we prepared the natural isomer \( (6R)-CH₂H₄folate \) following the procedure described by Bruce and Santi (22). No significant difference between the \[ K_m \] values of CH₂H₄folate for wild-type and mutant TS enzyme was observed using a racemic mixture or the pure natural isomer of cofactor (data not shown), indicating that the unnatural isomer does not inhibit significantly this enzyme activity, as was previously demonstrated for mouse TS (23).

DISCUSSION

Strategies—As expected, random mutagenesis by exposure of human sarcoma HT1080 cells to an alkylating agent (EMS) and selection with Thymitaq resulted in the generation of a large number of resistant colonies, as compared with Thymitaq selection without EMS pretreatment. Thymitaq was employed as an agent to select for TS mutant cells that expressed resistance to antifolate drugs.
Drug-resistant Mutants of Human Thymidylate Synthase

Amino acids in human TS that are highly conserved and important for ligand binding

Table III

| Positions in human and corresponding L. casei thymidylate synthase | Sequence conservation among 29 TS species | Interactions with CH₂H₄folate or dUMP |
|---------------------------------------------------------------|----------------------------------------|-------------------------------------|
| Lys⁴⁷/Lys⁸⁰                                                   | Invariant in vertebrates                | Arg⁶⁰ loop                          |
| Asp⁶⁹/Asp²²                                                   | Strictly invariant                      | Arg⁶⁰ loop                          |
| Arg⁹⁰/Arg²³                                                   | Highly conserved with two exceptions (Gly) | Hydrogen bond with dUMP and C terminus |
| Thr⁵¹/Thr²⁴                                                  | Highly conserved with one exceptions (Gln) | Arg⁶⁰ loop                          |
| Gly⁵⁹/His⁵⁵                                                  | Highly conserved with five exceptions (two His, Arg, Met, and Pro) | Arg⁶⁰ loop                          |
| Phe⁵⁹/Phe³²                                                  | Highly conserved with two exceptions (Met and Thr) | β-Sheet i, forming part of the substrate binding pocket |
| Glnⁱ¹⁴/Gln²¹⁷                                               | Highly conserved with one exception (Ala) | β-Sheet iii, a kick region for three β-sheet formation |

Tomudex (ZD1694), Thymitaq (AG337), BW1843U89, and 5-fluoro-2'-deoxyuridine (FdUrd) sensitivity in TS-negative cells transfected with wild-type and various mutant human TS cDNAs

Table IV

| Transfected TS-negative cells and its clone | Tomudex (ZD1694) | Thymitaq (AG337) |
|--------------------------------------------|------------------|------------------|
|                                            | IC₅₀ valuesᵃ     | Ratioᵇ           | IC₅₀ valuesᵃ     | Ratioᵇ           |
|                                            | ×10⁻⁹ μ          |                  | ×10⁻⁷ μ          |                  |
| Wild-type/clone (1)                        | 2.37 ± 0.18      | 0.65             | 1.64 ± 0.08      |                  |
| K47E/clone (2)                             | 1.53 ± 0.04      |                  | 8.25 ± 0.61      | 5.0              |
| D49G/clone (1)                             | 1.76 ± 0.09      | 0.74             | 66.7 ± 2.5       | 40               |
| G52S/clone (3)                             | 1.94 ± 0.05      | 0.83             | 19.1 ± 0.71      | 12               |
|                                            |                  |                  |                  |                  |
| Wild-type/clone (1)                        | 2.78 ± 0.12      | 0.71             | 2.90 ± 0.03      |                  |
| K47E/clone (2)                             | 7.55 ± 0.23      | 5.7              | 16.4 ± 4.3       | 5.7              |
| D49G/clone (1)                             | 14.7 ± 1.2       | 5.0              | 76.3 ± 1.9       | 26               |
| G52S/clone (3)                             | 8.4 ± 0.7        | 3.0              | 282 ± 28         | 97               |

ᵃ IC₅₀ values were obtained following 7-day exposures from full dose-response curves and represent the mean ± S.E. of at least two separate experiments involving duplicate samples from replicate cultures.
ᵇ The ratio is IC₅₀ value of mutant to wild type.

Kinetic parameters and drug binding affinities for wild-type and mutant human thymidylate synthases

Table V

| Parameter | Kₘ (dUMP) | Kₘ (CH₂H₄folate)ᵃ | kₘₐₜ | Kᵣ | Tomudex | BW1843U89 | Thymitaq | FdUrd |
|-----------|-----------|--------------------|-------|-----|---------|-----------|----------|-------|
|           | µM       | s⁻¹                |       | nM  |         |           |          |       |
| Wild type | 3.9       | 13                 | 1.7   | 13  | 0.34    | 8.4       | 2.6      |       |
| D49G      | 11        | 43                 | 0.57  | 50  | 1.1     | 55        | 14       |       |
| G52S      | 4.5       | 10                 | 3.5   | 45  | 0.98    | 41        | 52       |       |

ᵃ The values shown are corrected, as the racemic mixture of this cofactor was used.

a selective drug in as much as Thymitaq is not a substrate for folypolyglutamate synthetase and enters cells by passive diffusion, thus eliminating resistance mechanisms such as an altered reduced folate transporter and a decrease in folylpolyglutamate synthetase activity. Therefore, the expected major causes of resistance in the Thymitaq-resistant clones are over-expression and/or an alteration of the target human TS enzyme. The advantage of this approach is that mutations caused by EMS could occur anywhere in the entire TS gene and may not be limited to specific regions of the gene as in cassette or site-directed mutagenesis. However, as it is almost impossible to obtain more than one mutation in a nucleotide codon by random mutagenesis, some desirable amino acid substitutions are excluded by this approach.

Human TS Mutants Identified in EMS-exposed Cells—The presence of point mutations in EMS-exposed Thymitaq-resistant cells was determined by SSCP analysis using 6 pairs of primers to span the cDNA for TS; each amplified fragment was 150–260 bp, providing an 80–90% range of sensitivity (24). By screening most of the entire coding sequence of human TS gene from Thymitaq-resistant HT1080 sublines, shifted bands in addition to normal migrating bands were observed on SSCP gels, indicating the presence of wild-type and mutant TS genes in the sample. Previous studies demonstrated that polymorphisms could be detected when mutant DNA comprised as little as 3% of the total gene copies in a PCR mixture (25). However, SSCP cannot discriminate between pre-existing mutations and those mutations introduced by Taq polymerase errors during early-stage amplification.

TS fragments showing abnormal migration on SSCP gels were sequenced, and more than 20 mutations of human TS were identified in nine Thymitaq-resistant sublines, indicating that some cell lines contain more than one mutation in TS. This phenomenon was observed in other EMS-treated cell lines (17). We assumed that some or most of those mutations were generated by EMS exposure, based on following evidence: 1) none of the mutations were detected in parent HT1080 and EMS-untreated HT1080-A cells; and 2) most amino acid substitutions were found in highly conserved positions (Table III), suggesting that these variants likely led to drug resistance. We
Drug-resistant Mutants of Human Thymidylate Synthase

chosen eight mutations from 20 or more found for further investigation. Based on knowledge of the crystal structure of the enzyme, F59L and Q214R mutations were chosen since these amino acids are important for ligand binding and structural stability. Additionally, six other mutations (K47E, D49N, D49G, R50C, T51A, and G52S) located in the Arg$^{50}$ loop were chosen, as this loop becomes more ordered by movement and reorientation upon ligand binding (26). These eight TS mutants were expressed in mouse TS-negative cells to examine directly the ability of these mutations to allow growth in the absence of thymidine and confer resistance to Thymitaq. Three of these cDNAs allowed growth in the absence of thymidine, and cytotoxicity studies showed that D49G and G52S TS variants displayed resistance to the selective drug Thymitaq, providing additional proof that these mutations were involved in the Thymitaq-resistant phenotype.

The Arg$^{50}$ Loop and Drug Resistance—Of interest, of the 20 point mutations identified from random mutagenesis, six occurred in the highly conserved Arg$^{50}$ loop (amino acids 47–52). This loop connects elements of protein secondary structure, an α-helix A (residues 30–43) near the N terminus, and a β-sheet i (residues 54–66). Comparison of nearly identical crystal structures between the native unbound and dUMP-bound TS revealed that the only difference is that the mobile Arg$^{50}$ loop has less than 1.0-Å movement and undergoes reorientation upon Arg$^{50}$ binding to the phosphate moiety of dUMP, steps necessary to accept the incoming folate molecule. Once the ternary complex is formed, the carboxylate of the C-terminal residue and N-1 of CH$_2$H$_4$folate form hydrogen-bond networks with Arg$^{50}$ through fixed H$_2$O molecules. This flexible Arg$^{50}$ residue seems to be a bridge linking the enzyme C terminus, substrate, and cofactor (or antifolates) together. The movement of the Arg$^{50}$ residue is accompanied by adjustment and reorientation of its neighbor residues, indicating that the entire Arg$^{50}$ loop undergoes relocation and encompasses new interactions. For example, the hydrophobic atoms of Thr$^{51}$ has contacts with the buried Val$^{113}$ side chain after movement (6, 24, 27–30).

Besides structural studies, the residues in the conserved Arg$^{50}$ loop have been intensively studied by mutagenesis of E. coli and L. casei TS (1). The corresponding amino acids representing Arg$^{50}$, Asp$^{49}$, and Thr$^{51}$ in these proteins are quite distant from Arg$^{50}$. For example, the hydrophobic atoms of Thr$^{51}$ has contacts with the buried Val$^{113}$ side chain after movement.

Based on the above results and structural information of the Arg$^{50}$ loop, we postulate that the basis for reduced Thymitaq and FdUMP binding observed for D49S and G52S mutants results from impaired movement of the Arg$^{50}$ loop and resulting interaction of the loop with nucleotide or folate molecule. This is especially true for the Arg$^{50}$ residue which is involved in a hydrogen bond network with folate, dUMP, and enzyme C terminus. More drastic changes such as those in mutants R50C, D49N, and T51A result in inactive TS enzymes, whereas small structural perturbations of Arg$^{50}$ loop caused by mutants K47E, D49G, and G52S may be compensated for by the local reorientation of neighboring residues, which still maintain contacts with ligands in the new position. The modification of the loop Arg$^{50}$ causes changes in binding affinity leading to drug resistance, related not only to folate but also to nucleotide binding.

Kinetic Studies of D49G and G52S Human TS Mutants—The kinetic characterization studies of the highly purified altered enzymes obtained from an E. coli expression system are consistent with the cell growth inhibition results. The product of $K_{cat}/K_{m}$ has been suggested as a comparative index to represent both catalytic efficiency and drug inhibition (32). The values of $K_{cat}/K_{m}$ for wild type and G52S via FdUMP are 0.34 and 8.3 (s$^{-1} \times 10^3$), respectively, which correlates with the resistance displayed by the G52S TS transfectants to FdUrd over the wild-type TS transfectants in culture.

Future Applications in Gene Therapy—Treatment with intense dosages of chemotherapeutic agents, including fluorodeoxyuridine and antifolates, may increase the curvature of sensitive tumors (33). However, this approach is often limited due to myelosuppression. To overcome bone marrow toxicity from chemotherapy, transfer of drug resistance genes into hematopoietic progenitors is a promising approach. Introduction of drug resistance genes such as mutant dihydrofolate reductase, the multiple drug resistance gene (MDR-1), glutathione transferase, alkyl transferase, cytidine deaminase, and aldehyde dehydrogenase into murine hematopoietic cells has been shown to improve chemotherapy tolerance in vitro and in vivo (34–38).

The newly identified human TS mutants (D49G and G52S), because of their desirable properties that include catalytic function ($k_{cat}$) and resistance to FdUrd and Thymitaq, are excellent candidates for gene transfer studies. Preliminary transfection experiments of mutant human TS cDNA retroviral constructs have demonstrated that the G52S mutant functions in a dominant manner to protect murine bone marrow cells from FdUrd toxicity as compared with wild-type TS.$^2$

Acknowledgments—We thank Dr. Frank Maley for the human TS cDNA and anti-hTS antibody as well as for invaluable advice. We acknowledge the technical assistance of Dr. Saori Nakahara and Wen Chen.

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J. Biol. Chem. 1998, 273:11611-11618.
doi: 10.1074/jbc.273.19.11611

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