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Random Sequence Mutagenesis and Resistance to 5-Fluorouridine in Human Thymidylate Synthases*

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Thymidylate synthase (TS) catalyzes the methylation of dUMP to dTMP and is the target for the widely used chemotherapeutic agent 5-fluorouracil. We used random sequence mutagenesis to replace 13 codons within the active site of TS and obtain variants that are resistant to 5-fluorodeoxyuridine (5-FdUR). The resulting random library was selected for its ability to complement a TS-deficient *Escherichia coli* strain, and sequence analysis of survivors found multiple substitutions to be tolerable within the targeted region. An independent selection of the library was carried out in the presence of 5-FdUR, resulting in a more limited spectrum of mutations. One specific mutation, C199L, was observed in more than 46% of 5-FdUR-resistant clones. A 5-FdUR-resistant triple mutant, A197V/L198I/C199F, was purified to apparent homogeneity. Kinetic studies with the substrate dUMP indicate that this mutant is similar to the wild type in regards to k_{cat} and K_m values for dUMP and the cosubstrate CH_2H_4-folate. In contrast, equilibrium binding studies with the inhibitor, FdUMP, demonstrate that the dissociation constant (K_d) for FdUMP binding into the ternary complex was 20-fold higher than values obtained for the wild-type enzyme. This 5-FdUMP-resistant mutant, or others similarly selected, is a candidate for use in gene therapy to render susceptible normal cells resistant to the toxic effects of systemic 5-fluorouracil.

Thymidylate synthase (TS)[1] is a 72-kDa homodimer that catalyzes the formation of dTMP from dUMP using CH_2H_4-folate as both the single carbon unit source and the reductant. Thymidylate synthase is a central enzyme in DNA biosynthesis as it provides the only *de novo* source of dTMP. Inhibition of TS in mammalian cells results in depletion of cellular thymidylate, inhibition of DNA synthesis, and ultimately thymine-less death (1–3). In addition, TS activity is greatest in rapidly proliferating cells and is preferentially expressed at the onset of S phase (4). For these reasons, TS has been a key target for chemotherapeutic agents (5, 6).

The crystal structures of the human, *Escherichia coli*, and *Lactobacillus casei* TS have been solved and share multiple structural features (7–9). The enzyme is a symmetric dimer of structurally identical subunits. Deep within the active site cavity, three arginine residues and one serine residue coordinate the bound phosphate. Two of these arginine residues are donated from the other subunit, providing the structural basis for TS being an obligate dimer (10). In addition, structures of binary (*e.g.* TS-dUMP, TS-CH_2H_4-folate, or TS bound to novel inhibitors) and ternary complexes (*e.g.* TS-dUMP-folate analog, TS-dUMP analog-CH_2H_4-folate, or TS complexed with two analog inhibitors) have provided important information on key domains, chemical reactions, and conformational changes that occur during catalysis (10–14).

The active site of all known TS enzymes, with one exception, contains the amino acid sequence PCH. After formation of the reversible ternary complex with dUMP and CH_2H_4-folate, the reactive thiol of the cysteine (Cys-195) within this motif initiates the enzymatic reaction via nucleophilic attack at C-6 of dUMP. However, site-directed mutagenesis of these three residues has demonstrated that both Pro-194 and His-196 can tolerate specific substitutions in *L. casei* and *E. coli* without significant loss of catalytic activity (15, 16). The catalytic cysteine (Cys-195) was found to be immutable when tested by complementation in *L. casei* (17), but the constructed mutant C195S retains activity in *E. coli.* (7). Nucleotide sequence alignments indicate that TS is phylogenetically one of the most highly conserved enzymes known (18). However, it has become increasingly apparent that many amino acid residues that are conserved in nature are tolerant to a variety of substitutions *in vitro* (19).

In cells, 5-FU is metabolized to 5-FdUMP, which forms a stable inhibitory complex with thymidylate synthase and the cosubstrate CH_2H_4-folate (19–21). In the ternary complex, one covalent bond links the thiol group of Cys-195 to the C-6 of dUMP, and a second covalent bond links the C-5 of the pyrimidine ring to the one carbon unit (C11) of CH_2H_4-folate (19). Due largely to its effective inactivation of TS, 5-FU is widely used in the treatment of many common carcinomas including colon, breast, and ovarian cancers. Although 5-FU is a potent anti-tumor compound, its toxicity toward normal bone marrow and gastrointestinal tissues limits its use (22). Genetic transfer of a mutant TS enzyme that is resistant to inactivation by 5-FdUMP could protect these tissues from 5-FU toxicity. The basis for such a genetic rescue has been established for other proteins. For example, transfer of a mutant dihydrofolate reductase cDNA into mouse bone marrow stem cells has been shown to improve the survival of mice treated subsequently with methotrexate (23, 24). Recently, retroviral-mediated transfer of the gene encoding the human MGMT DNA repair

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[1] The abbreviations used are: TS, thymidylate synthase; 5-FdUR, 5-fluoro-2′-deoxyuridine; 5-FU, 5-fluorouracil; FdUMP, 5-fluoro-2′-deoxyuridine 5′-monophosphate; CH_2H_4-folate, (6*R*,*S*)-N^5,N^{10}-methylene-5,6,7,8-tetrahydrofolate; TES, *N*-tris[hydroxymethyl]methyl-2-aminoethane-sulfonic acid; bp, base pair(s); PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis.

TABLE I
Oligonucleotides used in this study

Oligomer designation	Nucleotide sequence
OLIGO1	5′-d(ATCATCATGTGCGCTTGGAATCCAAGAG ATCTTCCTCTGATGGCGCTGCCTCCATGC)-3′
OLIGO2[a]	5′-d(CCATGTCTC CGGATCTCTGGTACAGCTGGCAG GACAG CTCAC TGT TCAC**T**AC**G**TAGAACTGGCAGAGGGCATGGC ATGGAGGCAGCGCCĀTCĀG)-3′
DLTS2F	5′-d(ATCATCATGTGCGCTTGG)-3′
BSPE1DL	5′-d(GGCCCATGTCTCCGGATCTCTGGTACAGC)-3′
3′HSG	5′-d(TAACGCCAGGGTTTTCCCAG)-3′
DLTS3R	5′-d(AAAAAAAAACCATGTCTCCGGATCTCTGGTAC)-3′
TSNDE1	5′-d(AACGGTAAAGGAGCAGAG)-3′
TS-SAL1	5′-d(AAAAAAAAGTCGACCTAAACAGCCATTTCC)-3′

[a] Underlined nucleotides were subjected to randomization, constructed such that each nucleotide position will contain on average 86% wild-type sequence and 14% of the remaining three nucleotides. Double-underlined bold nucleotides indicate alterations that were introduced to create a silent *Sna*BI restriction site.

protein has been shown to confer protection of hematopoetic cells against alkylating agents *in vivo* and *in vitro* (25, 26).

In the present work, we illustrate a unique approach for obtaining mutants of TS that are catalytically active and are resistant to inhibition by 5-FdUMP. Since 5-FdUMP is structurally similar to the natural substrate dUMP, we lack adequate knowledge to design such mutants by site-directed mutagenesis; it is difficult to predict how single amino acid substitutions or multiple substitutions could restrict the binding of 5-FdUMP without affecting binding of dUMP. Random mutagenesis provides a combinatorial method to create altered enzymes without requiring detailed knowledge about amino acid interactions or effects of specific alterations. Based on sequence homology alignment and crystallographic information, we targeted a region for mutagenesis near the catalytic Cys-195 that assists in formation of the dUMP binding site. We used genetic complementation to obtain a collection of catalytically active mutants with amino acid replacements in the active site of TS. These active mutants were subsequently selected for their ability to confer growth of *E. coli* in the presence of 5-FdUR.

EXPERIMENTAL PROCEDURES

Cell Lines and Materials—CH_2H_4-folate was from Schircks Labs (Jona, Switzerland). 5-[6-^{3}H]FdUMP was supplied by Moravek Biochemicals (Brea, CA). [γ-^{32}P]ATP and Thermo Sequenase kits for DNA were obtained from Amersham Pharmacia Biotech. ABI Prism Dye Terminator Cycle Sequencing kits for fluorescent sequencing were the products of Perkin Elmer. *E. coli* DNA Pol I was from New England Biolabs. Plasmid DNA was isolated using the Maxiprep and Miniprep kits from Qiagen (Chatsworth, CA). 5-FdUR, 5-FdUMP, dUMP, TES, and all other reagents were from Sigma. *E. coli* NM522 (Stratagene) was used for cloning and library construction. *E. coli* χ2913recA (Δ*thy*A572, *rec*A56), kindly provided by Dr. Daniel Santi (University of California, San Francisco), is tetracycline resistant and was used in all complementation studies and in the purification of plasmid-encoded TS. Unless otherwise stated, all DNA oligomers were from Operon Technologies (Alameda, CA).

Plasmids—Plasmid pGCHTS-TAA, from D. Santi, contains the wild-type human thymidylate synthase cDNA modified by the addition of a 115-base pair 5′-untranslated region from the *L. casei* TS in a high copy number modified pUC vector background (8, 27). A unique translationally silent *Mro*I site was added at coding nucleotide 648 of the human TS vector pGCHTS by using the mega-primer method of oligo-directed mutagenesis (28–30). Briefly, a 113-bp PCR product was created by amplification with the primer DLTS2F with the 29-mer mutagenic primer BSPE1DL. DLTS2F is an 18-mer, which corresponds to nucleotides 529–546 of the sense strand, and BSPE1DL corresponds to the antisense nucleotides 644–661 with a G to A substitution at nucleotide 648. The 133-bp PCR product was purified and used as a mega-primer in a PCR reaction in conjunction with the primer 3′-HSG, a 20-mer corresponding to the antisense sequence of pGCHTS approximately 33 bp downstream of the *Sac*I site used to clone the human TS. The resulting 479-bp DNA fragment was subsequently digested with *Bgl*II and *Sac*I and ligated into the corresponding portion of the TS vector. A TS stuffer vector was created by replacing the TS open reading frame between coding nucleotides 555 (*Bgl*II site) and 646 (*Mro*I site) with a 1.3-kilobase DNA fragment derived from a modified pET3a vector (Novagen, Madison, WI). The DNA insert was prepared by digestion of pET3a with *Mro*I and *Bgl*II agarose gel purification of the appropriate fragment and ligation into the *Mro*I-*Bgl*II digested TS vector.

Construction of the TS Random Library—The TS random library was constructed by annealing two single-stranded DNA oligomers (*step 1*, Fig. 1). Oligomer 1 is a 57-mer that corresponds to the sense nucleotides 529–585 and contains a *Bgl*II site (nucleotide 555) for cloning. Oligomer 2 is the 94-mer that contains a mutated sequence corresponding to amino acids 197–200 and 205–213, with 14% random nucleotides and 86% wild-type nucleotides at each of the 39 randomized positions and an *Mro*I site for cloning (Table I). It was synthesized by Integrated DNA Technologies (Coralville, IA). The random oligomer contained on average 5.5 nucleotide changes, and thus we calculated that 0.3% of the oligomer population was wild type at the nucleotide level (0.86^{39} = 0.0027). Two silent mutations were introduced in the non-random region to create a unique *Sna*B1 site for subsequent identification based on digestion with the restriction enzyme. Oligomers 1 and 2 were annealed in 50 μl of 200 mM Tris-HCl, pH 7.5, 100 mM $MgCl_2$, 250 mM NaCl by incubation at 80 °C for 5 min, followed by 55 °C for 15 min, at 37 °C for 15 min, and at room temperature for 15 min (*step 1*, Fig. 1). The partial oligonucleotide duplex was extended in a 40-μl reaction mixture containing 10 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 7.5 mM dithiothreitol, 250 μM dNTPs, and 5 units of Klenow fragment of *E. coli* DNA Pol I for 2 h at 37 °C (*step 2*, Fig. 1). The double-stranded oligonucleotides were then amplified in a 100-μl polymerase chain reaction containing 20 mM Tris-HCl, pH 8.75, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100, 100 μg/ml bovine serum albumin, 1 μM primers DLTS2F and DLTS3R, and 2.5 units of *Pfu* DNA polymerase (Stratagene). The reaction mixture was heated for 30 cycles in a programmable thermal controller (MJ Research, Watertown, MA) at 95 °C for 1 min, 50 °C for 1 min, and 72 °C for 1 min (*step 3*, Fig. 1). DLTS2F, described above, corresponds identically to the 5′-end of oligomer 1. DLTS3R is a 32-mer corresponding to antisense nucleotides 636–658 and is identical to the 3′-end of oligomer 2 with the addition of a 5′-9-bp oligo-A overhang to facilitate subsequent restriction digestion. The amplified DNA was digested with *Bgl*II (New England Biolabs) and *Mro*I (an isoschizomer of *Bsp*E1; Boehringer Mannheim) (*step 4*, Fig. 1), separated via centrifugation through a Microcon-30 filter (Amicon) and extracted with phenol.

Replacement of Wild-type TS Sequence with the Random Library—The purified partially random oligonucleotides were used as inserts for construction of the human TS plasmid library. The stuffer vector was removed by digestion with *Bgl*II and *Mro*I, and the resulting 3.6-kilobase fragment was isolated from a 0.8% agarose gel and was ligated to the 95-bp restricted random insert using T4 DNA ligase (Life Technologies, Inc.) (*step 5*, Fig. 1). The ligation mixture was directly transformed (Bio-Rad Genepulser, 2 kV, 25 μFD, 400 Ohm) into electrocompetent NM522 cells (Stratagene) (*step 6*, Fig. 1) using 2 μl of the ligation mixture (containing 100 ng of backbone and a 5:1 molar ratio of random insert to cut plasmid DNA), and 100 μl of *E. coli* yielded on average $2–6 \times 10^4$ transformants. Thirty transformations were pooled to obtain a library size of 2×10^6 clones. The size of the library containing the TS plasmid was determined by plating an aliquot of the transformation mixture on media containing carbenicillin (50 μg/ml; Island Scientific, Bainbridge Island, WA). The remainder of the library was amplified by

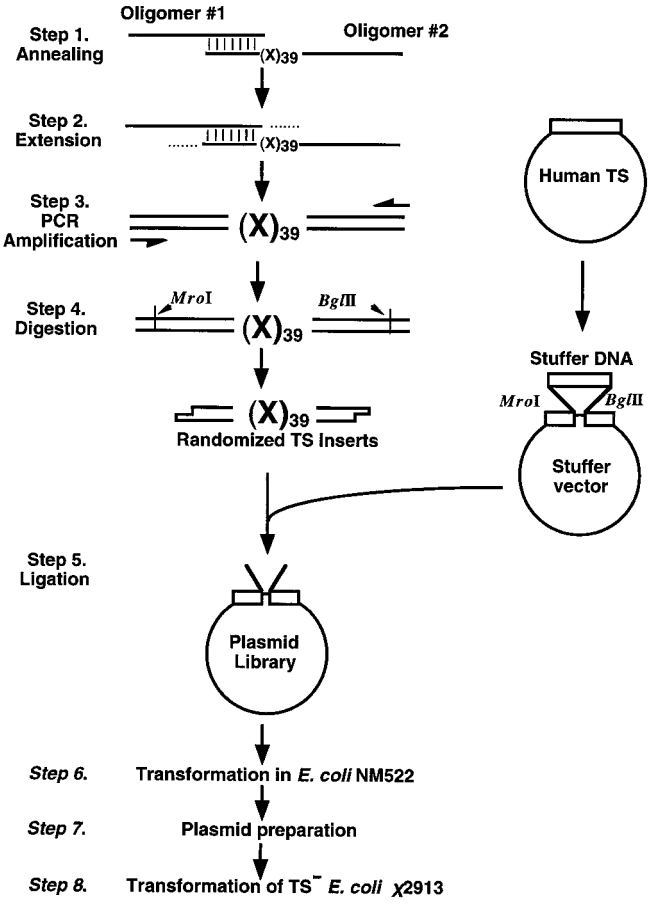

FIG. 1. **Scheme for random sequence mutagenesis of human TS.** Construction of a plasmid-borne library of TS variants containing random nucleotide substitutions is shown corresponding to residues 196–199 and 204–212. Synthesis of the random nucleotide-containing oligonucleotides (inserts) is illustrated in *steps 1–4*; $(X)_{39}$ in oligomer 2 denotes 39 non-contiguous residues containing 14% random nucleotides at each residue. Ligation of the inserts into the dummy vector, to replace the wild-type TS sequence at residues 196–199 and 204–212, is shown in *step 5*. Preparation of the plasmid library in TS^- *E. coli* is outlined in *steps 6–8*.

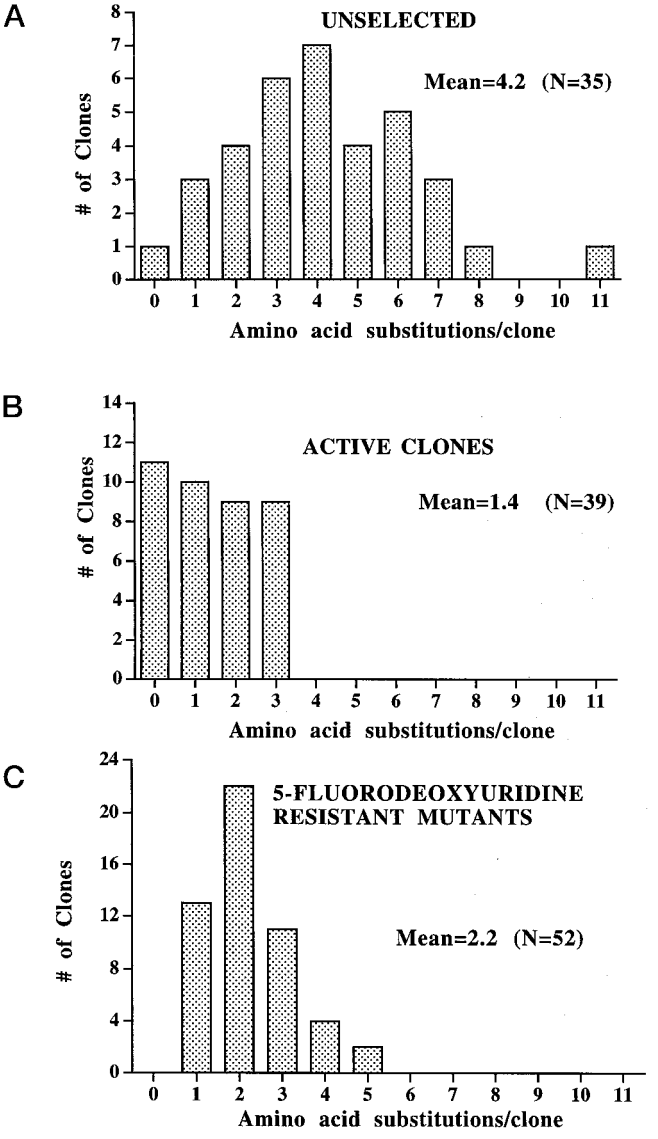

FIG. 2. **Number of amino acid substitutions.** *A*, nonselected mutants; *B*, active mutant library; *C*, 5-FdUR-selected mutant library. 35 clones were sequenced from the nonselected library, and it was determined, on average, that there were 5.7 nucleotide changes and 4.2 mutations per clone, including frameshift and nonsense mutations. 39 clones were sequenced from the pool of mutants that demonstrated the ability to complement the TS^- phenotype of χ2913 *E. coli* (comprising the active mutant library), and it was found that, on average, there were 3.0 nucleotide changes and 1.4 amino acid substitutions per mutant. 52 clones were sequenced from the 5-FdUR-selected library, demonstrating an average of 4.7 nucleotide and 2.2 amino acid substitutions per mutant.

growing the transformed NM522 cells overnight in 1 × YT media in the presence of carbenicillin and recovering the plasmid (*step 7*, Fig. 1). Transformation into χ2913 (TS^-) *E. coli* was conducted using 500 ng of the purified plasmid DNA library and 100 μl of electrocompetent cells. (Bio-Rad Genepulser, 1.8 kV, 25 μFD, 400 Ohm). Five separate transformations were pooled, and cells were grown overnight in nonselective medium containing 50 μg/ml carbenicillin, 10 μg/ml tetracycline, and 50 μg/ml thymidine and stored in aliquots at −80 °C in 10% glycerol (*step 8*, Fig. 1). The extent of randomization was verified by sequencing plasmid DNA from 35 clones grown on nonselective 2 × YT medium as described above.

Genetic Selection in E. coli—Mutants encoding active TS were selected by the method of Belfort and Pedersen-Lane (31). Only χ2913 *E. coli* cells that produce functional TS are capable of colony formation on media lacking thymidine. χ2913 cells containing the random library were grown overnight at 37 °C in 1 × YT medium containing the appropriate antibiotics. The culture was diluted 1:100 with the same medium and grown at 37 °C until the absorbance at 600 nm attained a value of 0.8 to 1.0. Aliquots of 1 ml of the exponentially growing cells were pelleted and resuspended in M9 salts, plated on minimal medium containing carbenicillin and tetracycline, and incubated at 37 °C for 36 h. Plasmids were isolated from 39 surviving colonies, and the inserted sequences containing the random region were sequenced to determine amino acid changes tolerable in maintaining catalytic activity of TS.

To select for library members that are resistant to killing with 5-FdUR, transformants were placed on minimal medium in plates with increasing amounts of 5-FdUR (0–150 nM 5-FdUR) and incubated at 37 °C for 36 h. Colonies that grew in 5-FdUR were isolated, and the plasmid was retransformed into fresh χ2913 *E. coli* to confirm the drug-resistant phenotype and eliminate false positives. Each retransformed mutant was then subjected to the same selection procedure. Appropriate dilutions were conducted ranging from 10^{-2} to 10^{-6} such that plates contained approximately 1000 colonies. DNA from those mutants that survived 125 or 150 nM FdUR (n = 53), which was lethal to *E. coli* harboring the wild-type TS, was sequenced.

Purification of Wild-type and Mutant TS—To construct a plasmid expressing the wild-type or a mutant TS enzyme (Mut 64) linked to a 6×-His polypeptide, the 981-bp TS-containing DNA fragment of pGCHTS-TAA and Mut 64 were PCR amplified by using the 5′ primer TS-NDE1 and the 3′ primer TS-SALI. TS-NDEI hybridizes to the N-terminal sequence of TS, which naturally contains an *Nde*I restriction site; primer TS-SALI contains a *Sal*I restriction site and a 5′-oligo-A tail and hybridizes to the sequence encoding the terminal 16 nucleotides of TS. The amplified DNA was cloned into pHis (a modified pUC12 vector provided by A. Hizi) and similarly digested with *Nde*I and *Sal*I. Cloning

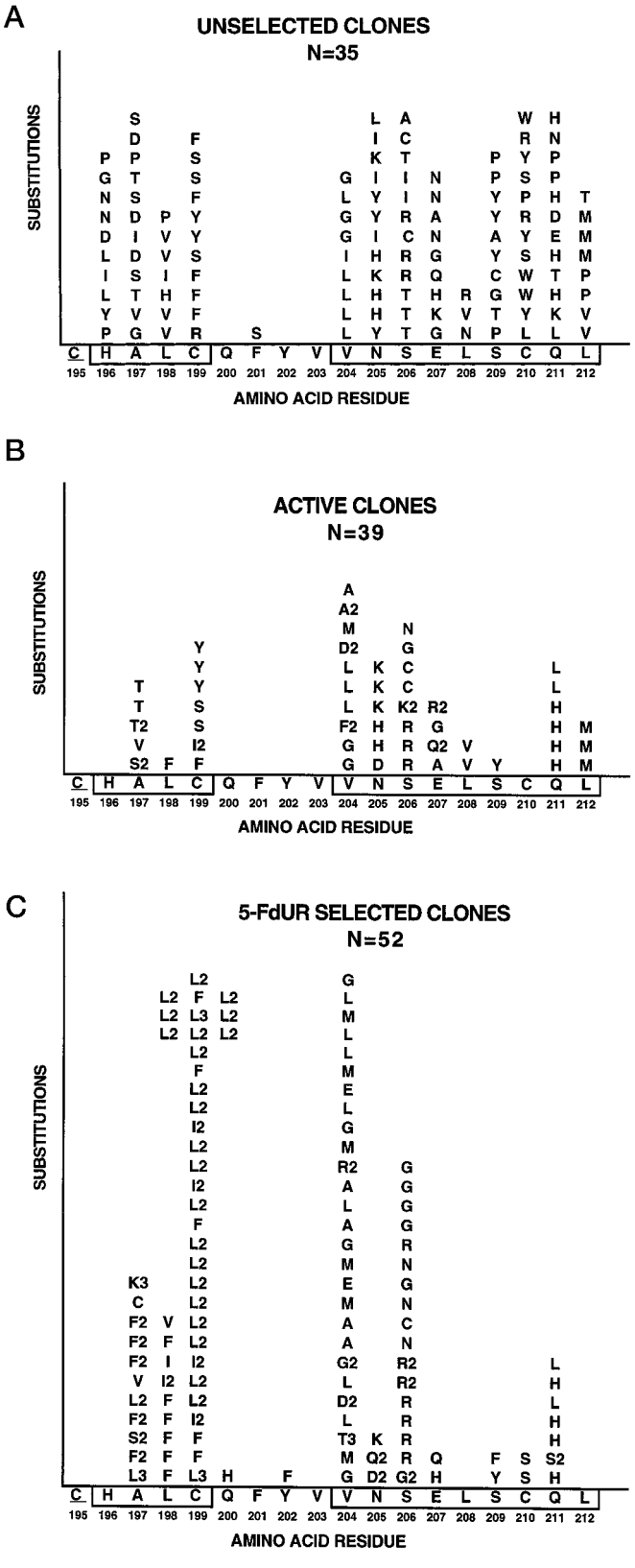

FIG. 3. **Amino acid substitutions.** *A*, nonselected; *B*, active mutant library; *C*, 5-FdUR-selected library. The wild-type TS sequence is shown *below* the *solid line*, and the substitutions observed at each position are indicated *above* each site. *Boxed residues* are those subjected to mutagenesis. The catalytic cysteine, Cys-195, is absolutely conserved (*underlined*). A *number* following a substitution indicates the number of nucleotides observed to be mutated in the corresponding codon; *no number* is shown if only one nucleotide was altered. *B*, active clones consisting of 2 amino acid substitutions include V204G/C199I, V204F/S206R, E207R/Q211H, A197T/L208V, and C199S/V204M. Active clones harboring triple substitutions include V204G/N205D/L212M, A197S/C199F/N205K, V204L/N205H/Q211H, V204L/N205K/S206K, and V204D/N205H/E207G. No active clones contained more than three amino acid alterations. *C*, in addition to the mutations shown, one of the variants encoded a mutation in the nonrandomized sequence P193S. The sequences of the individual 5-FU-resistant clones are depicted in Table III.

procedures were confirmed by both restriction analysis and DNA sequencing. The TS-6×-His fusion proteins were purified by a one-step metal chelation chromatographic procedure, using Ni^{2+} affinity resin and buffers (His-Bind resin and buffer kit, Novagen) according to a protocol modified from the supplier. TS containing χ2913 *E. coli* cells (250 ml), derived from overnight incubation of a single colony, was diluted 1:100 in fresh 2 × YT medium containing carbenicillin. After attaining an absorbance at 600 nm of 1.0, the bacteria were harvested by centrifugation, resuspended, and frozen at −80 °C in 30 ml of 1 × binding buffer (5 mM imidazole, 500 μM NaCl, 20 mM Tris-HCl, pH 7.9) and lysozyme (200 μg/ml, Sigma). Frozen cells were thawed and lysed on ice for approximately 3 h. The lysed cells were centrifuged (27,000 × *g*), and the supernatant was applied to a charged 2.5-ml His-Bind column (1 × 2.5 cm). The resin was prepared by successive washes with 30 ml of deionized water, 30 ml of 1 × charge buffer (50 mM $NiSO_4$), and 30 ml of 1 × binding buffer. All chromatographic steps were carried out at 4 °C at a maximal flow rate of 20 ml/h. Following application of the crude supernatant, the column was washed successively with 40 ml of 1 × binding buffer, 25 ml of a mixture containing 60% binding buffer and 40% wash buffer (60 mM imidazole, 500 μM NaCl, 20 mM Tris-HCl, pH 7.9), and 10 ml of 1 × wash buffer. TS was eluted with 30 ml of 1 × elution buffer (1 M imidazole, 500 μM NaCl, 20 mM Tris-HCl, pH 7.9), and about 90% of the recovered TS was released from the resin in the first 8 ml. Fractions (1.5 ml) containing purified human TS were analyzed by SDS-PAGE. Fractions containing TS were combined (3 ml) and dialyzed against 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 200 mM NaCl, and 10% glycerol for 16 h and then against the same buffer containing 1 mM dithiothreitol for an additional 16 h. The concentration of purified TS was determined by FdUMP binding according to the methods of Moran *et al.* (32).

TS Enzyme Assays—TS activity was monitored spectrophotometrically by the increase in absorbance at 340 nm that occurs concomitant with the production of H_2-folate ($\Delta\epsilon$ = 6400 M^{-1} cm^{-1}) (33, 34). The standard reaction buffer contained 50 nM TES, pH 7.4, 25 mM $MgCl_2$, 6.5 mM formaldehyde, 1 mM EDTA, and 150 μM 2-mercaptoethanol. When the concentration of dUMP was varied, (6*R*)-CH_2H_4-folate was added at 150 μM; when CH_2H_4-folate was varied, the concentration of dUMP was 100 μM (>20 K_m). Wild-type (10 nM) or Mut 64 (2.25 nM) TS was added to initiate the reaction. Steady-state kinetic parameters were obtained by a nonlinear least squares fit of the data to the Michaelis-Menten equation using Kalidegraph 3.0 software (Abelbeck Software, Reading PA).

Kinetic Analysis of FdUMP Binding—Equilibrium studies of FdUMP binding by wild-type TS were carried out in reaction mixtures that contained 3.5 nM enzyme, 0.3–10.8 nM [6-^{3}H]FdUMP, and 150 μM CH_2H_4-folate in TES buffer, pH 7.4. Equilibrium analysis of Mut 64 TS was conducted with 10 nM enzyme and 0.3–14 nM [^{3}H]FdUMP. Equilibrium binding mixtures were incubated for 6 h at 24 °C, and protein-bound [^{3}H]FdUMP was separated from free [^{3}H]FdUMP by centrifugation at 12,000 × *g* through a Microcon-10 filter (Amicon) and washed three times with TES buffer. The flow-through (500 μl) containing free [^{3}H]FdUMP was added to 5 ml of Scinti-Verse scintillation fluid (Fischer), and the radioactivity was quantitated. All data points were conducted in duplicate. Values of apparent dissociation constants (K_d) were determined by computer-assisted linear regression analysis of data graphed according to the Scatchard equation (35) and were the average of three separate determinations.

RESULTS

Construction of the Random Sequence Library—A library of 2×10^6 human (TS) mutants was created by random sequence mutagenesis. The human *TS* gene expressed in the plasmid, pGCHTS-TAA, was modified to include an *Mro*I restriction site at nucleotide 648. As illustrated in Fig. 1, a stuffer fragment was first inserted and was subsequently replaced by an insert containing random nucleotides. This allowed replacement of the targeted segment with a large nonfunctional stuffer frag-

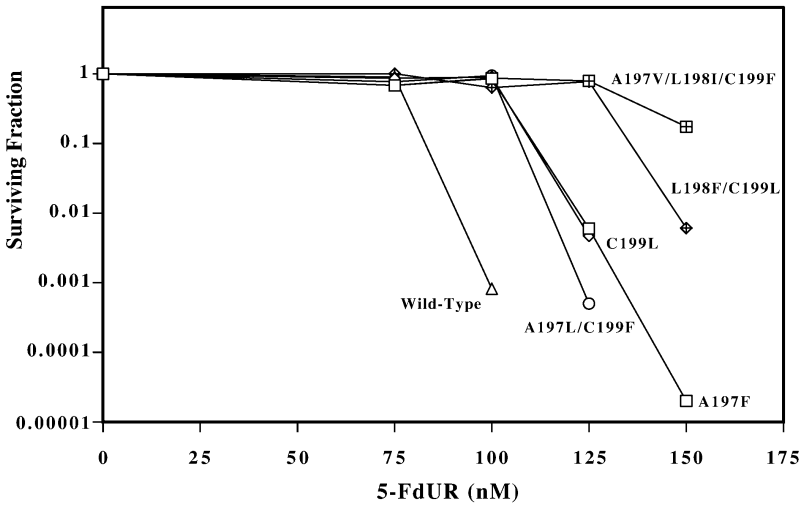

FIG. 4. **Survival of TS mutants incubated with 5-FdUR.** χ2913 cells expressing wild-type and TS mutants were grown on minimal media containing 5-FdUR for 36 h. Survival was determined by counting colonies at each dose of 5-FdUR and is expressed as a fraction of the survival of untreated cells. The triple mutant A197V/L198I/C199F is Mut 64.

ment. The insert was constructed by elongating a partially double-stranded oligonucleotide containing 39 random nucleotides using DNA polymerase. It was digested with restriction enzymes to produce staggered ends and ligated into the modified human TS plasmid to replace the stuffer fragment. The 39-base pair randomized region encoding for TS residues 196–199 and 204–212 was located on the C-terminal side of the catalytically active cysteine (Cys-195). Unlike the wild-type TS construct, the recombinant plasmid contains a silent *Sna*BI site that was used to confirm that any active clones with a wild-type DNA sequence are the result of selection from the library and not contamination.

Sequencing of Nonselected Clones to Determine Diversity of Library—Prior to selection, plasmid DNA was isolated from 35 transformed clones and sequenced. The number of substitutions per clone is presented in Fig. 2, and the types of substitutions are tabulated in Fig. 3. As seen in Fig. 2*A*, an average of 5.7 nucleotide changes and 4.3 amino acid changes per clone was detected. Amino acid substitutions in the nonselected clones were evenly distributed among the 13 residues encoded by the randomized nucleotides (Fig. 3*A*). 9 of the 35 nonselected clones analyzed (26%) contained at least one frameshift mutation, and 5 contained termination codons. The substitutions observed were in accord with the expected distribution in the plasmid population (36). Based on the number and frequency of random substitution, we calculated that the nonselected library should contain approximately 0.27% wild-type nucleotide sequences corresponding to 1% wild-type protein sequence (see "Discussion" for calculation). Therefore, it is not surprising that one wild-type molecule was detected among the 35 nonselected clones sequenced.

Selection and Sequencing of E. coli Expressing Active Thymidylate Synthase—The isolation of active enzymes from large plasmid libraries containing random sequences is facilitated by stringent positive genetic complementation. We first defined conditions under which there is a substantial difference in survival between bacteria expressing and not expressing active human thymidylate synthase. Whereas the wild-type TS construct was able to rescue the TS^- *E. coli* phenotype and formed colonies on minimal medium, *E. coli* harboring the stuffer vector that inactivates TS did not. Approximately 10% of the nonselected random library encoded functional enzymes (10^5 mutants). DNA from 39 mutants was sequenced without detecting any nucleotide substitutions or frameshift mutations. The corresponding amino acid substitutions are depicted in Fig. 3*B*. Wild-type protein sequence was observed in 11 clones (28%), and each contained the two silent mutations, indicating they did not result from contamination with wild-type plasmids.

TABLE II
Library size and percent wild type in the three mutant TS populations

Library	Library size (plasmid molecules)	Number clones sequenced	Wild-type protein
			%
Nonselected	2×10^6	35	1[a]
Active mutants	$\sim 10^5$	39	28
5-FdUMP selected	$\sim 10^2$	53	0

[a] Theoretical value.

Selection and Sequencing of E. coli Expressing Active TS Resistant to 5-Fluorodeoxyuridine—To isolate members of the random enzyme library demonstrating selective resistance to 5-FdUMP, an additional positive genetic selection was employed by supplementation of the minimal medium with a gradient of 5-FdUR concentrations. In preliminary experiments, we established that survival of the *E. coli* harboring wild-type TS was only modestly reduced to 90% at 75 nM 5-FdUR compared with controls in the absence of 5-FdUR. However, survival precipitously declined to 0.1% at 100 nM of the analog. No surviving wild-type clones were detected at dosages above 100 nM (Fig. 4). In contrast, approximately 0.1% of the *E. coli* harboring the random library formed colonies at 5-FdUR concentrations as high as 150 nM. In a typical experiment, colony formation by *E. coli* harboring plasmids with random inserts at 150 nM 5-FdUR was approximately 10^{-3} that of untreated *E. coli* (Table II). Individual colonies were isolated from plates containing 150 nM 5-FdUR ($n = 79$), and plasmid DNA was retransformed into fresh χ2913 to confirm the drug-resistant phenotype. DNA from those mutants that formed colonies upon a second exposure to 5-FdUR at a dose that was clearly lethal to the wild-type enzyme (125 or 150 nM, $n = 52$) was sequenced in the random region, and the corresponding amino acid changes were deduced (Fig. 3*C*). Although the wild-type enzyme was present in 28% of the active TS mutant library, no wild-type sequences were detected among the 5-FdUR-treated survivors. This suggests that we have indeed selected mutants that are more resistant to 5-FdUR than those encoded by the wild-type sequence.

Of the 52 mutants sequenced, no two mutants were identical at the nucleotide level. However, clones containing only the single mutation C199L or A197F were detected in multiple occurrences (6 and 2, respectively, Table III). All clones with multiple mutations at the amino acid level were unique. One mutant, Mut 64, demonstrated the highest survival at both 125

TABLE III

Human thymidylate synthase clones surviving 5-FdUR selection

The deduced amino acid changes in mutants resistant to 5-FdUR and the number of amino acid (AA) and nucleotide (nt) changes in each mutant are indicated. The wild-type sequence is indicated above the line, with the 13 randomized positions in underlined italics. In addition to the clones shown, one clone contained a mutation in the non-random sequence (Mut 18: P193S), yielding a total of 52 clones sequenced.

Clone number	195 C	196 *H*	197 *A*	198 *L*	199 *C*	200 Q	201 F	202 Y	203 V	204 *V*	205 *N*	206 *S*	207 *E*	208 *L*	209 *S*	210 *C*	211 *Q*	212 *L*	Number of mutations	
																			AA	nt
14	—	—	—	—	—	—	—	—	—	D	—	—	—	—	—	—	—	—	1	6
19	—	—	—	—	L	—	—	—	—	—	—	—	—	—	—	—	—	—	1	2
20	—	—	—	—	L	—	—	—	—	—	—	—	—	—	—	—	—	—	1	2
27	—	—	F	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1	4
31	—	—	—	—	L	—	—	—	—	—	—	—	—	—	—	—	—	—	1	4
34	—	—	—	—	L	—	—	—	—	—	—	—	—	—	—	—	—	—	1	2
59	—	—	—	—	—	—	—	—	—	A	—	—	—	—	—	—	—	—	1	2
69	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	L	—	1	2
73	—	—	—	—	—	—	—	—	—	L	—	—	—	—	—	—	—	—	1	3
75	—	—	—	—	L	—	—	—	—	—	—	—	—	—	—	—	—	—	1	3
76	—	—	—	—	L	—	—	—	—	—	—	—	—	—	—	—	—	—	1	3
87	—	—	F	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1	3
8	—	—	L	—	F	—	—	—	—	—	—	—	—	—	—	—	—	—	2	5
10	—	—	—	—	F	—	—	—	—	T	—	—	—	—	—	—	—	—	2	8
11	—	—	—	—	—	—	—	—	—	—	D	R	—	—	—	—	—	—	2	6
12	—	—	F	—	—	—	—	—	—	—	—	R	—	—	—	—	—	—	2	4
21	—	—	—	—	I	—	—	—	—	L	—	—	—	—	—	—	—	—	2	5
22	—	—	—	—	L	—	—	—	—	—	—	R	—	—	—	—	—	—	2	7
24	—	—	—	—	L	—	—	—	—	—	—	—	—	—	—	—	S	—	2	6
35	—	—	—	F	L	—	—	—	—	—	—	—	—	—	—	—	—	—	2	3
36	—	—	L	—	F	—	—	—	—	—	—	—	—	—	—	—	—	—	2	5
37	—	—	—	—	L	—	—	—	—	A	—	—	—	—	—	—	—	—	2	4
39	—	—	—	—	I	—	—	—	—	M	—	—	—	—	—	—	—	—	2	6
40	—	—	—	—	L	—	—	—	—	E	—	—	—	—	—	—	—	—	2	5
41	—	—	—	—	L	—	—	—	—	—	—	R	—	—	—	—	—	—	2	5
46	—	—	—	—	I	—	—	—	—	M	—	—	—	—	—	—	—	—	2	6
48	—	—	—	—	L	—	—	—	—	—	—	R	—	—	—	—	—	—	2	5
52	—	—	—	—	I	—	—	—	—	G	—	—	—	—	—	—	—	—	2	5
67	—	—	—	—	L	—	—	—	—	L	—	—	—	—	—	—	—	—	2	3
78	—	—	—	F	—	—	—	—	—	R	—	—	—	—	—	—	—	—	2	4
92	—	—	—	—	—	H	—	—	—	M	—	—	—	—	—	—	—	—	2	6
101	—	—	—	—	L	—	—	—	—	—	—	N	—	—	—	—	—	—	2	7
68	—	—	—	—	—	—	—	—	—	—	—	—	H	—	—	—	L	—	2	3
53	—	—	K	V	—	—	—	—	—	—	—	—	—	—	—	—		—	2	4
6	—	—	—	F	—	—	—	—	—	G	—	—	—	—	—	—	H	—	3	4
7	—	—	—	—	L	—	—	—	—	M	—	G	—	—	—	—	—	—	3	7
13	—	—	S	F	—	—	—	—	—	L	—	—	—	—	—	—	—	—	3	9
32	—	—	—	F	L	—	—	—	—	G	—	—	—	—	—	—	—	—	3	7
33	—	—	—	F	—	—	—	—	—	G	—	—	—	—	—	—	H	—	3	4
64	—	—	V	I	F	—	—	—	—	—	—	—	—	—	—	—	—	—	3	3
70	—	—	F	—	—	—	—	—	—	A	—	N	—	—	—	—	—	—	3	5
80	—	—	—	—	L	—	—	—	—	M	—	C	—	—	—	—	—	—	3	4
94	—	—	F	—		—	—	—	—	—	K	G	—	—	—	—	—	—	3	5
97	—	—	—	—	L	—	—	—	—	E	—	R	—	—	—	—	—	—	3	7
74	—	—	—	—	L	—	—	—	—	—	—	G	Q	—	—	—	—	—	3	4
38	—	—	—	I	—	—	—	—	—	A	—	R	—	—	—	—	H	—	4	7
84	—	—	—	—	L	—	—	—	—	M	—	G	—	—	—	—	H	—	4	5
88	—	—	—	—	L	—	—	—	—	L	Q	—	—	—	Y	—	—	—	4	6
98	—	—	—	—	L	—	—	F	—	L	—	N	—	—	—	—	—	—	4	4
82	—	—	—	—	L	—	—	—	—	L	—	G	—	—	F	S	—	—	5	6
99	—	—	C	—	F	—	—	—	—	G	—	G	—	—	—	S	—	—	5	7
																			2.2	4.7[a]

[a] Average (n = 52).

and 150 nM (Fig. 4) and was found to include three adjacent mutations: A197V, L198I, and C199F.

The spectrum of mutations observed in the 5-FdUR-selected library differed from the spectrum of active mutants at multiple key amino acid residues, most notably Ala-197 and Cys-199. Among the active TS library, Ala-197 was found to be mutable to Thr, Val, and Ser. Notably, A197F was not detected, indicating that it was not selected solely on the basis of activity. By contrast, A197F was found in 5 of 11 (45%) mutants in the 5-FdUR-selected library. Similarly, Cys-199 was altered to Tyr, Ser, Ile, or Phe in the active mutant library, whereas in the drug-resistant library, the mutation C199L was detected in 24 of the 52 (46%) 5-FdUR-resistant clones. The nucleotide substitutions observed generating the C199L mutations were predominantly double (and one triple) nucleotide changes. Three clones contained only the single mutation C199L, yet each of the three differed by the presence of silent nucleotide changes.

Purification and Kinetic Analysis of Wild-type and Mut 64 Thymidylate Synthase—To study the mechanism of FdUR resistance, we purified wild-type and Mut 64 enzymes and conducted kinetic analysis. Both were subcloned, resequenced, and expressed in *E. coli* as N-terminal fusions with a histidine tag. This allowed a one-step purification using nickel chelation chromatography. SDS-PAGE analysis demonstrated a single predominant TS band at 36 kDa with an estimated purity of the wild-type enzyme of >95% and Mut 64 TS >85% (Fig. 5). The mutant enzyme preparation contained two minor protein bands of approximately 29 and 9 kDa. The sum value of their molecular mass (~38 kDa) as well as their equimolar ratio indicate they may represent TS degradation products.

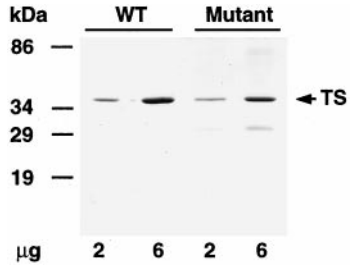

FIG. 5. **Analysis of purified TS by 12% SDS-PAGE.** Preparations of the wild-type (WT) and the triple mutant, Mut 64, were purified by Ni^{2+} chelation chromatography. Either 2 or 6 μg of each purified protein was subjected to SDS-PAGE and detected by Coomassie Blue staining.

As seen in Table IV, although kinetic parameters of Mut 64 for the normal substrates were nearly identical to that of the wild-type enzyme, the dissociation constant for 5-FdUMP was nearly 20 times greater. The K_m value for dUMP for Mut 64 (2.5 ± 0.4 μM) was not significantly different than that of the wild-type enzyme (1.8 ± 0.7 μM); the K_m for CH_2H_4-folate (28 ± 9 μM) was at most marginally increased in the mutant enzyme relative to wild-type TS (14 ± 5 μM). The steady-state rate of the reaction, k_{cat}, was found to be essentially unchanged in the FdUR-resistant mutant relative to the wild-type protein (Table IV). k_{cat}/K_m ratios of both dUMP and CH_2H_4-folate demonstrate that the triple mutant is an equally efficient enzyme compared with the wild type. To determine the relative binding of the covalent inhibitor 5-FdUMP into TS, equilibrium binding studies measuring the formation of the ternary complex were conducted. A Scatchard plot of the binding data (Fig. 6) demonstrated that both TS forms contain a single class of FdUMP binding sites. The K_d values were determined by averaging three different experiments, and the mutant form was found to have a K_d for FdUMP of $2.3 \pm 0.9 \times 10^{-9}$ M, whereas the wild-type K_d was determined to be $1.1 \pm 0.4 \times 10^{-10}$ M (Table IV). The 20-fold increase in K_d of Mut 64 is likely representative of the increased discrimination of the mutant between uridylate and its fluorinated counterpart. These kinetic data indicate that Mut 64 is functionally a quite similar enzyme to the wild type, differing primarily in its behavior toward 5-FdUMP.

TABLE IV
Rate constants for the wild-type and mutant forms of TS

Parameter	Wild type (n)[a]	Mutant 64 (n)[a] (A197V,V198I,C199F)
K_m dUMP[b]	1.8 ± 0.7 μM (3)	2.5 ± 0.4 μM (3)
K_m CH_2H_4-folate[c]	14 ± 5 μM (3)	28 ± 9 μM (3)
k_{cat}[b]	3.9/s	3.4/s
k_{cat}/K_m dUMP[d]	2.2 μM^{-1} s^{-1}	1.4 μM^{-1} s^{-1}
k_{cat}/K_m CH_2H_4-folate[d]	0.28 μM^{-1} s^{-1}	0.12 μM^{-1} s^{-1}
K_d FdUMP	$1.1 \pm 0.4 \times 10^{-10}$ M (3)	$23 \pm 9 \times 10^{-10}$ M (3)

[a] n = number of independent determinations.
[b] Determined at 150 μM CH_2H_4-folate.
[c] Determined at 100 μM dUMP.
[d] k_{cat} divided by the average K_m value.

DISCUSSION

As a first step toward creating novel TS variants, we used random sequence mutagenesis to alter the active site of the human TS. We generated a large library of TS substitutions spanning residues 196–199 and 204–212. Based on the crystal structure of the *E. coli* and human TS, many of these residues are likely to form the wall of the active site cavity, and, therefore, amino acid substitutions are likely to cause subtle changes in binding pyrimidine-based analogs. Absolutely conserved residues, such as Pro-194 and Cys-195, were left unaltered. Although completely conserved in nature, His-196 has been mutated in both *E. coli* (His-147) and *L. casei* (His-199) to non-bulky residues, resulting in active enzymes that function in a genetic complementation assay (15, 16). Hence, His-196 was included in the randomized region. To emphasize the most critical residues, the number of random substitutions should be minimized. Residues 200–203 were left unrandomized because they are either absolutely (Gln-200 and Val-203) or highly (Phe-201 and Tyr-202) conserved, allowing for a contiguous stretch of four residues to remain unaltered. To our knowledge, among residues in our random region, only His-196 has been subjected to mutagenesis by other groups (15, 16). No alterations in this residue have been screened for 5-FdUR resistance.

Thymidylate synthase has been subjected to extensive mutagenesis by many laboratories. A thorough review of these active mutants has been conducted by Carreras and Santi (19). However, less is known about alterations that can confer selective resistance to 5-FdUR. Zhang *et al*. (37) have created a mutation in a conserved loop guarding the active site of the mouse TS (R44V), which demonstrates a large reduction in the binding of both dUMP and FdUMP, and a 100-fold reduction in the catalytic activity (37). Although several authors have reported the discovery of altered TS molecules that demonstrate 5-FdUR resistance (38–43), in only one such mutant has the substituted amino acid been identified. A 5-FU-resistant HCT116 colonic tumor cell line was found to encode for the single mutant Y33H. This mutant has a K_d value approximately 3–4-fold higher than the wild-type enzyme in the absence of any large alterations in catalytic efficiency or affinity for dUMP or CH_2H_4-folate (44).

Substitutions That Retain Activity Differed in Many Respects Compared with the Nonselected Random Library—The members of the random library that were selected by their ability to complement a TS^- *E. coli* contained on average far fewer amino acid substitutions than the nonselected library (Fig. 2, *A* and *B*). This is probably a reflection that a large number of substitutions are intolerable in the active site of TS. All frameshift and nonsense mutations were eliminated (Fig. 3, *A* and *B*). Wild-type protein sequence was observed in 28% of the clones, approximately 20 times that observed in the nonselected library. In addition, differential survival of the library on medium with and without thymidine demonstrated that approximately 90% of the nonselected library is unable to complement the TS^- *E. coli*. Both pieces of data indicate the active mutant library contains approximately 10^5 unique clones. Compared with the nonselected library, the substitutions catalogued in the selected population were more conservative. However, all residues in the random region, with the exception of His-196 and Cys-210, tolerated substitutions, indicating the high degree of plasticity of the selected region. Substitutions at His-196 may not have been detected in our sequencing of 39 of approximately 10^5 TS variants, and two substitutions observed in Cys-210 in the drug-selected library indicate this residue is not immutable. By far, most of the amino acid substitutions were the result of single nucleotide substitutions (44 of 53 substitutions), as expected by the greater probability of creating one nucleotide change relative to two or more.

The 5-FdUR-selected Library Is Further Restricted—In the drug-treated library, the average number of amino acid substitutions is greater than that in the active library (mean = 2.2 *versus* 1.4). Not only is the wild type eliminated, but there are a greater proportion of double mutants (Table III, Fig. 2*C*). The 5-FdUR-selected library essentially consists of only a small subset of the active library mutant population; there was approximately a 1000-fold killing observed between the non-drug-treated active mutant library and the library exposed to 150 nM

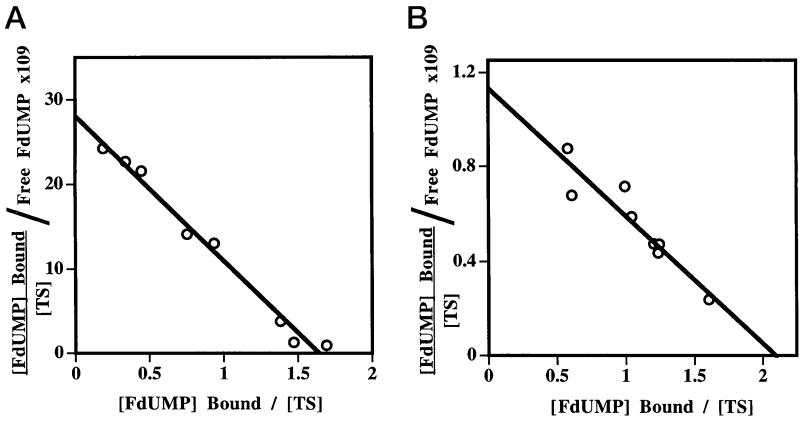

FIG. 6. **Determination of the dissociation constant for FdUMP binding to wild-type and Mut 64 TS.** Purified wild-type TS (3.5 nM) or Mut 64 (10 nM) was incubated for 6 h at 24 °C with 150 μM CH_2H_4-folate and increasing amounts (0.3–14.4 nM) of [6-^{3}H]FdUMP. The TS-CH_2H_4-folate-[6-^{3}H]FdUMP complex was resolved as described under "Experimental Procedures." Values of dissociation constant, K_d, were inferred from the negative reciprocal of slopes of data plotted according to the Scatchard equation (35). Shown are typical plots obtained with wild-type TS (*A*) and Mut 64 (*B*).

5-FdUR. In addition, the spectrum of mutations differed. For example, C199L was not detected in any of the 39 clones sequenced from the active library, yet it was present in 46% of the 5-FdUR-selected library (24 of 52 clones). The frequency of double and triple nucleotide substitutions at residue Cys-199 demonstrates the stringency of 5-FdUR selection. Any alteration in Cys-199 resulting in a leucine (TGC to CTN, TTA, or TTG) requires at least two mutations. The number of double nucleotide mutations that create a C199L alteration in a library of 10^5 active clones can be calculated using the equation $P_{C\rightarrow L} = \Sigma\ P_{TGC\rightarrow CTN} + P_{TGC\rightarrow TTA} + P_{TGC\rightarrow TTG} = (r/3)^2(1) + (1 - r)(r/3)^2 + (1 - r)(r/3)^2 = 0.0060$, where $P_{C\rightarrow L}$ is the probability of residue 199 being altered to leucine, $P_{TGC\rightarrow NNN}$ is the probability of a given double nucleotide alteration, and r is the percent randomness, *i.e.* the probability that one particular nucleotide is substituted for any of the three remaining bases (14%). From a library of 10^5 active mutants, we would expect only 600 clones to contain a C to L substitution (0.6%); yet, we have detected 24 clones in the drug-resistant library after sequencing just 52 survivors (46%). Even more striking is the number of clones that contain only the single substitution alteration C199L. Assuming that there are on average 3 codons per amino acid, $a_x = 1 - (1 - r)^2\ (1 - r/3) = 0.295$, where a_x is the probability of substitution of an amino acid at a given residue (36) and r is 14% as described above. In the 13 randomized residues, a wild-type protein will occur at a frequency of $(1–0.295)^{13} = 1\%$. The probability of obtaining a clone with only the mutation C199L would be $(1–0.295)^{12}\ (0.0060) = 9 \times 10^{-5}$. In a library of 10^5 mutants, we expect only 9 clones to contain only the C200L mutation in the absence of selection (0.0006%). Remarkably, we have detected six unique C200L mutants in our sequencing of just 52 drug-resistant mutants (12%). Parallel arguments can be made for substitutions for other residues, indicating that over-representation of certain amino acid substitutions in the drug-selected library is associated with increased ability to survive 5-FdUR treatment in *E. coli* .

Kinetic Analysis of Mut 64 Reveals Decreased Binding of FdUMP Relative to the Wild Type—Of the TS variants surveyed, the triple mutant Mut 64 was the most resistant to 5-FdUR. Not only was its survival rate consistently higher at each drug dose, the colony size of *E. coli* harboring Mut 64 was on average larger and less heterogeneous than that formed by *E. coli* expressing the other mutants. This mutant carried alterations in three adjacent residues next to the active site conserved PCH sequence. The substitutions, A197V/L198I/C199F, were conservative relative to hydrophobicity. Valine occurs naturally in position 197 in Saimirine herpesvirus I, Ateline herpesvirus 2, and *Candida albicans* TS enzymes (19). Although Ile-198 has not been reported in nature, it is a conservative change. Phenylalanine at position 199 is found in both *E. coli* and *Bacillus subtilis* TS.

The similarity in K_m values for both dUMP and the cosubstrate CH_2H_4-folate between Mut 64 and the wild type indicate that the triple alterations in Mut 64 do not significantly affect interactions with either substrate. In addition, the k_{cat} of Mut 64 is only slightly decreased compared with the wild type. In fact, the overall efficiency of the mutant enzyme, as measured by the k_{cat}/K_m ratio, indicates that Mut 64 is very similar to that of the wild-type enzyme (Table IV). Yet, equilibrium binding studies have determined that Mut 64 displays a marked decrease in the dissociation constant compared with wild type. The K_d obtained for the wild-type TS ($1.1 \pm 0.4 \times 10^{-10}$ M) is in agreement with the published value (38). The K_d for Mut 64 ($2.3 \pm 0.9 \times 10^{-9}$ M) is 20 times that of the wild-type enzyme, indicating that alteration of the three adjacent residues may alter the structure of TS in subtle ways such that the enzyme can discriminate between the fluorinated and non-fluorinated pyrimidine ring.

Potential Applications—Random sequence mutagenesis coupled to a positive genetic selection provides a novel approach to creating altered TS molecules with desired properties; here we have presented the engineering and identification of mutants of TS that demonstrated resistance to 5-FdUR. An analogous approach using random mutagenesis and genetic complementation in *E. coli* can be developed for other clinically relevant TS inhibitors as they are developed. Although site-directed mutagenesis of TS has been used to identify residues required for catalytic activity, this approach is limited because residues are ultimately tested one at a time, and we lack rules to predict the effects of multiple substitutions. Effective site-directed mutagenesis requires detailed knowledge about amino acid interactions and predictions regarding the effects of specific alterations. Random mutagenesis provides a combinatorial alternative in which a detailed understanding of the changes necessary to create variants is not required.

Despite its efficacy in the treatment of many human malignancies, the use of 5-FU has been limited by toxicity to bone marrow, gastrointestinal, and other tissues (22). The introduction and expression of variants of TS that function in the presence of systemic 5-FU treatment could protect normal cells from the cytotoxic side effects of this drug or conversely allow

the use of increased amounts of 5-FU to be safely administered. In addition, understanding the mechanisms by which TS can become 5-FU resistant is of importance not only for potential uses in gene therapy; tumors have been identified that are refractory to treatment with 5-FU due to mutations (39–43) and overexpression of TS (45–47). A better understanding of the structural basis of drug resistance could perhaps lead to the design of more clinically effective pyrimidine or anti-folate inhibitors of TS.

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