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Genetic Complementation and Resistance to 5-Fluoro-2′-deoxyuridine in Thymidine Auxotrophs Expressing a Highly Defective Mutant of Human Thymidylate Synthase

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ABSTRACT. A mutant human thymidylate synthase (TS) has been created in which a glutamine residue at position 214 has been replaced by glutamate. Glutamine at position 214 is postulated to be involved in maintaining the enzyme in a conformation that facilitates the binding of the substrate dUMP. Although the k_{cat}/K_m of the mutant protein for the substrate, dUMP, is 10^3 lower than that of wild-type TS, the mutant TS confers thymidine prototrophy on a TS-deficient bacterial strain when expressed at high levels. In the present investigation, a TS-deficient Chinese hamster lung cell line was transfected with DNA encoding the defective protein. Thymidine prototrophs were isolated that expressed the defective protein at levels that were physiologically relevant. The activities of the enzymes expressed endogenously in representative prototrophs were consistent with the activities observed for the purified proteins. At similar levels of TS expression, thymidine prototrophs expressing Glu214 TS were 8-fold more resistant to 5-fluoro-2′-deoxyuridine (FdUrd) cytotoxicity than are prototrophs expressing Gln214 TS. FdUrd is a prodrug of the tight-binding TS inhibitor, 5-fluoro-2′-deoxyuridine-5′-monophosphate (FdUMP). The resistance to FdUrd was associated with a significant decrease in the binding of FdUMP to the purified mutant enzyme. The data are consistent with the interpretation that TSs that are highly defective are capable of sufficient dTMP production for cell survival and optimal growth, yet may confer resistance to TS-directed inhibitors. BIOCHEM PHARMACOL **58**;6:973–981, 1999.

KEY WORDS. thymidylate synthase; thymidine; protein deficiency; floxuridine; drug resistance; gene transfer

The enzyme TS** (EC 2.1.1.45) catalyzes a reaction in which a nucleotide, dUMP, and a folate, CH_2H_4folate, are substrates. During the reaction, a proton at the 5-position of dUMP is replaced with the methylene group of CH_2H_4folate. The methylene group is reduced to a methyl group by a hydride ion from the pterin ring of CH_2H_4folate, resulting in formation of the nucleotide dTMP [1, 2]. The enzymatic reaction provides the sole *de novo* source of dTMP, a precursor required for DNA biosynthesis. Because of its pivotal role in DNA biosynthesis, TS is a target for chemotherapeutic intervention in neoplastic and parasitic disease. The 5-fluoropyrimidine drugs, FUra and FdUrd, have been utilized in the therapy of cancers of the breast, gastrointestinal tract, and head and neck. Whereas these agents are cytotoxic through several mechanisms of action, a major action associated with clinical response is inhibition of TS (reviewed in Ref. 3). The fluoropyrimidines are metabolized to the nucleotide, FdUMP, which is an analog of dUMP. It is postulated that FdUMP proceeds along the same reaction pathway as dUMP and that CH_2H_4folate functions similarly with either nucleotide [4, 5]. At the step in which a proton at the 5-position of dUMP is abstracted to initiate product formation and release, the substitution of a fluorine atom at this position in FdUMP precludes further catalytic progress, resulting in the formation of a ternary complex devoid of catalytic activity. It is presumed that the inhibitory ternary complex is analogous in structure to an intermediate formed among TS, dUMP, and CH_2H_4folate during catalysis. The inhibitory ternary complex is highly stable since it is comprised of two covalent linkages, one between an enzyme cysteine residue and the 6-position of FdUMP and the second between the methylene group of CH_2H_4folate and the 5-position of FdUMP [4–6].

Upon formation of either the catalytic or the inhibitory ternary complex, TS undergoes large changes in conforma-

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** *Abbreviations*: TS, thymidylate synthase; CH_2H_4folate, 5,10- methylenetetrahydrofolate; FUra, 5-fluorouracil; FdUrd, 5-fluoro-2′-deoxyuridine; FdUMP, 5-fluoro-2′-deoxyuridine-5′-monophosphate; dUrd, 2′-deoxyuridine; DMEM, Dulbecco's modified Eagle's medium; CHLTS$^-$, Chinese hamster lung RJK88.13 cell line deficient in thymidylate synthase; and K_d, dissociation constant.

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tion. This has been deduced by spectroscopic, hydrodynamic, and X-ray crystallographic analyses [4, 5, 7, 8]. During the conformational change, numerous residues are displaced toward the active site, thus compressing the enzyme onto the ligands. This displacement has been termed segmental accommodation [8]. One region of the enzyme that is postulated to function as a hinge during residue displacement is designated the β-kink [8]. This is a region of the β-sheet backbone of TS in which a bulge occurs in three of the β-sheets that form the central core of the polypeptide [9, 10]. The highly conserved Gln214 of human TS is located at the bulge in one of the β-sheets [11, 12]. Recent studies of mutant proteins with substitutions at position 214 indicated that this residue is involved in maintaining the enzyme in a conformation that is important in nucleotide binding and catalysis [13].

The mutant protein with a glutamate residue at position 214 is of particular interest since glutamate and glutamine have similar side chain volumes but differ in hydrogen bonding and electrostatic interactions. Expression of the mutant protein in a TS-deficient strain of *Escherichia coli* revealed that the protein is catalytically active, since bacterial transformants are capable of growth in medium lacking thymidine; however, analysis of kinetic constants of the mutant protein revealed that the k_{cat}/K_m for dUMP is decreased by 10^3, relative to that of Gln214 TS [13]. That a highly defective mutant protein confers thymidine prototrophy on a TS-deficient bacterial transformant is presumably a consequence of high levels of expression of the protein. The effects of expression of the mutant protein under conditions of physiological relevance have been investigated in a TS-deficient mammalian cell line transfected with cDNAs encoding either Gln214 or Glu214 TS. The data revealed that transfectants expressing Glu214 TS can be isolated, albeit at a lower frequency than transfectants expressing Gln214 TS. Moreover, transfectants expressing Glu214 TS were significantly more resistant to FdUrd, a prodrug of FdUMP, than were transfectants expressing Gln214 TS.

MATERIALS AND METHODS

Materials

[5-^{3}H]dUMP (20–23 Ci/mmol), [5-^{3}H]dUrd (20 Ci/mmol), and [6-^{3}H]FdUMP (20 Ci/mmol) were obtained from Moravek Biochemicals. Bovine calf serum supplemented with iron was obtained from HyClone Laboratories. (6*RS*) CH_2H_4folate was prepared as described previously [14]. Diaminobenzidine, FdUMP, FdUrd, folinic acid, thymidine, dUMP, and neutral red dye were obtained from the Sigma Chemical Co.

Mutagenesis

Plasmid pKB169, containing the human TS cDNA, was obtained from F. G. Berger (University of South Carolina) [15]. The bacterial expression vector pTS080, which contains the human TS cDNA under the transcriptional control of the T7 RNA polymerase promoter, was provided by W. S. Dallas (Wellcome Research Laboratories) [16]. A G to C mutation was produced at position 640 in the TS cDNA sequence by site-specific mutagenesis using the oligonucleotide, 5′-CCCGATCTCTCGTACAGCTG-3′, which is complementary to the sense strand of human TS cDNA at positions 631 to 650 [17]. For mutagenesis of pKB169, the T7-InVitrogen mutagenesis kit (US Biochemical Corp.) was utilized. Transformants expressing the mutated plasmid derived from pKB169, designated pJZ199, were isolated by screening with oligonucleotides complementary to positions 631 to 650 of the sense strand, which contain either a G or C at position 640. The Transformer site-directed mutagenesis kit (CLONTECH Laboratories) was utilized for mutagenesis of pTS080 and for selection of mutated TS cDNAs. All mutated plasmids were sequenced by dideoxy chain termination using modified T7 DNA polymerase (US Biochemical Corp.) to confirm the presence of the mutation [18].

Plasmid Construction

The wild-type and mutant TS cDNAs were excised by digestion of pKB169 and pJZ199 with the restriction endonucleases *Acc*I and *Hpa*I. The *Acc*I/*Hpa*I fragments were cloned into the eukaryotic expression vectors pSV2 and pMAMneo (CLONTECH Laboratories), as described previously [15]. The TS cDNAs were inserted in the normal and reverse orientations in pMAMneo. pSV2 contains an SV40 early promoter, whereas pMAMneo contains an RSV-enhancer-activated MMTV promoter and a selectable marker gene, neomycin phosphotransferase. The designations of the plasmids resulting from insertion of TS cDNA in the normal orientation, relative to the promoter, are shown in Table 1.

Cell Cultivation

The Chinese hamster lung cell line RJK88.13 is a subline of V79 cells that is deficient in TS [19] (provided by R. L. Nussbaum, National Human Genome Research Institute). Parental V79 cells were maintained as monolayers in DMEM (Gibco Laboratories) supplemented with 10% fetal bovine serum; CHLTS$^-$ cells were maintained in the same medium supplemented with FACT (0.02 μM folinic acid, 1 μM aminopterin, 0.1 μM cyanocobalamin, and 10 μM thymidine). The FACT-supplemented medium prevents the growth of cells that have reverted to thymidine prototrophy [20]. Cells were monitored routinely for the absence of *Mycoplasma* by the Mycotrim detection system (New England Nuclear).

Selection and Subcloning of Transfectants

Prior to DNA transfections, cells were cultured for 6 days in DMEM containing 10% fetal bovine serum and 10 μM

TABLE 1. Cell lines derived from Chinese hamster lung TS-deficient sublines transfected with human TS cDNA

Promoter	Plasmid	Transfectant	Residue at 214*	Clones/Population†
MMTV	pKB180§	CHL-MhTSQ	Gln	150
MMTV	pJZ203	CHL-MhTSE	Glu	50
SV40‡	pJZ205	CHL-ShTSQ	Gln	150
SV40	pJZ207	CHL-ShTSE	Glu	100

*Amino acid residue at position 214 in human TS polypeptide.
†Number of cell clones arising from the selection process that comprise the mass population.
‡Early promoter.
§Obtained from F. Berger, University of South Carolina.

thymidine. Cells (4×10^5) were transfected by calcium-phosphate precipitation [21] using 20 μg of plasmid DNA. Controls were transfected with vector containing no TS cDNA or TS cDNA inserted in the reverse orientation relative to the promoter. After transfection, cells were grown for 48 hr in medium containing 10 μM thymidine and 10 μM folinic acid before shifting to selective medium. Cells transfected with pMAMneo constructs were subjected to a three-step selection procedure. Cells were cultured initially in medium containing 800 μg/mL of Geneticin (Gibco Laboratories), 10 μM thymidine, and 10 μM folinic acid. Cells surviving in this medium were selected in medium containing 10 μM folinic acid. Finally, cells were selected in medium with no drug supplements. Cells transfected with pSV2 constructs were subjected to a two-step selection procedure. Cells were cultured initially in medium containing 10 μM folinic acid, and then in medium with no drug supplements. Cell clones surviving the selections were combined and maintained as mass populations in DMEM supplemented with 10% bovine calf serum. Transfectant populations were subcloned by dilution in 96-well microplates (Costar) utilizing maintenance medium containing 33% conditioned medium and 15% fetal bovine serum.

Cytotoxicity

Cells (500 cells/well) were placed into 96-well microplates in DMEM supplemented with dialyzed 10% bovine calf serum. After 24 hr, the medium was replaced with medium supplemented with 0.05 to 100 nM FdUrd. Growth was continued for 5–6 cell generations. Cell growth was determined by a modification of a neutral red dye assay [22]. Briefly, culture medium was removed and replaced with DMEM supplemented with 40 mM HEPES, pH 6.6, 3% DMSO, and 0.02% (w/v) neutral red dye. After incubation for 2 hr, samples were analyzed as described previously [22].

Immunoblot Analysis of TS

Cells in logarithmic growth were harvested in phosphate-buffered saline (~1×10^8 cells/mL). Cell pellets were suspended in ice-cold 100 mM Tris–HCl, pH 7.4, containing 20 mM 2-mercaptoethanol, 25 mM $MgCl_2$, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 50 μg/mL of aprotinin, 20 μg/mL of leupeptin, 10% glycerol, and 0.01% Triton X-100. After sonication, the suspension was centrifuged at 25,000 *g* for 1 hr at 4°. Protein was determined by the Bradford method using bovine serum albumin as a protein standard [23]. Extract protein (150–340 μg) was separated on 10% SDS-polyacrylamide gels, transferred to Immobilon-PVDF membranes (Millipore), and detected as described previously [13]. The intensity of immune complexes was determined by densitometric scanning.

Isoelectric Focusing Gel Electrophoresis

Cell extracts were prepared as described for immunoblot analyses. Complexes were prepared among TS in cell extracts, (6*RS*) CH_2H_4folate, and [^{32}P]FdUMP as described previously [14]. The complexes were separated by isoelectric focusing gel electrophoresis as described previously [14].

TS Level Determination by FdUMP Binding

TS levels in cell extracts were determined by modification of a previously described procedure [24]. Cell extracts were prepared as described for immunoblot analyses. Extracts (50 μg of soluble protein) were incubated in 50 mM Tris–HCl, pH 7.4, containing 150 mM KCl, 20 mM 2-mercaptoethanol, 160 μg/mL of bovine serum albumin, 6 mM CH_2O, 400 μM (6*RS*) CH_2H_4folate, and 0.25 μM [5-^{3}H]FdUMP. Samples were incubated for 2–4 hr at 30°. Unbound label was removed by adsorption onto charcoal, and protein-bound radiolabel was determined by liquid scintillation counting. Nonspecific binding was determined by incubating the extracts with a 500-fold excess of unlabeled FdUMP. TS levels for cells expressing Gln214 TS were calculated by using the assumption that 1.7 mol of FdUMP are bound per mol of TS. TS levels for cells expressing Glu214 TS were estimated from the FdUMP binding data by fractional saturation analysis:

$$\textit{Fraction [FdUMP] Bound} = \frac{\dfrac{[FdUMP]x}{[FdUMP] + K_{d1}} + \dfrac{[FdUMP]y}{[FdUMP] + K_{d2}}}{x + y}$$

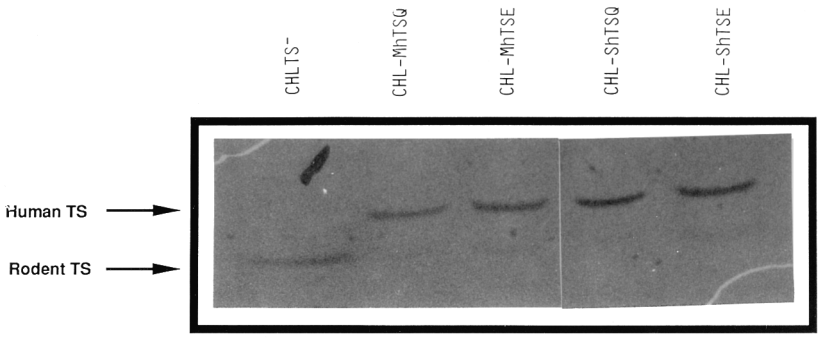

FIG. 1. Immunoblot analysis of TS in cell extracts. Cell protein (340 μg, CHLTS⁻; 170 μg, transfectants) was separated on 10% SDS-polyacrylamide gels, transferred to PVDF membranes, and detected by successive incubation with murine anti-human TS monoclonal antibody, goat anti-murine IgG-peroxidase, and diaminobenzidine/H_2O_2 as described in Materials and Methods. The arrows denote the migration of the human and hamster enzymes. Cell designations are explained in Table 1.

where x = fraction of binding sites with K_{d1}, and y = fraction of binding sites with K_{d2}. The values for x, y, K_{d1}, and K_{d2} were determined from equilibrium binding studies utilizing purified Glu214 TS.

TS Activity

Cell extracts were prepared as described for immunoblot analysis. TS activity in extracts was determined by a tritium release assay utilizing [5-^{3}H]dUMP as described previously [25]. For transfectants expressing Glu214 TS, the concentration of dUMP in the assay was increased to 2 mM. Calculation of k_{cat} values was based on enzyme quantitation by immunoblot analysis.

TS Purification

The TS-negative strain of *E. coli*, TX61 (provided by W. S. Dallas, Wellcome Research Laboratories), was transformed with pTS080 constructs containing DNA encoding either Gln214 or Glu214 TS. Recombinant human TS was purified to apparent homogeneity as described previously [13]. Subsequent to purification, protein fractions were analyzed by 12% SDS-polyacrylamide gels.

FdUMP Dissociation Constant

Apparent dissociation constants for the binding of FdUMP into a ternary complex with purified TS and (6*RS*) CH_2H_4folate were determined as described previously [25], except that 0.1 to 20 μM [6-^{3}H]FdUMP was utilized in the studies of Glu214 TS. The equilibrium binding data were fit to one- and two-site binding models of the Langmuir binding isotherm with the software program SlideWrite Plus 5.0. The data were linearized by the method of Scatchard utilizing the software program SigmaPlot 3.0 [26].

RESULTS

To investigate the effect of expression of a highly defective TS on cell survival, a Chinese hamster lung cell line was utilized that exhibits thymidine auxotrophy and no detectable TS activity [19]. Of particular importance to the use of this subline as a functional assay system for TS is the observation that the thymidine auxotrophy is phenotypically stable during long-term cultivation in nonselective medium [19]. FdUMP binding studies revealed that the cells exhibited no detectable FdUMP binding activity (data not shown). Cells were transfected with human TS cDNAs expressed from either a MMTV or SV40 promoter. Transfectants were selected in two or three steps to maximize the recovery of thymidine prototrophs. Transfectants were grown for a minimum of 2 weeks at each step. Folinic acid was included in the selections since high levels of exogenous folate have been reported to support the growth of cells expressing mutant TSs in the absence of thymidine [27]. No significant loss of transfectants was observed when folinic acid was removed from the selection medium. Cells transfected with pMAMneo constructs containing TS cDNA in the reverse orientation survived only in medium containing thymidine. The number of transfectant clones isolated in the absence of thymidine is shown in Table 1. Clones arising from cells transfected with cDNA encoding Glu214 TS occurred at a lower frequency, regardless of the expression system. Clones obtained from each transfection were combined and maintained as a cell population (Table 1). Cell populations transfected with pSV2 constructs exhibited similar growth rates in medium supplemented with dialyzed or nondialyzed serum (data not shown). Immunohistochemical analysis of CHL-ShTSQ and CHL-ShTSE utilizing a monoclonal antibody against proliferating cell nuclear antigen indicated no significant differences in immunostaining between or within cell populations (data not shown).

Since the transfectants exhibited thymidine prototrophy, this indicated that the cells were expressing human TS and that the levels of TS were sufficient for optimal cell growth. To confirm that human TS was expressed in the transfectants, cellular proteins were separated by SDS–PAGE and examined by immunoblot analysis utilizing a murine monoclonal antibody against human TS. As shown in Fig. 1, all transfectants expressed two proteins that cross-reacted with the human TS antibody. The major species, approximately 36 kDa, migrated to a position consistent with the calculated value of M_r for the human TS polypeptide, 35,706 [17]. The minor species co-migrated with a protein detected

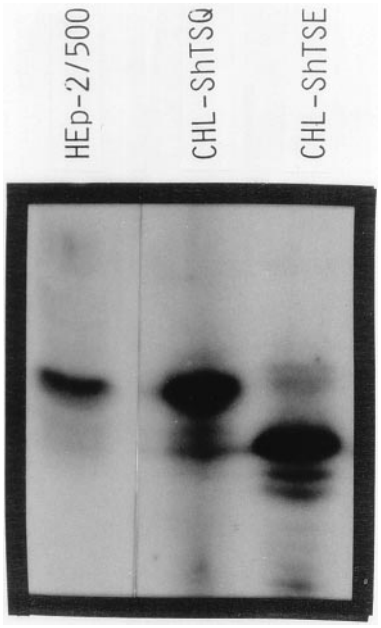

FIG. 2. Isoelectric focusing gel electrophoresis of ternary complexes. Cell extracts were incubated with 670 nM [^{32}P]FdUMP and 400 μM (6*RS*) CH_2H_4folate; the resulting ternary complexes were denatured and separated by isoelectric focusing in 9 M urea/4% acrylamide gels containing 0.67% (v/v) each of ampholines: pH 4–6, pH 6–8, and pH 3.5–10. Labeled complexes were detected by autoradiography. The amount of TS loaded onto the gel was: HEp-2/500, 59 fmol; CHL-ShTSQ, 126 fmol; and CHL-ShTSE, 126 fmol. The cell line designation is explained in Table 1.

in TS-deficient hamster V79 cells (Fig. 1) and in parental V79 cells that express TS (data not shown).

SDS–PAGE indicated that the major cross-reacting protein in the transfectants was of human origin. Since it is expected that TSs containing either a glutamate or glutamine residue at position 214 would differ in charge, the structure of TS expressed in the transfectants was examined by isoelectric focusing. TS in cell extracts was radiolabeled specifically by ternary complex formation utilizing [^{32}P] FdUMP and CH_2H_4folate. The complexes were denatured and separated by isoelectric focusing gel electrophoresis (Fig. 2). The mobility of the complex prepared from cells transfected with the vector encoding Gln214 TS was similar to that prepared from human HEp-2/500 cells. TS expressed in HEp-2/500 cells contains a glutamine residue at position 214 [28]. The complex prepared from cells transfected with the vector encoding Glu214 was more acidic than the complex prepared from cells transfected with the vector encoding Gln214. This is consistent with the expected charge difference resulting from the amino acid substitution. No ternary complex was detected in the TS-deficient hamster subline (data not shown). These data suggested that the transfectants were expressing either the Gln214 or Glu214 human TSs.

The levels of TS in the transfectants were estimated by immunoblot analyses utilizing a monoclonal antibody that is monospecific for human TS [13]. Previous studies with

TABLE 2. TS levels in transfectant cell extracts

	TS levels	
Transfectant*	**Immunoblot†**	**FdUMP binding‡**
CHL-MhTSQ	1	0.80 ± 0.1
CHL-MhTSE	1.6 ± 0.3	1.2§
CHL-ShTSQ	2.0 ± 0.5	2.7 ± 0.30
CHL-ShTSE	2.4 ± 0.7	2.9§

*Cell line designation is given in Table 1.

†TS levels were estimated by densitometric scanning of immunoblots as described in Materials and Methods. The data are relative to the mean of the intensities of immune complexes formed with CHL-MhTSQ extracts. Data are the means (±SD) of 3 separate determinations.

‡TS levels were determined by measuring the binding of FdUMP to extract protein as described in Materials and Methods. The data, expressed as picomoles of TS per milligram of soluble protein, are the means (±SD) of 5–14 separate determinations.

§The data were estimated by fractional saturation analysis, based on the assumption of two binding sites with K_d values of 0.26 and 1.48 μM for FdUMP.

purified Gln214 and Glu214 TSs revealed that the proteins exhibit similar cross-reactivity with D3B31, the murine anti-human TS monoclonal antibody utilized in the immunoblot analyses [13]. Relative levels of TS were obtained by densitometric scanning of immunoblots. Relative to TS expressed in CHL-MhTSQ, the levels of TS were 1.6-, 2.0-, and 2.4-fold higher in CHL-MhTSE, CHL-ShTSQ, and CHL-ShTSE, respectively (Table 2). The levels of TS in the transfectants also were assessed by measuring the binding of FdUMP to extract protein. FdUMP is a stoichiometric inhibitor of TS in the presence of CH_2H_4folate, and is utilized frequently to quantitate TS in cell extracts. The extent of binding of FdUMP to extract protein was determined by utilizing 250 nM FdUMP. While this concentration is adequate for quantitation of Gln214 TS, it will underestimate the levels of Glu214 TS, which has a significantly reduced affinity for FdUMP (see below). Unfortunately, concentrations of FdUMP that were adequate for quantitation of Glu214 TS produced unacceptably high backgrounds in cell extracts. To estimate the levels of Glu214 TS in the transfectants, the data were adjusted by utilizing the K_d values for FdUMP binding to the purified Glu214 TS (see below). The levels of TS, as determined by FdUMP binding, are shown in Table 2. Comparison of the data obtained by immunoblot and FdUMP binding analyses revealed that, within each expression system, the levels of TS were similar. Cells transfected by pSV2 constructs exhibited 2- to 3-fold higher levels of TS than cells transfected by pMAMneo constructs. The data also revealed that TS was expressed at levels that were of physiological relevance, since the levels of TS in parental Chinese hamster lung cell lines were 2.5 pmol/mg of soluble protein.

Since the levels of TS were similar in transfectants expressing TS from the same promoter, the levels of endogenous TS activity were determined. For these studies, cell populations expressing TS from pSV2 constructs were subcloned, and TS activities of individual clones were determined. The activities of the enzymes expressed in

TABLE 3. TS activity of transfectant cell clones*

Transfectant clone	k_{cat} (sec^{-1})
CHL-ShTSQ-Cl 1	1.8
CHL-ShTSQ-Cl 2	1.9
CHL-ShTSQ-Cl 3	2.8
CHL-ShTSE-Cl 1	0.40
CHL-ShTSE-Cl 2	0.13
CHL-ShTSE-Cl 3	0.11

*TS activity was determined in cell extracts of clonal derivatives of transfected cell populations. Data are the means of 3 separate determinations. The data were converted to k_{cat} values as described in Materials and Methods.

three clones of each transfectant population are shown in Table 3. The mean k_{cat} values determined in cell extracts were 2.2 and 0.21 sec^{-1} for Gln214 and Glu214 TSs, respectively. The k_{cat} values for the purified proteins are 3.1 and 0.27 sec^{-1} for Gln214 and Glu214 TSs, respectively [13]. Thus, *in situ* activities correlated reasonably well with the catalytic constants of the purified enzymes.

As a measure of the effect of expression of a highly defective TS, the response of the transfectants to FdUrd was examined. FdUrd is a prodrug of FdUMP, which is a mechanism-based inhibitor of TS and a sensitive probe of the relationship between TS structure and function. The cytotoxic effects of FdUrd were examined in cell populations of the transfectants to reduce the effect of cell heterogeneity on the observed response. Within each vector system, the transfected populations expressed similar levels of TS (Table 2). Since FdUMP is a tight-binding inhibitor of TS, it is expected that the levels of FdUMP required for complete inhibition of TS will be proportional to the levels of TS. Thus, in cells with a similar genetic background, the higher the level of TS, the higher the concentration of FdUrd required for cytotoxicity. For this reason, it is important to analyze FdUrd sensitivity in cells with similar levels of TS. The effect of FdUrd on the growth of cells expressing the wild-type and mutant TSs is shown in Fig. 3. Within each vector system, cells expressing Gln214 TS were approximately 8-fold more sensitive to FdUrd than cells expressing Glu214 TS. The IC_{50} values for FdUrd were 3 ± 1 and 24 ± 3 nM for CHL-MhTSQ and CHL-MhTSE, and 4 ± 1 and 31 ± 2 nM for CHL-ShTSQ and CHL-ShTSE, respectively. Untransfected TS-deficient cells exhibited no cytotoxicity even at 300 nM FdUrd (data not shown). Thus, the data revealed that expression of the mutant protein conferred resistance to FdUrd.

In addition to TS levels, the affinity of the enzyme for FdUMP is a determinant of TS inhibition by FdUMP. Since transfectants expressing Glu214 TS were relatively resistant to FdUrd, the affinity of the mutant protein for FdUMP was determined. In these studies, the apparent K_d for FdUMP binding was determined for the recombinant human enzyme purified from a TS-deficient bacterial strain. Previous investigations revealed that recombinant wild-type human TS isolated from bacteria exhibits a single class of binding sites for FdUMP, with an apparent K_d of 0.45 nM

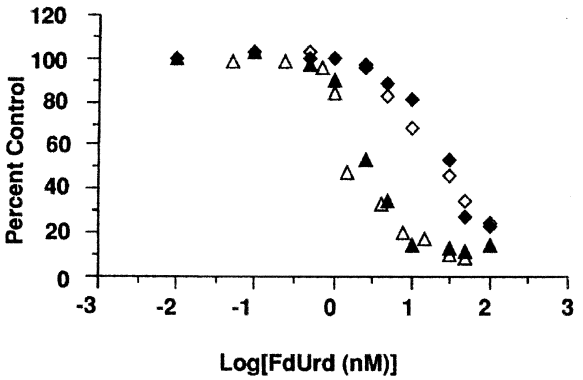

FIG. 3. Growth response of transfectants to FdUrd. Cells in monolayer were exposed to FdUrd (0.05 to 200 nM) for 5–6 cell generations. Cells were stained by neutral red dye and quantitated by absorbance as described in Materials and Methods. The data are expressed as the percent of untreated control cell growth and are the means of 6 independent measurements. Cell designations are explained in Table 1 (symbols are Δ, CHL-MhTSQ; ▲, CHL-ShTSQ; ◇, CHL-MhTSE; and ◆, CHL-ShTSE).

[13]. FdUMP binding studies with Glu214 TS revealed the existence of two classes of binding sites, with apparent K_d values of 0.26 ± 0.03 and 1.48 ± 0.16 μM, respectively (Fig. 4).

DISCUSSION

A Chinese hamster lung V79 subline deficient in TS was utilized to investigate the effect of expression of a defective TS on cell survival. Although the molecular basis for the TS deficiency is unknown, studies conducted in this laboratory revealed that the subline expresses endogenous TS mRNA (data not shown) and endogenous TS cross-reacting protein. FdUMP binding activity was not detected in

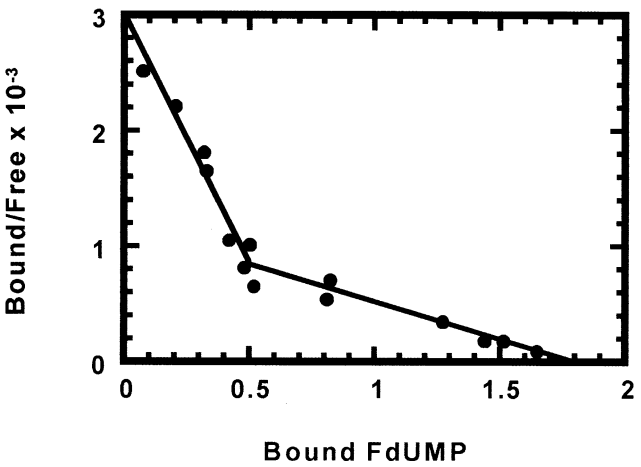

FIG. 4. FdUMP binding to Glu214 TS. Glu214 TS was purified as described in Materials and Methods. Enzyme (1 nM) was incubated with 0.1 to 20 μM [6-^{3}H]FdUMP and 150 μM CH_2H_4folate for 6 hr at 24° as described in Materials and Methods. The data were analyzed by the Scatchard equation, and K_d values were estimated by linear regression analysis. The data are the means of 3 separate determinations.

cell extracts, nor was TS activity detected *in situ* (data not shown). Furthermore, previous studies revealed that the subline undergoes no reversion to thymidine prototrophy during cultivation in medium lacking thymidine [19]. These results indicated that the TS-deficient subline is suitable for investigation of the expression of heterologous TS proteins.

Two mammalian expression systems were employed in this investigation, with the aim of isolating transfectants with different levels of TS expression. The levels of TS were 2- to 3-fold lower in cell populations expressing TS from a MMTV promoter. Interestingly, the yield of thymidine prototrophs expressing Glu214 TS was 2-fold lower in cell populations expressing the protein from a MMTV promoter, relative to a SV40 promoter. No promoter effect was observed upon the frequency of isolation of thymidine prototrophs expressing Gln214 TS. Although the k_{cat}/K_m for dUMP is 1000-fold less for Glu214 TS than Gln214 TS, transfectants expressing the proteins from a SV40 promoter exhibited similar growth rates at levels of TS that are physiologically relevant. In addition, the activities of the enzymes expressed in clonal derivatives of the transfectants were reasonably close to the catalytic efficiencies of the purified proteins. The data indicate that the capacity for dTMP production significantly exceeds demand in these mammalian cells.

Previous studies have indicated that the level of activity of TS is in considerable excess, relative to the requirement for dTTP in mammalian cells. Studies of conditional thymidine auxotrophs that express temperature-sensitive TSs revealed that near-normal growth rates are achieved at levels of TS approximately 10% of the level in parental mouse FM3A cells [29]. A similar conclusion was reached in studies of the recovery of TS during continuous exposure of mouse L1210 cells to FdUrd. In these studies, drug-exposed cells, with levels of TS 10–20% of that of cells with no FdUrd exposure, exhibited growth rates similar to untreated controls [30]. In the present studies, cells expressing TSs that differ by 10-fold in catalytic activity exhibited similar growth rates. Since the K_m for the mutant enzyme is 230 μM and the intracellular levels of dUMP are 5–10 μM in mammalian cells that have been examined, the data indicate that the capability for dTMP production is compromised significantly in cells expressing Glu214 TS [13, 31, 32]. Thus, studies employing three different approaches to decrease endogenous TS activity lead to the conclusion that TS activity in mammalian cells is significantly higher than is necessary to maintain DNA biosynthesis and cell viability.

The cytotoxic effect of the TS-directed drug FdUrd was analyzed in cell populations expressing a highly defective TS. The TS-directed cytotoxicity of FdUrd is dependent on its metabolic activation to FdUMP and on the levels of CH_2H_4folate, which is required for tight binding of FdUMP to TS. To reduce the effect of intercellular variation in metabolism on FdUrd cytotoxicity, cell populations of the transfectants were utilized to investigate FdUrd cytotoxicity. Thus, differences in response to FdUrd should be due to differences in either TS levels or affinity for ligands involved in inhibition, rather than to cell heterogeneity. TS levels are an important determinant of FdUrd cytotoxicity since FdUMP, in the presence of CH_2H_4folate, can bind stoichiometrically to TS. The relationship between the concentration of a tight-binding inhibitor and enzyme concentration has been described by Cha [33]: $I_{50} = 1/2\ E_t + K_d$.

In this equation, I_{50} is the concentration of the inhibitor required for 50% inhibition, E_t is the concentration of total enzyme, and K_d is the dissociation constant for enzyme–inhibitor complex. For a tight-binding inhibitor such as FdUMP, the equation predicts that the levels of FdUMP required for complete enzyme inhibition are proportional to TS concentration, as long as the K_d remains constant. A *priori*, it is expected that the conversion of FdUrd to FdUMP is similar in the transfected cell populations. Thus, this relationship translates to a requirement for higher levels of FdUrd in cells with higher levels of TS or that express TS with a lower affinity for FdUMP. In the present studies, immunoblot and FdUMP binding analyses indicated that, within each expression system, transfectant populations expressed the proteins at similar levels. Within each expression system, cells expressing Glu214 TS were 8-fold more resistant to FdUrd than cells expressing Gln214 TS. If one assumes that the metabolism of FdUrd to FdUMP is similar among the transfectant populations, the K_d for FdUMP binding is predicted from the equation to be 8-fold higher for Glu214 TS than for Gln214 TS. In previous studies of the cytotoxicity of FdUrd in TS-deficient Chinese hamster lung transfectants, cell populations expressing a mutant human TS that exhibits a K_d for FdUMP binding that is 4-fold higher, relative to that of wild-type TS, are 4-fold more resistant to FdUrd [15].

FdUMP binding studies revealed that Glu214 TS exhibits biphasic binding of FdUMP, with K_{d1} and K_{d2} values 600- and 3500-fold higher than the apparent K_d for FdUMP binding to Gln214 TS. The deviation from the relationship between IC_{50} and K_d predicted by the equation of Cha is likely to be due to at least two issues. The equation describes the relationship between the two values for a tight-binding inhibitor. Replacement of glutamine with a glutamate residue alters the binding interaction between TS and FdUMP so that the inhibitor is no longer a tight-binding inhibitor. Based on previous studies of the effect of substitution at position 214 on dUMP binding to TS, it is likely that glutamate at position 214 significantly increases the rate of dissociation of FdUMP from the enzyme [13]. In fact, dUMP binding to Glu214 TS could not be detected in transient kinetic studies under conditions in which dUMP induces pronounced changes in fluorescence in Gln214 TS (Steadman D and Spencer T, unpublished results). A second issue that is likely to contribute to the deviation between IC_{50} and K_d for transfectant populations expressing Glu214 TS derives from the low k_{cat}/K_m for dUMP for this protein. Expression of the

mutant protein is expected to result in low levels of dTTP in the transfectants. dTTP exerts feedback inhibition on the activity of thymidine kinase, the enzyme which catalyzes the synthesis of FdUMP from FdUrd [34]. Thus, the levels of FdUMP may be higher in transfectants expressing Glu214 TS than Gln214 TS.

The studies of FdUMP binding indicate that Glu214 TS displays a different binding mode for FdUMP than does Gln214 TS. A Scatchard plot of the binding data revealed that two classes of binding sites for FdUMP exist in Glu214 TS. A single class of FdUMP binding sites was observed with Gln214 TS and with TSs with alanine, asparagine, glycine, histidine, serine, or leucine at position 214 [13]. Similarly, a single class of FdUMP binding sites was observed in TSs isolated from several human cell lines [25, 35]. The basis for the existence of two classes of FdUMP binding sites in Glu214 TS is unknown. Recent studies have revealed that a mutant human TS protein with substitution of a histidine for a tyrosine residue at position 33 exhibits an enhanced negative cooperativity between the two subunits, with respect to FdUMP binding [36]. Perhaps the presence of a glutamate at position 214 alters the subunit interaction in a similar manner.

During the last year, several investigations focusing on mutant human TSs have appeared in the literature [13, 37, 38]. The interest is derived, in part, from gene therapy approaches in which mutant TSs are transferred into normal tissues, such as the hematopoietic system, for protection from the cytotoxic effects of high-dose cancer chemotherapy. An ideal candidate for gene therapy is a mutant TS that confers resistance to TS-directed inhibitors but maintains sufficient production of dTMP for cell viability. The results of this investigation reveal that even highly defective proteins can maintain normal cell growth. It will be of interest to examine the response of transfectants expressing Glu214 TS to TS inhibitors that are analogs of the folate substrate. In addition, it will be of interest to examine the effect of expression of other mutant TSs with substitutions at this position, since these enzymes exhibited a broad range of kinetic constants and FdUMP binding activities [13].

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