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A Naturally Occurring Tyrosine to Histidine Replacement at Residue 33 of Human Thymidylate Synthase Confers Resistance to 5-Fluoro-2′-deoxyuridine in Mammalian and Bacterial Cells

KAREN W. BARBOUR, DIANA K. HOGANSON, SONDRA H. BERGER, and FRANKLIN G. BERGER

Departments of Biological Sciences (K.W.B., F.G.B.) and Basic Pharmaceutical Sciences (D.K.H., S.H.B.), University of South Carolina, Columbia, South Carolina 29208

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SUMMARY

Structural changes in the macromolecular targets of pharmacological agents can result in alterations in the efficacy of these agents. In previous studies, we identified a variant structural form of thymidylate synthase (TS) that is associated with relative resistance to 5-fluoro-2′-deoxyuridine, in a human colonic tumor cell line. We now report on the use of DNA transfer techniques to examine directly the effects of each TS form on drug response. TS cDNA constructs, corresponding to the normal or variant TS mRNA, were expressed in Chinese hamster lung cells or in *Escherichia coli*, and response to 5-fluoro-2′-deoxyuridine was determined. We observed that expression of the variant TS, which differs from the normal form by a tyrosine to histidine substitution at residue 33, confers a 4-fold level of drug resistance in the mammalian cells, as well as in bacteria. The possible role of Tyr-33 in 5-fluoropyrimidine-mediated inhibition of TS is discussed.

Genetic variation in macromolecular structure, a phenomenon commonly found in all living species, can result in altered interactions between pharmacological agents and their intracellular targets. This may generate changes in the efficacy of these agents. A full understanding of drug action must, therefore, take into account the presence of naturally occurring genetic variation and its impact upon drug response. As a model for this phenomenon, we have been examining responses to 5-fluoropyrimidines in mammalian cells, with emphasis on the role of variation in the structure of the enzyme TS (EC 2.1.1.45), an intracellular target for this class of drugs.

TS catalyzes the methylation of dUMP by $CH_2H_4PteGlu$, to form TMP and dihydrofolate; this reaction is indispensable for the *de novo* biosynthesis of TMP and is, therefore, required for DNA replication (1–3). 5-Fluoropyrimidines, particularly 5-fluorouracil and FdUrd, have been widely used in the chemotherapy of solid tumors. A primary mechanism of action of these agents involves deprivation of TMP through inhibition of TS, although other mechanisms may also be important (1–3). Inhibition of the enzyme by 5-fluoropyrimidines occurs via the formation of FdUMP, which enters into a stable, covalent, ternary complex with the enzyme and its co-substrate, $CH_2H_4PteGlu$ (4–6).

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It can be predicted that alterations in TS structure might perturb the interaction between FdUMP and the enzyme, generating alterations in response to 5-fluoropyrimidines. This prediction has been borne out in a variety of studies (7–9). Recently, we reported that the human colonic tumor cell line HCT116 produces a variant structural form of TS, in addition to the common form found in all colonic cell lines tested (10). The novel form has a more basic pI and was originally identified by isoelectric focusing-gel electrophoresis (10). Among TS-overproducing derivatives of HCT116, cells overexpressing the novel form are more resistant to FdUrd, compared with cells overexpressing the normal form (11). Such an association between drug response and altered TS structure led us to suggest that the novel TS form, which is encoded by a variant structural gene, confers relative resistance to FdUrd (11); this was supported by preliminary kinetic data indicating that this form has a reduced affinity for FdUMP (11).

Comparative sequence analysis of cDNAs corresponding to the normal and variant TS mRNAs showed the presence of a U to C transition mutation in the coding region of the variant mRNA, resulting in replacement of an evolutionarily conserved tyrosine by a histidine at residue 33 of the TS polypeptide (13); this mutation represents the only difference between the two TS forms and must account for the structural and functional differences between them.

ABBREVIATIONS: TS, thymidylate synthase; $CH_2H_4PteGlu$, 5,10-methylenetetrahydrofolate; FdUrd, 5-fluoro-2′-deoxyuridine; FdUMP, 5-fluoro-2′-deoxyuridylate; dUrd, 2′-deoxyuridine; dUMP, 2′-deoxyuridylate; PCR, polymerase chain reaction; MMTV, mouse mammary tumor virus; SV40, simian virus 40; RFLP, restriction fragment length polymorphism; kb, kilobase(s); bp, base pair(s); RSV, Rous sarcoma virus.

The conclusion that the novel TS confers a FdUrd-resistant phenotype is an indirect one, derived primarily from an observed association between drug response and the form of TS expressed in TS-overproducing HCT116 cells (11). As a more direct test of this conclusion, we have utilized DNA transfer techniques to express each of the TS forms in Chinese hamster lung cells and in *Escherichia coli*; we find that, in both systems, cells expressing the novel form are more resistant to FdUrd than are cells expressing the normal form. Thus, the Tyr-33 to His-33 replacement within the TS polypeptide confers resistance to FdUrd in both mammalian and bacterial cells.

Experimental Procedures

TS cDNA plasmids. Full length human TS cDNAs were constructed from partial cDNAs, because we were unable to isolate the appropriate clones directly from libraries; this was in large part due to poor protection of the *Eco*RI cleavage site at nucleotides 301/302 during construction of the libraries.[1] Plasmid pKB103, containing the region between nucleotide 302 and the poly(A) stretch, was isolated from a cDNA library prepared in phage λ using poly(A)-containing RNA of cell line HCT116/200–11 (11, 13); the cDNA was subcloned into pBluescript SK(−) DNA (Stratagene, Inc.). Plasmid pKB161 contains the 5′ portion of the variant TS cDNA, spanning the region between nucleotides 28 and 301. Plasmid pKB147 spans the region between nucleotides 25 and 301 of the normal TS cDNA and was isolated and subcloned from a library prepared with RNA of cell line HCT116/200–1 (11, 13). To construct full length TS cDNAs, the *Eco*RI site at the 3′ end of the insert of pKB103 was removed by nested deletion (14) to produce pKB177; the cDNA insert of either pKB147 or pKB161 was directly cloned into the single *Eco*RI site of pKB177, in the proper orientation. The resulting plasmids were pKB170, containing the region between nucleotides 28 and 1354 of the variant cDNA, and pKB169, containing the region between nucleotides 25 and 1354 of the normal cDNA. The TS cDNAs of these two plasmids differ by a single T to C mutation at codon 33 (see text and Ref. 13), as verified by DNA sequencing.

For expression of TS cDNAs in mammalian cells, *Acc*I/*Hpa*I fragments of pKB170 and pKB169, containing the TS cDNAs to nucleotide 1236, were cloned into two expression vectors. They were inserted into the *Sal*I site of pMAMneo (Clontech, Inc.) by blunt-end ligation; this vector drives transcription of inserted sequences through the action of an RSV enhancer-activated MMTV promoter. Resulting plasmids were pMMTS-A, containing the normal cDNA, and pMMTS-B, containing the variant cDNA. The TS cDNA fragments were also cloned into pSV2, which contains an SV40 viral promoter. The vector was digested with *Hind*III and *Hpa*I, and the cDNAs were inserted by blunt-end ligation; this resulted in plasmids pSVTS-A and pSVTS-B, carrying the normal and variant cDNAs, respectively.

Expression of human TS in *E. coli* was accomplished with plasmid pDHTS-S1, which was a kind gift of Dr. Daniel Santi of the University of California, San Francisco. This plasmid contains a full length normal TS cDNA modified for high level expression in *E. coli* (15). For expression of the variant TS, the appropriate mutation was introduced into pDHTS-S1 by site-directed mutagenesis (16), using a kit from Amersham, Inc.; introduction of the T to C mutation at nucleotide 190 resulted in plasmid pKB246.

Blot analysis of DNA and RNA. Cellular DNA and RNA were isolated by standard methods (17). For Southern blot analysis, 10 μg of DNA were digested with *Rsa*I, and resulting fragments were fractionated by agarose gel electrophoresis and transferred onto nylon membranes. Blots were hybridized to a TS-specific probe, which was the 277-bp *Eco*RI fragment of pKB147 (see above) labeled by nick translation; after hybridization, the blots were washed and visualized by autoradiography.

For Northern blot analysis, 10-μg samples of RNA were subjected to electrophoresis through formaldehyde-containing agarose gels, transferred onto nylon membranes, and hybridized to a nick-translated probe. The probe was either the TS-specific 277-bp *Eco*RI fragment of pKB147 or the 350-bp *Eco*RI/*Cla*I fragment of pMAMneo containing the RSV enhancer and upstream region of the MMTV promoter (see Fig. 4). Blots were washed and visualized by autoradiography.

PCR amplification. TS structural gene sequences were amplified by PCR, as described in the GeneAmp reagent kit (Perkin Elmer Cetus). Reaction mixtures contained 1.0 μg of DNA in a total volume of 100 μl; inclusion of 10% dimethylsulfoxide was necessary for formation of the correct product. Both PCR primers were from within the first exon of the TS structural gene (18). The 5′ primer (5′-ACCACTTGGCCTGCCTCCGT-3′) corresponds to the region between nucleotides 1 and 20 in the TS mRNA (12) and is part of a 28-base tandem direct repeat at nucleotides 1–28, 29–56, and 57–84. The 3′ primer (5′-CTGCATGCCGAATACCGACA-3′) is complementary to the region between nucleotides 260 and 279. Amplification was performed in a Perkin Elmer Cetus model 480 thermal cycler.

Introduction of DNA into mammalian cells. Cell line RJK88.13, kindly provided by Dr. Robert Nussbaum of the University of Pennsylvania, was used as the host for DNA transfections. This cell line, which is a TS-deficient derivative of V79 Chinese hamster lung cells (19), was routinely maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum and 10 μM thymidine. Transfections were performed by calcium phosphate precipitation (20), using 25 μg of DNA/culture. Two days after transfection, cells were placed in selective medium, which contains no thymidine; colonies having the ability to grow in the absence of thymidine were pooled and propagated as a mass culture. Extracts for TS level determinations were made as described previously (11).

Transformation of bacteria. A *thyA*$^-$ strain of *E. coli*, χ2913 (21), was kindly provided by Dr. Daniel Santi; it was maintained in medium containing 50 μg/ml thymine. Transformation was by the $CaCl_2$ method, with selection in medium containing 100 μg/ml ampicillin and no thymine. Extracts for TS level determinations were prepared by sonication in 0.1 M Tris·HCl, pH 7.4, 25 mM $MgCl_2$, 1 mM Na_2EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, 50 μg/ml aprotinin.

Determination of TS protein concentrations. The concentrations of TS in cellular extracts were determined by FdUMP binding, which measures the formation of covalent ternary complexes among the enzyme, [^{3}H]FdUMP (Moravek Biochemicals, Inc.), and CH_2H_4PteGlu (4, 5). Binding assays were conducted according to the methods of Moran *et al.* (22), except that the concentration of FdUMP was raised to 0.31 μM. For calculations, binding of FdUMP was assumed to be 1.7 pmol/pmol of TS.

Dose-response analysis of FdUrd sensitivity. To determine the response of transfected mammalian cells to FdUrd, approximately 100,000 cells were seeded in 25-cm^2 tissue culture flasks, in drug-free medium. After the cells were allowed to attach overnight, various concentrations of FdUrd (0–50 nM) were added, and the number of cells in each flask was counted 72 hr later, with a hemocytometer.

To measure the effects of FdUrd on bacterial cell transformants, cells were inoculated into 10 ml of minimal medium (15) containing various concentrations of drug (0–5 μM). The densities of the cultures were determined at various times, in a Klett-Summersen colorimeter.

Results

Identification of an RFLP that characterizes the novel TS gene. The Tyr-33 to His-33 replacement in the variant TS polypeptide is generated by a U to C transition at nucleotide 190 within the TS mRNA (13).[1] Inspection of this region of the

[1] The numbering for human TS mRNA nucleotides is according to that in GenBank Entry X02308, which is the sequence reported by Takeishi *et al.* (12).

mRNA sequence (Fig. 1) indicates that the mutation occurs at an *Rsa*I site in the corresponding DNA; thus, the gene encoding the normal TS should contain an *Rsa*I site at this location, whereas that encoding the novel TS should not.

To test this, and to provide additional proof that the identified mutation does exist within the HCT116 genome, DNAs from several cell lines of human origin were digested with *Rsa*I, and TS-specific fragments were identified by Southern blotting. The 277-bp *Eco*RI fragment of cDNA plasmid pKB147 was used as a probe; this probe contains the region between nucleotides 25 and 301 of the TS mRNA and spans the *Rsa*I site at codon 33. As seen in Fig. 2, a hybridizing fragment about 1.6-kb long was observed in all DNA samples; this most likely represents a TS pseudogene (23). DNA from cell line CBS, which expresses only the normal TS form (10), contains additional fragments, with lengths of about 1.3 kb and 0.7 kb (Fig. 2, *lane A*). The smaller of these corresponds to the region between the *Rsa*I sites in the 5′ flanking region and at codon 33 (18). The 1.3-kb fragment spans the region between the *Rsa*I sites at codon 33 and within the first intron (18). DNA from cell line HCT116, which expresses both TS forms (10), contains the pseudogene fragment, the two fragments of the normal gene, and a unique 2.0-kb fragment (Fig. 2, *lane B*); the latter is derived from the variant gene and is the size expected when the *Rsa*I site at codon 33 is lost.

DNA from cell line HCT116/200–1, which overproduces the normal TS form by gene amplification (11), showed intense hybridization to the 1.3-kb and 0.7-kb fragments (Fig. 2, *lane C*), indicating selective amplification of the normal TS allele in this cell line. Similarly, DNA from cell line HCT116/200–11, which overproduces the novel TS form (11), showed intense hybridization to the 2.0-kb fragment (Fig. 2, *lane D*), indicating specific amplification of the variant gene.

Thus, as predicted from cDNA sequence data (13), the two forms of TS are encoded by two structural gene alleles that are distinguished by the presence or absence of an *Rsa*I restriction site at codon 33. The normal allele contains an *Rsa*I site at this location, whereas the novel allele does not.

PCR amplification of genomic DNA was used as an alternative method of detecting the RFLP that distinguishes the two TS alleles. DNA from cell lines HCT116/200–1 and HCT 116/200–11 were amplified using two primers from within the first exon of the TS structural gene (18). The 5′ primer corresponds to tandem direct repeats at nucleotides 1–28, 29–56, and 57–84 in the TS mRNA (12). Each of these could theoretically act as a primer binding site for the PCR reaction, generating products with expected lengths of 279, 251, and 223 bp. As seen in Fig. 3, a 250-bp product was generated with both DNAs, indicating that the primer site at nucleotides 29–56 predominates. The PCR product from HCT116/200–1 DNA was digested by *Rsa*I into 160-bp and 90-bp fragments (Fig. 3), both of which are predicted from the known locations of the PCR primers and the *Rsa*I site at codon 33 within the normal TS gene. In contrast, the product from HCT116/200–11 DNA was resistant to *Rsa*I (Fig. 3), reflecting the presence of the T to C mutation.

The results, in total, demonstrate that the structural gene encoding the novel TS form has lost an *Rsa*I site. This has occurred at the location of the T to C mutation that causes the amino acid substitution characterizing the variant form of TS.

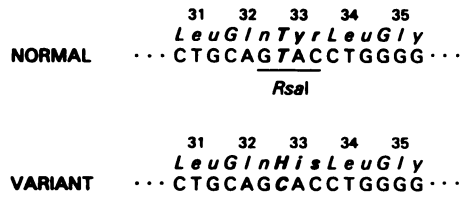

Fig. 1. Comparison of the sequences of the normal and variant TS forms. The mRNA and polypeptide sequences between codons 31 and 35 are shown for both forms of TS. The *Rsa*I site that is destroyed by the T to C point mutation in the variant form is *underlined*.

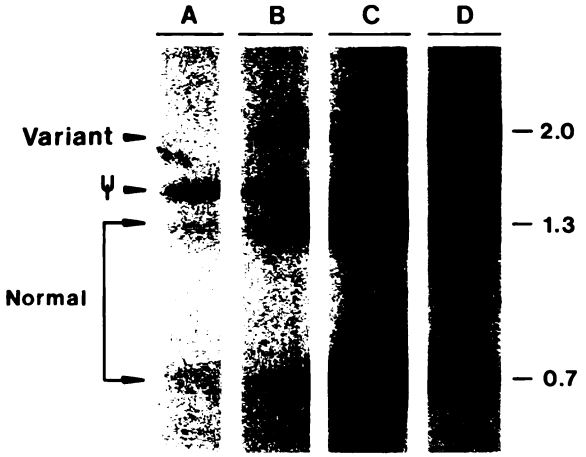

Fig. 2. Southern blot analysis of the RFLP that distinguishes the normal and variant TS structural genes. Cellular DNAs were digested with *Rsa*I, and fragments were fractionated on agarose gels, blotted onto nylon membranes, and hybridized to a radiolabeled TS-specific probe. The DNAs were from the following cell lines: CBS, which produces the normal form (*lane A*); HCT116, which produces both forms (*lane B*); HCT116/200–1, which overproduces the normal form (*lane C*); and HCT116/200–11, which overproduces the variant form (*lane D*). Bands corresponding to the normal and variant TS gene fragments are indicated to the *left*. Ψ, Pseudogene fragment. Band sizes (in kb) are indicated to the *right*.

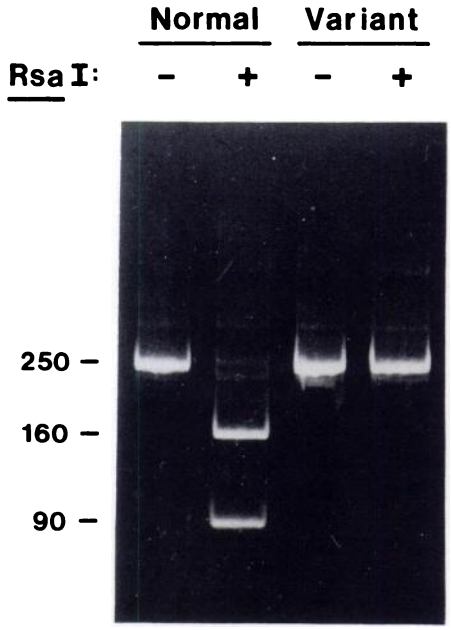

Fig. 3. PCR analysis of the RFLP that distinguishes the normal and variant TS structural genes. Cellular DNAs were amplified by PCR, using primers described in the text. The products, either before (–) or after (+) *Rsa*I digestion, were examined by agarose gel electrophoresis and ethidium bromide staining. DNA for the normal enzyme was from cell line HCT116/200–1, whereas that for the variant was from cell line HCT116/200–11. Band sizes (in bp) are indicated to the *left*.

Expression of the two TS forms under control of the MMTV promoter in transfected mammalian cells. To assess directly the effects of the Tyr-33 to His-33 replacement upon FdUrd response, we used DNA transfection to generate cells expressing one or the other TS form. TS cDNAs, corresponding to the normal or variant mRNA and differing by only the T to C change at codon 33, were cloned into the expression vector pMAMneo (Fig. 4); in this vector, transcription of the cDNA is driven by the MMTV promoter. Plasmids pMMTS-A and pMMTS-B, containing the normal and variant TS cDNAs, respectively, were transfected into the TS-deficient Chinese hamster lung cell line RJK88.13 (19). After the transfection, cells were cultured in G418 in the absence of thymidine, to select for expression of both the *neo* gene and TS. No colonies appeared in mock-transfected cells or in cells transfected with vector alone, even after prolonged (i.e., 1.5 months) culture in selective medium. With the normal cDNA, healthy colonies appeared after approximately 2 weeks; these were pooled to form cell line MMTS-A. The variant cDNA also gave rise to healthy colonies, although they were significantly fewer in number; these were also pooled, generating cell line MMTS-B. The striking difference in transfection efficiency between the normal and variant cDNAs was observed in several experiments. We have not quantified the transfection frequency difference, nor have we studied it in any further detail.

The concentrations of TS in crude extracts were very different between cell lines MMTS-A and MMTS-B. MMTS-A contained 0.14 ± 0.015 pmol of TS/mg of protein, as measured by [^{3}H]FdUMP binding; in contrast, MMTS-B contained 0.59 ± 0.10 pmol of TS/mg of protein. Total TS activity levels in the transfectants were assessed by an *in situ* assay. Whole cells were incubated with [5-^{3}H]dUrd, which is converted to TMP intracellularly, a process that results in formation of [^{3}H]H_2O. Whereas parental RJK88.13 cells had no detectable activity in this assay, both transfectants exhibited equal levels of activity *in situ* (data not shown). The fact that cellular activities were equal in the two transfected cell lines, yet TS polypeptide concentrations were not, suggests that the two TS forms differ with regard to catalytic efficiency (see Discussion).

TS mRNA levels in the transfected cell lines were quantitated by Northern blotting, using a human TS-specific cDNA probe. Two hamster-specific transcripts, 1.3 and 1.0 kb in length, were visible in all lanes (Fig. 5). These were the only RNAs detected in untransfected host cells (Fig. 5). MMTS-A contained several additional RNA species, including a prominent one of 1.8-kb length. This is the expected size of the fusion

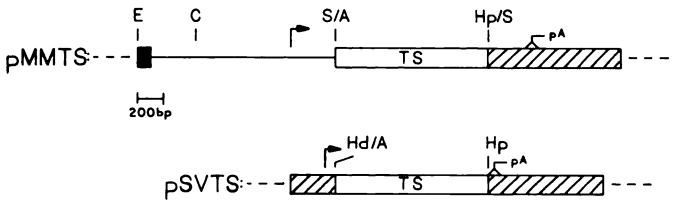

Fig. 4. Constructs for transfection and expression of TS cDNAs. TS cDNAs corresponding to the normal or variant mRNA were cloned into the mammalian expression vectors pMAMneo and pSV2, generating plasmids pMMTS and pSVTS, respectively. *Dashed lines*, vector sequences; *solid lines*, MMTV sequences; *filled box*, RSV enhancer; *cross-hatched rectangles*, SV40 sequences; *open rectangles*, TS cDNAs; *pA*, SV40 polyadenylation/splicing region; *bent arrow*, transcription initiation sites. Restriction sites are *Hin*dIII (Hd), *Eco*RI (*E*), *Cla*I (*C*), *Sal*I (*S*), *Acc*I (*A*), and *Hpa*I (*Hp*).

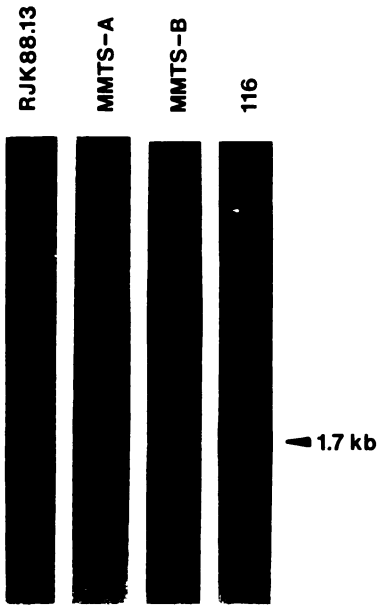

Fig. 5. Northern blot analysis of human TS mRNA in cell lines MMTS-A and MMTS-B. Total RNAs from cell lines MMTS-A and MMTS-B, as well as HCT116 and the parental line RJK88.13, were fractionated on agarose gels, blotted onto nylon membranes, and hybridized to a radiolabeled TS-specific probe. The length of human TS mRNA is indicated to the *right*.

transcript initiated at the MMTV promoter and polyadenylylated at the SV40 poly(A) addition site within the expression vector (see Fig. 4). MMTS-B also contained several additional transcripts (Fig. 5), two of which were quite large (i.e., about 3–3.5 kb) and very abundant, relative to the others. The level of TS-specific RNA in MMTS-B was about 20–50-fold greater than that in MMTS-A.

The abundant, high molecular weight RNAs in MMTS-B could be due to transcription initiation upstream of the MMTV start site. A probe corresponding to the RSV enhancer, along with the upstream region of the MMTV promoter, was found to hybridize to only the large RNAs of MMTS-B; no hybridization was detected with RNA from MMTS-A (data not shown). Thus, the large RNAs in MMTS-B result, at least in part, from upstream initiation. It is likely that the transfected plasmid integrated into the host genome in a fashion that brought the TS cDNA under control of strong endogenous promoters (see Discussion).

The availability of transfected cells expressing exclusively one TS form or the other allows a direct test of the effects of the Tyr-33 to His-33 mutation on FdUrd sensitivity. Dose-response analysis of FdUrd toxicity was conducted for each cell line. The ID_{50} values were 1.2 nM for MMTS-A and 19 nM for MMTS-B (Fig. 6). In several independent experiments, MMTS-B was observed to be 15–20-fold more resistant to drug than MMTS-A. Thus, expression of the variant TS form renders cells less sensitive to FdUrd, a notion consistent with the earlier suggestion that the variant form of TS is associated with a reduced drug response (11).

Expression of the two TS forms under control of the SV40 promoter in transfected mammalian cells. In the transfection experiments described in the previous section, it was uncertain whether the relative FdUrd resistance of MMTS-B cells was due to the Tyr-33 to His-33 replacement, the higher levels of TS expression, or both. In order to obtain transfectants

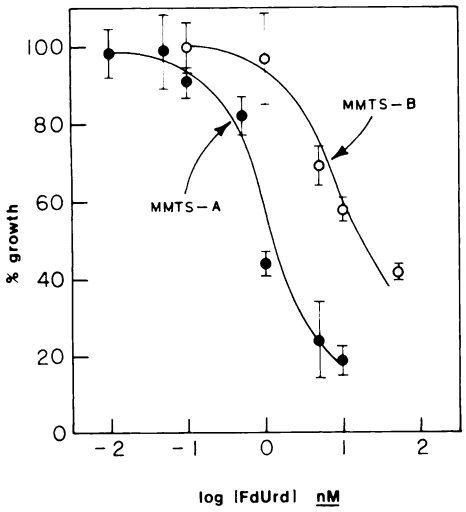

Fig. 6. FdUrd response of MMTS-A and MMTS-B cells. Drug response was measured as described in Experimental Procedures. At each concentration of FdUrd, the final number of cells on the dish was expressed as a percentage of the number obtained in the absence of drug. Each *point* represents the average of triplicate determinations; *vertical bars* are standard errors.

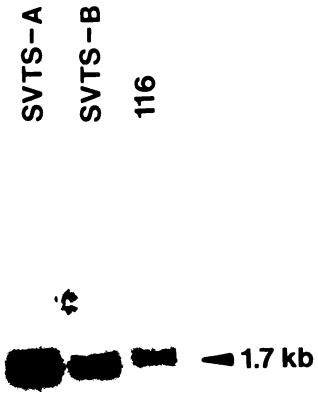

Fig. 7. Northern blot analysis of human TS mRNA in cell lines SVTS-A and SVTS-B. Total RNAs from cell lines SVTS-A, SVTS-B, and HCT116 were fractionated by agarose gel electrophoresis, blotted onto a nylon membrane, and hybridized to a radiolabeled TS-specific probe. The size of human TS mRNA (1.7 kb) is indicated to the *right*.

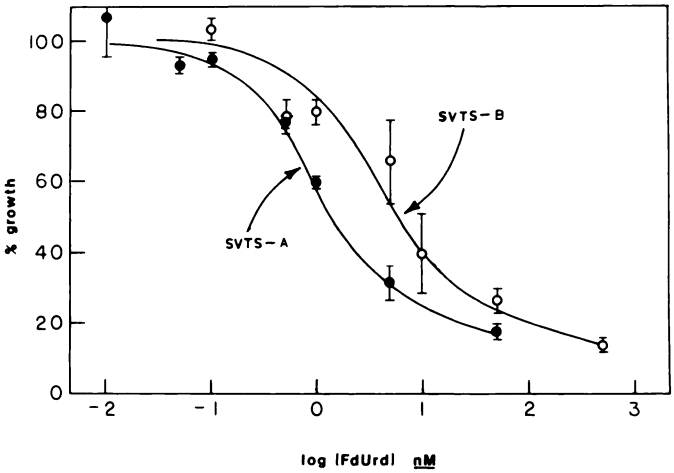

Fig. 8. FdUrd response of SVTS-A and SVTS-B cells. Analysis was exactly as described in the legend to Fig. 6.

expressing equal levels of the two TS forms, we tested constructs where TS expression is driven by a different promoter. The TS cDNAs were cloned into the expression vector pSV2, which contains the SV40 promoter (Fig. 4); plasmids pSVTS-A and pSVTS-B contain the normal and variant TS cDNAs, respectively. RJK88.13 cells were transfected with these plasmids and selected in medium lacking thymidine. Both constructs gave rise to a large number of colonies, with no apparent difference in transfection frequency between the two. Transfectants expressing the normal cDNA were pooled to form cell line SVTS-A, whereas those expressing the variant cDNA were pooled to form cell line SVTS-B.

The transfected cell lines each contained about 0.13–0.17 pmol of TS/mg of protein; thus, in contrast to the MMTV promoter constructs, SV40 promoter constructs resulted in nearly equal expression of the two TS forms. Each cell line produced a single TS mRNA, having the expected length of 1.4 kb (Fig. 7); SVTS-A cells contained about 2–3-fold higher concentrations of this mRNA, compared with SVTS-B cells. FdUrd sensitivities were determined, and the ID_{50} values were found to be 1.4 nM for cell line SVTS-A and 6.3 nM for SVTS-B (Fig. 8). Thus, under circumstances where cellular concentrations of TS are equal, the variant TS leads to a 4–5-fold higher level of FdUrd resistance, compared with the normal TS. This indicates that the tyrosine to histidine mutation at residue 33 causes a reduced drug response. The 15-fold resistance of cell line MMTS-B, relative to MMTS-A (Fig. 6), is most likely derived from the combined effects of both altered TS structure and increased enzyme concentration.

Expression of the two TS forms in bacteria. To generate *E. coli* cells expressing the normal or the variant TS, a *thyA*$^-$ strain (χ2913), which lacks TS and requires thymine for growth (21), was transformed with constructs containing human TS cDNAs in a bacterial expression vector. Plasmid pDHTS-S1 contains the normal cDNA modified for high level expression in *E. coli* (15). Plasmid pKB246, containing the novel cDNA, was generated by site-directed mutagenesis of pDHTS-S1 (see Experimental Procedures). Both plasmids transformed χ2913 cells to thymine prototrophy, indicating expression of the human TS and complementation of the *thyA*$^-$ mutation in the host strain.

TS concentrations, assayed by FdUMP binding in soluble extracts, were 4–6 pmol of enzyme/mg of protein for both the normal and novel TS-expressing transformants. In previous studies, TS expression in pDHTS-S1 transformants was reported to be about 1.6% of total protein (15). Assuming that the FdUMP-binding activity of pure human TS is about 27 nmol of nucleotide/mg of protein, we expected that the transformants should express the enzyme at concentrations of >400 pmol/mg of protein; this is about 100-fold more than we observed. Recent results suggest that human TS concentrations are very sensitive to growth conditions in these bacteria, perhaps as a consequence of fluctuations in plasmid copy number. However, we are not able to explain fully the discrepancy between the levels of TS we have observed and those observed by Davisson *et al.* (15).

FdUrd sensitivities for the human TS-expressing bacterial cells were determined and are shown in Fig. 9. Transformants expressing the normal form of TS exhibited an ID_{50} of 0.27 μM, whereas those expressing the variant form exhibited an ID_{50} of 0.84 μM. Thus, compared with the normal form, the variant TS form conferred a 3–4-fold resistance to FdUrd in bacterial cells. The fact that the two TS forms were expressed at nearly identical concentrations indicates that the differential response to drug is caused by the Tyr-33 to His-33 substitution.

Discussion

In earlier work, we suggested that the altered structural form of TS in cell line HCT116 confers relative resistance to FdUrd (11). This suggestion was based upon the finding that derivatives of HCT116 that overexpress the variant form of TS are more resistant to drug than are derivatives that overexpress the normal form (11). Interpretation of this observation is complicated by the fact that, although the TS-overproducers accumulate high levels of one or the other TS form, they also maintain low level production of the alternative form. We embarked on the current experiments in order to examine the effects of each TS form independently of the other.

Chinese hamster cell lines expressing TS cDNAs under control of either the MMTV or the SV40 promoter were produced by DNA transfection. Interestingly, with the MMTV promoter, transfectants expressing the variant TS form contained higher concentrations of enzyme than transfectants expressing the normal form; such was not the case with the SV40 promoter. Indeed, cell line MMTS-B, which expresses the variant TS, contained some 20–50-fold higher concentrations of TS mRNA and 3–4-fold higher levels of TS protein, compared with cell line MMTS-A, which expresses the normal enzyme. Because we analyzed only uncloned mass cultures of transfectants, this difference in TS expression must be prevalent among cells within the two populations.

Additional observations with the MMTV promoter constructs are of note. First, the transfection frequency for pMMTS-B, carrying the variant TS cDNA, was significantly less than that for pMMTS-A, carrying the normal cDNA.

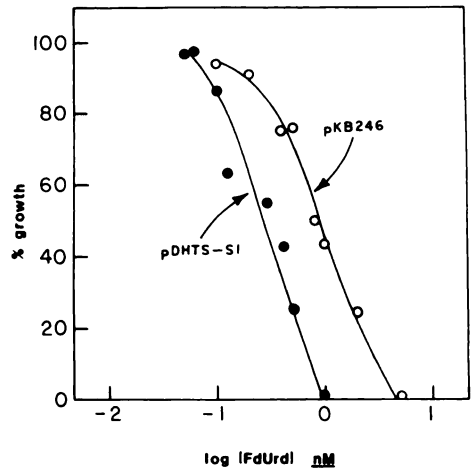

Fig. 9. FdUrd response of *E. coli* cells expressing human TS forms. Bacterial transformants expressing either the normal (pDHTS-S1) or the variant (pKB246) form of TS were inoculated into minimal medium containing various concentrations of FdUrd, and the generation times were determined. At each concentration, the generation time is expressed as a percentage of that in the absence of drug.

Second, most of the human TS-specific mRNA in cells expressing the variant form is initiated at some strong endogenous promoter(s), rather than at the MMTV promoter within the expression vector (Fig. 5). We suspect that, in cells transfected with the variant cDNA, thymidine-independent growth requires higher levels of TS mRNA than can be generated from the MMTV promoter. Thus, to survive the selective conditions, the plasmid must integrate into the genome in a manner that places the cDNA under control of an appropriate hamster promoter; this may explain the low transfection frequency with the variant cDNA construct.

As mentioned above, these effects were not observed with the SV40 promoter constructs. There were no differences in transfection frequency between the normal and variant cDNAs under control of the SV40 promoter. In addition, nearly identical TS enzyme and mRNA concentrations were present in transfectants expressing the normal or the variant enzyme. The reasons for the different behaviors of the MMTV and the SV40 constructs are not clear, but we surmise that subtle events at the post-transcriptional or translational level may be involved.

In drug-response studies, we found that cell line MMTS-B required about 15–20-fold higher FdUrd concentrations for 50% growth inhibition than did cell line MMTS-A (Fig. 6). This is not due solely to higher levels of TS expression in MMTS-B, because cell line SVTS-B was about 3–4-fold more resistant than cell line SVTS-A, even though the two express similar levels of TS (Fig. 8). Thus, the Tyr-33 to His-33 replacement must be responsible for a significant (i.e., about 4-fold) level of drug resistance in mammalian cells.

In *E. coli*, the level of TS expression was far less than expected from previous studies with the identical vector and bacterial strain (15). However, we did observe that cells expressing the novel TS were about 3–4-fold more resistant to FdUrd, compared with cells expressing the normal TS, extending the observations made with mammalian cell transfectants.

The amino acid coding regions of the TS cDNAs corresponding to the normal and variant TS mRNAs differ by only the T to C mutation at nucleotide 190. We conclude, therefore, that the novel TS form confers a FdUrd-resistant phenotype to both mammalian and bacterial cells as a consequence of the tyrosine to histidine substitution at residue 33. It is rather striking that this substitution resulted in a similar 3–4-fold resistance in each of several distinct circumstances where the normal and variant TS forms were expressed at equal levels. Such a level of resistance was observed in TS-overproducing HCT116 cells (11), in transfected mammalian cells (Figs. 6 and 8), and in transformed bacteria (Fig. 9).

Most likely, the Tyr-33 to His-33 substitution results in a reduced affinity of the enzyme for the active metabolite, FdUMP. In earlier kinetic experiments with crude enzyme preparations, we did observe that the binding constant for FdUMP association with TS is lower for the variant enzyme, compared with the normal enzyme (11). Recent detailed analyses using purified preparations of TS have shown that the variant form exhibits a k_{cat} that is reduced by about 8-fold and a K_a for FdUMP association that is reduced by about 4-fold (24).[2] Thus, the Tyr-33 to His-33 mutation does alter the interaction between enzyme and ligands.

[2] C. T. Hughey, F. G. Berger, and S. H. Berger, Biochemical characterization of Human thymidylate synthases. Manuscript in preparation.

It will be of interest to understand the mechanism(s) by which the Tyr-33 to His-33 replacement perturbs the affinity of the enzyme for FdUMP. Tyr-33 is conserved among a variety of prokaryotic and eukaryotic species (25). Recent X-ray structural determinations have shown that the hydroxyl group of Tyr-33, which is located in an amphipathic helix (helix A), participates in a hydrogen bond with the main-chain carbonyl oxygen of Met-219, located at the base of helix J (25, 26).[3] Helix J is part of the active site proper and contains several amino acids that participate directly in ligand binding (25, 26). Hydrogen bonding between the A and J helices probably functions in stabilizing the conformation of the active site. A similar bond is formed between Tyr-4 and Val-170 in the *E. coli* enzyme, between Tyr-4 and Val-138 in the phage T4 enzyme, and between Tyr-6 and Ile-222 in the *Lactobacillus caseii* enzyme.[3] Further studies will have a bearing on the issue of spatially distant amino acid residues and how they communicate with, and transfer structural information to, the active sites of enzymes.

Finally, the fact that the Tyr-33 to His-33 mutation is naturally occurring raises questions concerning its prevalence in the human population and its impact, if any, on tumor response to 5-fluoropyrimidines in a clinical setting. Although relatively modest in its effects on TS function, the mutation may have significant consequences *in vivo*, with potential for being a major determinant of therapeutic success. The existence of an RFLP that identifies the T to C change underlying the amino acid replacement provides a convenient marker that may be useful in addressing such issues in the future.

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[3] C. Schiffer and R. Stroud, personal communication.

Send reprint requests to: Franklin G. Berger, Department of Biological Sciences, University of South Carolina, Columbia, SC 29208.