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Single Amino Acid Substitution Defines a Naturally Occurring Genetic Variant of Human Thymidylate Synthase

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SUMMARY

Previously, we identified an altered structural form of thymidylate synthase (TS) in a human colonic tumor cell line. This form, which is encoded by a variant structural gene, renders cells relatively resistant to 5-fluoro-2′-deoxyuridine as a result of the reduced affinity of the enzyme for the active metabolite 5-fluoro-2′-deoxyuridylic acid. We have isolated a cDNA clone specific to the altered TS and have determined its sequence. Two point mutations distinguish the normal from the altered TS mRNAs. One, a (A→G) change, is located within the 3′-untranslated region; the other, a T→C change within the amino acid-coding region, predicts replacement of tyrosine by histidine at residue 33 of the polypeptide. This sequence change was confirmed by direct analysis of cDNA amplified by the polymerase chain reaction and was further verified using allele-specific oligonucleotides as probes in Northern blots. These results, along with studies by other laboratories showing Tyr^{33} to be evolutionarily conserved, suggest that this residue plays an important role in TS function.

TS (EC 2.1.1.45) catalyzes the methylation of dUMP by $CH_2H_4PteGlu$, to generate dTMP and dihydrofolate. Because the enzyme is required for the *de novo* synthesis of dTMP, it has been an important target at which antineoplastic agents are directed (1). Fluoropyrimidine analogs such as 5-fluorouracil and FdUrd exert their cytotoxic effects primarily through formation of FdUMP, which inhibits TS by entering into a stable, covalent, ternary complex with the enzyme and $CH_2H_4PteGlu$ (2–5). Complex formation precludes enzyme catalysis, leading to cessation of DNA biosynthesis, presumably as a consequence of dTMP deprivation.

A variety of studies have shown that the structure and concentration of TS are important determinants of cellular sensitivity to fluoropyrimidines (6–8). Recently, we reported that the human colon tumor cell line HCT116 is relatively resistant to FdUrd, due to expression of an altered form of TS (9, 10). This form, which is encoded by a variant structural gene, has a more basic pI and exhibits reduced affinity for both FdUMP and $CH_2H_4PteGlu$ (10). These properties are presumably due to one or more alterations in primary structure. In the present communication, we have identified a single amino acid change that distinguishes the normal and variant TS polypeptides. A T→C transition in codon 33 of TS mRNA results in substitution of tyrosine by histidine; this change accounts for the observed structural and functional differences between the two TS forms.

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Experimental Procedures

Materials. Oligonucleotides were prepared on an Applied Biosystems 380B synthesizer in the Oligonucleotide Synthesis Facility of the Institute for Biological Research and Technology, University of South Carolina. They were used without further purification.

Cell culture. All cell lines are TS-overproducing derivatives of colonic tumor cell line HCT116 and have been described previously (9, 10). They were maintained as monolayers in Dulbecco's modified Eagle's medium supplemented with 10 μM folinic acid and 10% fetal bovine serum.

Cloning and sequencing of cDNA. Poly(A)$^+$ RNA was isolated from HCT116/200-11 cells by standard methods. Double-stranded cDNA was prepared by the protocol of Gubler and Hoffman (11). Libraries of this DNA were made in either λgt10 or λZAP II (Stratagene); materials for packaging and transfection were obtained from Stratagene. TS cDNA clones were identified by the method of Benton and Davis (12); probes for screening were nick-translated mouse TS cDNA clone pMTS3 (13) or an end-labeled oligonucleotide (5′-GATCCAACACATCCTCCGCT-3′) corresponding to residues 201–220 of the TS mRNA. Inserts in λgt10 clones were subcloned into pT_3T_718 DNA by standard methods, whereas those in λZAP II clones were directly converted to pBluescript SK(−) by automatic excision (14). DNA sequencing was by the dideoxy chain termination method (15), using modified T7 DNA polymerase (US Biochemical Corporation).

Direct amplification and sequencing of cDNA. A 1-μg sample of the double-stranded cDNA used for cloning was subjected to ampli-

ABBREVIATIONS: TS, thymidylate synthase; $CH_2H_4PteGlu$, 5,10-methylenetetrahydrofolate; FdUrd, 5-fluoro-2′-deoxyuridine; FdUMP, 5-fluoro-2′-deoxyuridylic acid; PCR, polymerase chain reaction.

fication by the PCR (16), as described in the GeneAmp reagent kit (Perkin Elmer Cetus); reaction volumes were 100 μl. The forward primer for PCR (5′-ACCACTTGGCCTGCCTCCGT-3′) corresponds to residues 1–20 of TS mRNA, whereas the reverse primer (5′-TGATAAACCACAGCAACTCC-3′) is complementary to residues 351–370. Amplification was performed in an Ericomp thermal cycler and consisted of 20 cycles of denaturation at 94° for 3 min, annealing at 55° for 2.5 min, and extension at 72° for 2.5 min; this was followed by 20 cycles using the same conditions except that annealing was at 50°. Final extension was at 72° for 15 min. The 370-base pair product was gel-purified onto DEAE-paper, eluted from the paper, and sequenced.

Northern blotting. Total RNA (10 μg) was fractionated on a 1.2% agarose gel containing 2.2 M formaldehyde, transferred to a nylon membrane, and hybridized to end-labeled oligonucleotide probes. Oligo(A) (5′-TGCCCCAGGTACTGCAGCTC-3′) is complementary to residues 181–200 of the normal TS mRNA; oligo (G) (5′-TGCCCCAGGTGCTGCAGCTC-3′) is complementary to residues 181–200 of the variant TS mRNA. A control probe (5′-TGATAAACCACAGCAACTCC-3′) is complementary to residues 351–370, a region that does not differ between the two TS mRNAs. Hybridizations were carried out at 61° for the oligo(A) probe, 63° for the oligo(G) probe, and 53° for the control probe; all were in 0.6 M sodium chloride, with washes at 0.3 M sodium chloride. Hybridizing RNAs were observed by autoradiography.

Results

Isolation and sequence analysis of TS cDNA clones. To determine the primary sequence of the altered TS form, we isolated and sequenced a cDNA clone corresponding to the altered TS mRNA. Cell line HCT116/200-11 overproduces the variant mRNA as a consequence of gene amplification (10). Poly(A)$^+$ RNA from HCT116/200-11 cells was used to prepare a cDNA library, and TS-specific cDNA clones were identified by hybridization to a mouse TS cDNA probe (13). Among 120,000 clones, 6 were specific to TS. One such clone was purified, and its cDNA insert was subcloned into a plasmid vector to generate pKB103. The sequence of this insert was determined and compared to the full length sequence of a human fibroblast TS mRNA previously reported by Takeishi *et al.* (17). The insert of pKB103 extends from codon 70 (nucleotide 302), through the termination site following codon 313 (nucleotide 1035), to the poly(A) stretch; its sequence is identical to that reported by Takeishi *et al.* (17), except for an A→G base change at nucleotide 1124 in the 3′-untranslated region. Thus, the deduced primary structure of the variant TS polypeptide is identical to that of the normal TS between residues 71 and 313, a region that includes the folate binding site (amino acids 71–90) and the nucleotide binding site (amino acids 190–233).

None of the cDNA clones isolated in the first screen were full length. This was the result of poor protection of the *Eco*RI site within codon 70 (nucleotide 301) during construction of the library. To identify clones containing 5′ sequences of the TS mRNA, a second library was prepared and screened with an oligonucleotide corresponding to residues 201–220 of the fibroblast TS cDNA (17). One clone was identified, and its insert was subcloned to generate pKB120. Sequence analysis of pKB120 showed that it spans the region from codon 12 (nucleotide 127) to the poly(A) stretch; it differs from the fibroblast TS cDNA at nucleotide 190, where it contains a C instead of a T (Fig. 1). This T→C base change converts codon 33 from UAC to CAC and predicts replacement of a tyrosine by a histidine in the variant TS polypeptide. The two point mutations that distinguish the normal and variant TS mRNAs are highlighted in Fig. 2.

To verify and extend the analysis of the altered TS, direct sequencing of cDNA amplified by the PCR (16) was used. Total cDNA was prepared by reverse transcription of HCT116/200-11 poly(A)$^+$ RNA and subjected to PCR; the forward primer corresponded to nucleotides 1–20, whereas the reverse primer corresponded to nucleotides 351–370 (17). The resulting 370-base pair DNA fragment (Fig. 3) was sequenced between nucleotide 86 (which is 8 residues 5′ to the translational start site) and nucleotide 240 and was found to be identical to the fibroblast TS cDNA, except for a single T→C difference at nucleotide 190, confirming the results of analysis of cDNA clones.

Northern blot analysis of mRNAs encoding the normal and altered TS forms. As an independent confirmation of the T→C base difference between the two TS mRNAs, Northern blot analysis using allele-specific oligonucleotides was performed. The nucleotides were complementary to residues 181–200, with one [oligo (A)] containing an A at the site complementary to residue 190 and the other [oligo(G)] containing a G at this site. Each was hybridized to Northern blots of RNA from cell lines overproducing the normal and variant TS mRNAs (10). Fig. 4 shows that oligo(A) hybridized preferentially to RNA from cell lines overproducing the normal TS, whereas oligo(G) hybridized preferentially to RNA from cells overproducing the variant TS; a control probe complementary to a region that is identical in the two TS mRNAs did not distinguish between the two types of TS-overproducing cell lines. This reinforces the notion that the T→C point mutation at codon 33 distinguishes mRNAs encoding the two TS forms.

Discussion

The structure of the macromolecular target of a cytotoxic drug is a critical determinant of cellular sensitivity to that drug. Our results show that a single naturally occurring change, a Tyr→His replacement, in the primary structure of the TS molecule confers relative resistance to the TS-directed antimetabolite FdUrd in cultured cells (10). The Tyr^{33}→His^{33} mutation is the only difference between the altered form of TS and the normal form and, therefore, must be responsible for the diminished effectiveness of the enzyme as a drug target (9, 10). Tyr^{33} is one of 55 residues that are invariant among eight species that have been examined (18); the altered TS characterized in the present study is the only one with an amino acid other than tyrosine at this evolutionarily conserved site.

The data in the present study, together with previously published experiments (9, 10), strongly favor the notion that the Tyr^{33}→His^{33} mutation is responsible for the relative FdUrd resistance of cell line HCT116. However, as noted earlier (10), it is quite possible that factors in addition to this mutation contribute to the phenotype of HCT116 and other colonic cell lines.

Amino acid substitutions affecting the function of drug targets have been described previously. For example, Melera *et al.* (19, 20) identified an altered form of dihydrofolate reductase in Chinese hamster cells. This form contains two amino acid changes; an Asp^{95}→Asn^{95} substitution alters the electrophoretic mobility of the enzyme, whereas a Leu^{22}→Phe^{22} change results in poor affinity for methotrexate. Thus, amino acid substitu-

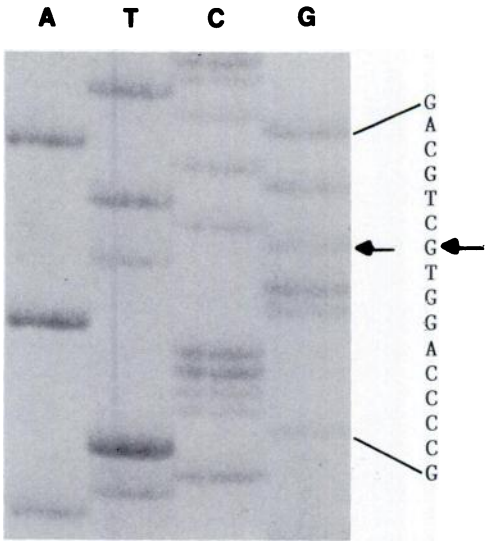

Fig. 1. Identification of a point mutation in the amino acid coding region of the altered TS mRNA. A sequencing gel for the complementary strand of the altered TS mRNA between nucleotides 180 and 200 is shown on the *left*. The *arrow* indicates the position of the point mutation that distinguishes the altered from the normal mRNA. The predicted amino acid sequence encoded by this region within each mRNA is shown on the *right*.

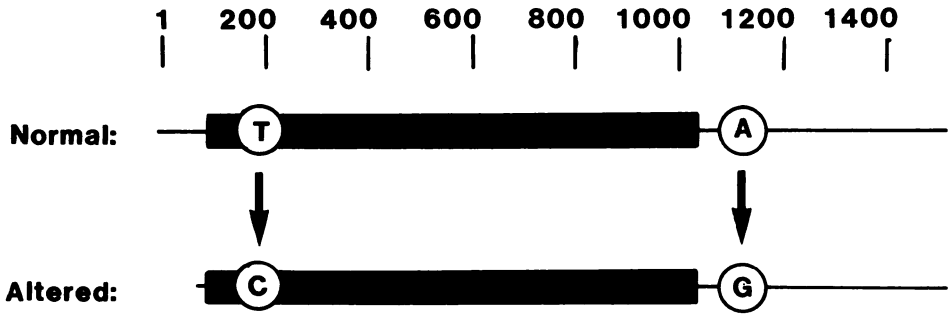

Fig. 2. Comparison of mRNAs encoding the normal and altered TS forms. Sequenced regions of the normal and altered TS mRNAs are aligned. Numbering is based upon the sequence of the normal mRNA (17). The amino acid-coding region is indicated by a *thick line*. The T→C difference responsible for the Tyr^{33}→His^{33} substitution is highlighted, as is the A→G difference in the 3′-untranslated region.

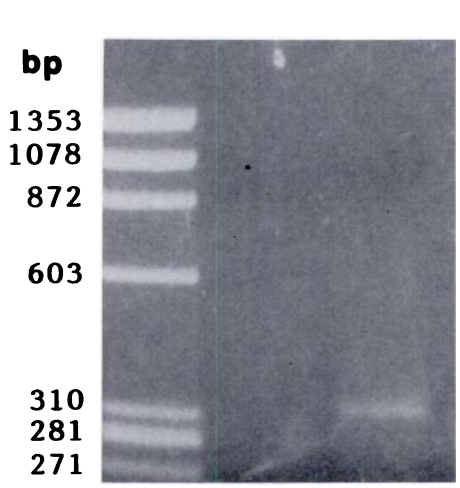

Fig. 3. PCR amplification of TS mRNA. Double-stranded cDNA prepared from HCT116/200-11 poly(A)$^+$) RNA was subjected to PCR amplification between nucleotides 1 and 370 (see Experimental Procedures). The 370-base pair product was purified and an aliquot was analyzed by agarose gel electrophoresis and ethidium bromide staining. Size markers (*HaeIII* fragments of ϕx174 RF DNA, *lane M*) are indicated to the *left*.

tions may be a relatively common source of variable drug response among human tumor cells.

High resolution X-ray structural analysis has shown that, in the TS from *Lactobacillus casei*, Tyr^6, which is analogous to Tyr^{33} in the human enzyme, is located within an amphipathic α-helix that flanks the active site cavity (18). It is likely that a similar amphipathic helix occurs between residues 29 and 44 of human TS, although an atomic structure for this enzyme is not yet available. A "helical wheel" depiction of this putative helix is shown in Fig. 5. The hydrophobicity index (21) for the polar face of the helix totals −3.4 kcal mol^{-1}, whereas that for the apolar face totals +5.3 kcal mol^{-1}. The Tyr→His change at residue 33 lowers the index of the apolar face to +4.6 kcal mol^{-1}.

The role of Tyr^{33} in TS function is not known. It is possible that a specific interaction between this residue and another amino acid located elsewhere in the polypeptide is critical to maintaining proper structure at the active site; such an interaction, which could involve a hydrogen bond, might be destroyed by a Tyr^{33}→His^{33} change. Alternatively, Tyr^{33} may play a more general role, deriving from its contribution to the amphipathic nature of the putative α-helix located between residues 29 and 44 (Fig. 5). This helix may form an interface between hydrophilic and hydrophobic environments within the protein; in reducing the amphiphilicity of the helix, the Tyr^{33}→His^{33} alteration may disturb the structural integrity of this region of the TS molecule. It will certainly be important to compare the two forms of human TS at the level of atomic structure.

The frequency of the Tyr^{33}→His^{33} mutation in the normal human population is unknown. As discussed previously (10), the altered TS may exist as a polymorphism in humans; alternatively, it may have spontaneously arisen during tumorigenesis or during establishment of the cell lines in culture. Distinguishing among these possibilities will be of great utility in assessing whether variant forms of TS identified in cultured cell lines are segregating in the human population and have an

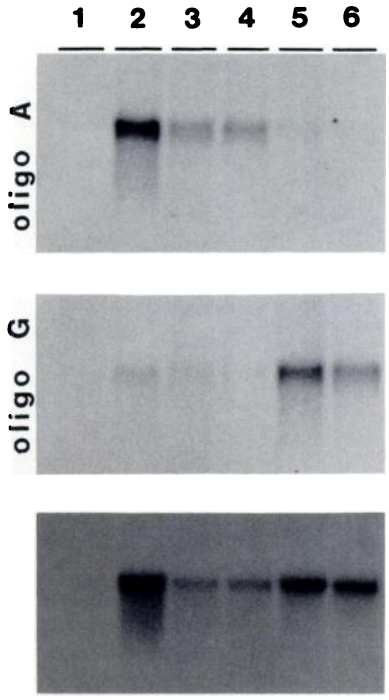

Fig. 4. Northern blot analysis of TS mRNAs using allele-specific oligonucleotides. Total RNAs from cell line HCT116 (*lane 1*) as well as derivatives that overproduce the normal (*lanes 2–4*) and the variant (*lanes 5* and *6*) forms of TS were fractionated on agarose gels, blotted onto nylon membranes, and hybridized to oligonucleotides specific to the normal mRNA [oligo(A)] (*upper*), to the variant [oligo (G)] (*middle*), or to both mRNAs (control) (*bottom*). The TS-overproducing cell lines were HCT116/200-1 (*lane 2*), HCT116/200-9 (*lane 3*), HCT116/200-10 (*lane 4*), HCT116/200-11 (*lane 5*), and HCT116/200-15 (*lane 6*). All have been described previously (10).

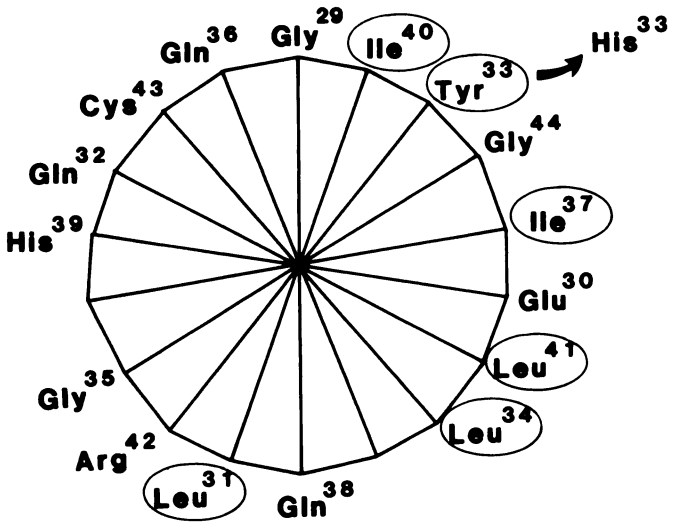

Fig. 5. Helical wheel representation of the putative amphipathic α-helix between residues 29 and 44 of human TS. Hydrophobic amino acids are *circled*; the position of the Tyr→His change that characterizes the altered TS form is indicated.

impact upon clinical response to 5-fluorouracil therapy in cancer patients.

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