Effects of 5-Fluorouracil Substitution on the RNA Conformation and in Vitro Translation of Thymidylate Synthase Messenger RNA*

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In vitro transcribed thymidylate synthase (TS) mRNA which is 100% substituted with 5-fluorouracil (FUra) was analyzed for changes in mRNA secondary structure, for alterations in translational efficiency, and for evidence of RNA-directed miscoding in vitro. FUra substitution in TS mRNA results in an altered migration pattern in non-denaturing RNA gels and in decreased hyperchromicity in RNA melting temperature studies, consistent with a change in mRNA secondary structure. However, no change in the translational efficiency of FUra-substituted TS mRNA is seen compared to control TS mRNA in either rabbit reticulocyte lysate or wheat germ extract in vitro translation systems. Analysis of the in vitro translation product of FUra-substituted TS mRNA by Western immunoblotting, isoelectric focusing, and TS catalytic activity experiments shows no differences compared to control TS mRNA. We conclude that the in vitro translation products of FUra-substituted and control TS mRNA are identical. Our findings do not support the hypothesis that changes in the mRNA template are responsible for the RNA-directed cytotoxicity of FUra.

For the past 30 years, 5-fluorouracil (FUra) has been the most active agent in the chemotherapy of colorectal cancer. Despite this experience, the exact mechanism of action of FUra cytotoxicity has not been precisely defined. The best characterized biochemical effect of FUra is the ability of its active metabolite, FdUMP, to inhibit the enzyme thymidylate synthase (TS; EC 2.1.1.45) in the presence of 5,10-methylenetetrahydrofolate, thus, interfering with DNA synthesis. However, FUra can also be metabolized to 5-fluorouridine 5'-monophosphate (FUTP) which can incorporate into RNA leading to alterations in TS mRNA which was synthesized in an identical manner except that FUTP was used in place of UTP in the reaction mixture. The rate of the in vitro transcription reaction was measured by adding 3 μCi of [3,8-3H]ATP to the reaction mixture and subsequently removing 3-μl aliquots at various time points. The aliquots were precipitated by spotting on 1-cm² pieces of filter paper (Whatman No. 3, Maidstone, England) and boiling for 10 min in 10% (w/v) trichloroacetic acid, followed by two washes each in water, ethanol, and acetone. The filter papers were then air-dried and placed in 10 ml of 3a/07B scintillation mixture (RPI, Mount Prospect, IL), and the tritium radioactivity was measured in a Beckman LS801 scintillation counter. The synthesis of completely FUra-substituted TS mRNA was confirmed by hydrolysis of the RNA and subsequent analysis for uracil and FUra bases by gas chromatography/mass spectrometry (16).

Although the exact biochemical lesion responsible for this RNA-directed FUra cytotoxicity is not known, FUra can replace uracil in all types of RNA resulting in numerous changes in RNA metabolism (7–10). Alterations in mRNA translation may represent a potentially important mechanism of FUra cytotoxicity. Studies in both bacterial and mammalian systems suggest that FUra incorporation in mRNA may decrease translational efficiency (11) or cause miscoding during protein synthesis (12–14). The mechanisms by which FUra might cause these effects are not known. One possibility is that FUra-induced translational errors are caused by base mispairing resulting from FUra-substitution in the mRNA template leading to the synthesis of altered or inactive proteins. Alternatively, FUra substitution in mRNA could potentially decrease the efficiency of translation requiring the cell to devote greater resources to protein synthesis. To examine these possibilities, we synthesized TS mRNA which was 100% substituted with FUra and analyzed it for changes in mRNA secondary structure, for alterations in translational efficiency, and for evidence of translational miscoding in vitro. TS was selected because it represents an important target enzyme for FUra activity which is essential for cell division. Furthermore, if FUra incorporation into TS mRNA alters the structure and function of newly synthesized TS protein, then this RNA-directed drug effect could potentially affect the ability of FdUMP to bind to and inhibit the TS enzyme.

MATERIALS AND METHODS

Reagents—[5-3H]dUMP (20 Ci/mmol), [6-3H5-FdUMP (18 Ci/mmol), and [3,8-3H]ATP (18 Ci/mmol) were purchased from Moravek Biochemicals (Brea, CA). FUTP was synthesized by Sierra Biochemicals (Tucson, AZ). All other chemicals, unless otherwise specified, were reagent grade and were purchased from Sigma.

Preparation of in Vitro Synthesized TS mRNA—Full-length TS mRNA (1525 bp) was transcribed from human TS cDNA using SP6 RNA polymerase (Promega, Madison, WI) according to the Promega protocol as described previously (15). Completely FUra-substituted TS mRNA was synthesized in an identical manner except that FUTP was used in place of UTP in the reaction mixture. The rate of the in vitro transcription reaction was measured by adding 3 μCi of [3,8-3H]ATP to the reaction mixture and subsequently removing 3-μl aliquots at various time points. The aliquots were precipitated by spotting on 1-cm² pieces of filter paper (Whatman No. 3, Maidstone, England) and boiling for 10 min in 10% (w/v) trichloroacetic acid, followed by two washes each in water, ethanol, and acetone. The filter papers were then air-dried and placed in 10 ml of 3a/07B scintillation mixture (RPI, Mount Prospect, IL), and the tritium radioactivity was measured in a Beckman LS801 scintillation counter. The synthesis of completely FUra-substituted TS mRNA was confirmed by hydrolysis of the RNA and subsequent analysis for uracil and FUra bases by gas chromatography/mass spectrometry (16).

RNA Gel Electrophoresis and RNA Melting Curves—TS mRNA (1 μg) was analyzed by electrophoresis on 12-cm horizontal 1.5% agarose gels using 20 mm MOPS, 50 mm sodium acetate, 1 mm EDTA, pH 7.0, as running buffer. Denaturing gels included 7% formaldehyde and were
run at 75 mA for 45 min. Overall, RNA migrated much more slowly in nondenaturing gels which were run at 75 mA for 2 h. Gels were stained with ethidium bromide and were photographed under UV illumination using an Eagle Eye frame integrator (Stratagene, La Jolla, CA).

Absorbance melting curve analysis of TS mRNA was performed using the method of Puglisi and Tinoco (17). Briefly, 1 μg of TS mRNA was dissolved in 0.01 M sodium chloride, 10 mM sodium phosphate, pH 7.0, and 0.1 mM EDTA, pH 8.0, and the UV absorbance was measured at 260 nm over a range of 28-70 °C using a Hewlett-Packard 8452A spectrophotometer equipped with a digitally controlled HP 89090A Peltier temperature control accessory cuvette holder.

In Vitro Translation—Translation reaction mixtures (final volume, 30 μl) containing 10 μl of rabbit reticulocyte lysate, 66 mM potassium acetate, 542 μM magnesium acetate, 50 μCi of [3H]methylmethionine, and 13 μl of translation mixture, all supplied in an in vitro translation system kit (Du Pont-New England Nuclear), were incubated with TS mRNA at 37 °C for 40 min, and the products were analyzed by SDS-PAGE (12.5% acrylamide) as previously described (15). Unincorporated radioactive label was removed by washing the gel three times in 100 ml of 40% methanol, 10% acetic acid (v/v) for 20 min. The gel was then soaked in 250 ml of Enlightning (Du Pont-New England Nuclear) containing 3% glycerol for 2 h at room temperature. After drying for 2 h, the translation products were visualized by autoradiography. Wheat germ extracts were resolved by 12.5% acrylamide gel electrophoresis with a Tricine running buffer and were transferred to a nitrocellulose membrane with an LKB multiphor electrophoresis unit. Antibody staining was performed with a TS polyclonal antibody (18) at a dilution of 1:1000 in Blotto buffer. A horseradish-conjugated antibody (Bio-Rad) at a dilution of 1:1000 in Blotto buffer was used as the secondary antibody.

Isoelectric Focusing Analysis—Aliquots (10 μl) of the rabbit reticulocyte lysate in vitro translation reaction mixtures were analyzed on Ampholine PAG plates (1 mm thickness), pH range 3.5-9.5 (Pharmacia LKB Biotechnology Inc.) on a LKB Multiphor II isoelectric focusing apparatus set at a current of 50 mA for 1.5 h. After isoelectric focusing was completed, the proteins were visualized by autoradiography.

RESULTS

Synthesis of FURA-substituted Thymidylate Synthase mRNA—In vitro transcription of a plasmid containing full-length human TS cDNA using bacteriophage SP6 RNA polymerase results in a 1.5-kb transcript of human TS mRNA. FURA-substituted TS mRNA was synthesized by replacing the UTP with FUTP in the transcription reaction. The efficiency and the time course of the in vitro transcription reaction were quantified by adding [3,8-3H]ATP to the reaction mixture and measuring the tritium label accumulating in the acid-precipitable (RNA-containing) fraction over time (Fig. 1). The overall yield of TS mRNA is decreased to 82% when FUTP replaces UTP in the reaction mixture; however, the relative rates of the RNA polymerase reactions are similar with 50% of the maximal product formed after approximately 20 min for each reaction (Fig. 1). Complete hydrolysis of the in vitro transcribed TS mRNA and analysis of the pyrimidine bases by gas chromatography and mass spectrometry confirm the complete (>99.9%) replacement of uracil with FURA in the FURA-substituted TS mRNA. The fidelity of the in vitro transcription reaction utilizing FUTP was not directly examined; however, the results of our subsequent experiments on the translation of TS mRNA suggest an equivalent degree of fidelity of the SP6 RNA polymerase using either UTP or FUTP as substrate.

Altered Electrophoretic Migration of FURA-substituted TS mRNA in Nondenaturing Agarose Gels—Changes in RNA conformation and secondary structure of FURA-substituted TS mRNA were examined by gel electrophoresis in denaturing (formaldehyde-containing) and nondenaturing agarose gels. Under denaturing conditions, the electrophoretic migration patterns of wild-type and FURA-substituted TS mRNA are identical (Fig. 2A). In contrast, under nondenaturing conditions, FURA-substituted TS mRNA migrates more slowly than control TS mRNA (Fig. 2B) consistent with a difference in the RNA molecular conformation.

RNA Melting Curve Analysis—To further examine the difference in RNA secondary structure between FURA-substituted and control TS mRNA, RNA melting temperature experiments were performed. Heat denaturation of the ordered, base-paired

![Fig. 1. Efficiency of in vitro transcription of human TS cDNA. Incorporation of [3,8-3H]ATP into TS mRNA was measured in reactions which utilized either UTP (●) or FUTP (○) as described under “Materials and Methods.”](image-url)
regions in an RNA molecule increases its UV absorption (hyperchromicity), and the change in UV absorbance is proportional to the number and type of base pairs broken (17). The melting curve of TS mRNA is markedly changed by FURA incorporation resulting in less hyperchromicity (6.3%) compared to wild-type TS mRNA (10.8%) (Fig. 3). This result is consistent with a decrease in RNA base pairing in FURA-containing TS mRNA, and it suggests that FURA-incorporation alters RNA secondary structure.

**In Vitro Translation of FURA-substituted mRNA**—The effects of FURA substitution on mRNA translation were examined by incubating TS mRNA in either rabbit reticulocyte lysates (Fig. 4A) or in wheat germ extracts (Fig. 4B). Translation of FURA-substituted and control TS mRNA both yield a 35-kDa protein product in either *in vitro* translation system. The efficiency of translation in rabbit reticulocyte lysates was measured by determining the amount of 35S radioactivity accumulating in the trichloroacetic acid-precipitable fraction over time. Identical results are observed for both FURA-substituted and control TS mRNA (Fig. 5).

To further characterize the 35-kDa protein product of translation of FURA-substituted TS mRNA, a Western immunoblot analysis was performed using a rabbit polyclonal TS antisera (18). *In vitro* translation of both control and FURA-substituted TS mRNA yields a 35-kDa protein product which is recognized by rabbit polyclonal TS antisera (Fig. 6). No qualitative or quantitative differences between the FURA-substituted or control TS mRNA translational protein products are observed.

**Isoelectric Focusing Analysis**—Further analysis of the translational protein products was performed by isoelectric focusing. Aliquots of the rabbit reticulocyte lysate *in vitro* translation mixture were placed on an isoelectric focusing apparatus, and the proteins were separated on the basis of their isoelectric points. The *in vitro* translation products of FURA-substituted and control TS mRNA resolve at the same isoelectric point, consistent with identical amino acid sequences for both proteins (Fig. 7).

**FdUMP-binding Assay and TS Catalytic Activity**—Immunologic and electrophoretic evidence suggests that the *in vitro* translational product of FURA-containing TS mRNA is identical to wild-type TS. However, amino acid substitutions might still be present which would be detected only by using more sensitive tests of enzyme structure or function. Therefore, we examined the ability of TS protein synthesized from FURA-containing TS mRNA to form a ternary complex with FdUMP and 5,10-methylenetetrahydrofolate. Specific FdUMP-binding activity, approximately 2.5–3-fold higher than background, is detected only when either FURA-substituted or control TS...
exogenous RNA was resolved by SDS-PAGE. The gel was electroblotted onto FUra-substituted TS mRNA added RNA and analyzed by isoelectric focusing under "Materials and Methods." TS protein is indicated by the amount of [\(^3\)H]FdUMP bound by the lysate mixtures containing mRNA is added to rabbit reticulocyte lysates (Table I). These values are slightly higher than the previously reported TS enzyme \(K_m\) values for dUMP measured under optimized conditions (\(K_m = 3.4 \mu M\)) (21).

<table>
<thead>
<tr>
<th>RNA</th>
<th>[(^3)H]FdUMP binding activity</th>
<th>TS catalytic activity</th>
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<tbody>
<tr>
<td>Control TS mRNA</td>
<td>0.08 ± 0.01</td>
<td>9.9 ± 0.9</td>
</tr>
<tr>
<td>FUra TS mRNA</td>
<td>0.6 ± 0.03</td>
<td>9.2 ± 0.4</td>
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* No significant difference, \(p > 0.05\).

**DISCUSSION**

Evidence that FUra incorporation in mRNA causes miscoding during translation initially came from studies in bacteria. Nakada and Magasanik (12) found that FUra readily incorporated into *Escherichia coli* mRNA resulting in the synthesis of an altered, inactive \(\beta\)-galactosidase enzyme. This altered protein was postulated to result from the translation of FUra-containing mRNA. Additional evidence came from studies by Rosen *et al.* (13) on the amber alkaline phosphatase mutation in *E. coli*. The replacement of uracil by FUra changed the nonsense mutant amber codon, UAG, to FUra-AG, which was read during translation as CAG, resulting in the phenotypic reversal of the amber mutation. Evidence for translational miscoding in mammalian cells came from a series of experiments by Dolnick and Pink (14) in human KB cells which overexpress dihydrofolate reductase (DHFR). In this system, FUra exposure did not change the rate of synthesis of DHFR as measured by immunoprecipitation; however, a significant decrease in enzyme activity was observed. This apparent paradox was examined further by purifying DHFR mRNA from drug-treated cells and translating it in *vitro* in rabbit reticulocyte lysates. No change in the *in vitro* translational activity of the FUra-containing mRNA was seen. However, the DHFR enzyme isolated from these cells after FUra exposure had altered affinities for binding methotrexate and anti-DHFR antisera. These changes in enzyme structure and function were attributed to amino acid substitutions resulting from mispairing of FUra in mRNA during translation. Under the conditions used in this study, the amount of FUra substitution in total cellular RNA was estimated at 2%; however, the degree of substitution in the specific DHFR mRNA undergoing translation was not quantified. In addition, the protein product of the *in vitro* translation reaction was not characterized. Nonetheless, these observations supported the theory that translational miscoding was an important mechanism of RNA-directed FUra cytotoxicity.

Our experiments do not demonstrate any change in mRNA function as a result of FUra incorporation in TS mRNA. No difference is seen in the translational efficiency of FUra-containing TS mRNA compared to wild-type TS mRNA, and both
yield similar 35-kDa protein products. Further analysis by Western immunoblots, isoelectric focusing, FdUMP-binding, and TS catalytic activity shows no difference in the protein products of translation of FUra-substituted and wild-type TS mRNA leading us to conclude that the proteins are identical. There is no evidence that FUra incorporation into the mRNA template causes miscoding during translation. Our findings are in agreement with others who have failed to observe FUra-induced alterations in mRNA translation. Grunberg-Manago and Michelson (11) found that the in vitro translation of polyfluorouridyl acid RNA resulted exclusively in the synthesis of polyphenylalanine, which is the same protein encoded by polyuridylic acid RNA. No evidence for translational miscoding was observed; however, in contrast to our findings, the efficiency of in vitro translation of polyfluorouridyl acid RNA was decreased by over 90% compared to polyuridylic acid RNA. In other experiments, Glazer and Hartman (22, 23) found that FUra incorporation into total cellular polyadenylated RNA only minimally affected its in vitro translational activity, even after exposure to toxic levels of FUra. They concluded that FUra-induced changes in mRNA were not important mechanisms of drug action.

However, our findings do not completely exclude FUra effects on mRNA as a mechanism of cytotoxicity. Potentially important drug-induced alterations in mRNA metabolism may still occur at the level of nuclear mRNA processing rather than during translation. FUra-substituted precursor mRNA can be blocked from reaching the cytoplasmic translation apparatus by a number of mechanisms, including impaired nuclear processing (24), increased nuclear degradation (25), or decreased nuclear transport (10). The best evidence for a FUra effect on mRNA processing comes from an elegant series of experiments by Doong and Dolnick (26). FUra substitution in precursor DHFR mRNA resulted in the formation of abnormal splicing products in an in vitro mRNA processing system. However, over 80% substitution with FUra was required before processing changes were observed. An alternative mechanism for FUra-induced miscoding has been proposed by Gleason and Fraenkel-Conrat (27). 5-Fluorocytosine, which can be generated in some cells by amimation of FUra, incorporated into RNA in the place of cytosine and was subsequently read as uracil during protein synthesis. Different cell lines varied in their ability to convert FUra to 5-fluorocytosine which may explain why translational miscoding was inconsistently observed. This would also account for the lack of translational miscoding seen in our in vitro system. A final possibility is that translational miscoding may result from FUra actions on other RNA species, such as tRNA (7), tRNA (28), or uracil-rich small nuclear RNA (snRNA) (10, 29). Although these mechanisms were not examined in our study, they should be pursued in further investigations.

The complete replacement of uracil with FUra in TS mRNA in our experiments results in an altered migration pattern in non-denaturing RNA gels and in increased hyperchromicity in RNA melting temperature studies. These observations suggest that FUra substitution in TS mRNA induces a change in RNA secondary structure, a finding which is in agreement with other studies of FUra incorporation in RNA. Armstrong et al. (10) reported that FUra altered the secondary structure of snRNA as evidenced by the slower migration of certain snRNA bands on non-denaturing polyacrylamide gels. Likewise, Danenberg et al. (30) examined the effects of FUra-incorporation on the self-splicing activity of Tetrahymena rRNA. Substitution with 100% FUra moderately inhibited the autocatalytic, self-splicing reaction of this RNA, and it greatly increased the pH and temperature sensitivity of the process. This inhibition was attributed to a conformational change in the RNA molecule resulting from weaker base pairings between FUra and adenine. The physicochemical differences between FUra and uracil in their ability to form base pairs are incompletely understood (31). Although the physical structure of the FUra-adenine base pair is similar to uracil-adenine, its rate of opening is enhanced (31), resulting in greater thermal instability (11). This difference has been attributed to the enhanced acidity of the N-3 hydrogen in FUra leading to a higher degree of ionization at physiologic pH; the pKₐ of FUra is 7.8 and uracil is 10.1 (30). However, high resolution nuclear magnetic resonance studies of oligodeoxynucleotide base pairs suggest that abnormal FUra base pairing is caused by decreased stacking of the FUra residues and not by enhanced acidity (31).

In summary, we studied the effect of 100% replacement of uracil by FUra on the RNA conformation and in vitro translation of TS mRNA. Despite evidence that FUra incorporation alters RNA secondary structure, no difference in the translational efficiency of FUra-containing TS mRNA is observed. The protein product of translation of FUra-substituted TS mRNA is identical to control TS. These findings do not support the hypothesis that FUra-incorporation into mRNA causes miscoding during translation. Thus, it is unlikely that changes in the mRNA template are responsible for the RNA-directed cytotoxicity of FUra. Further studies are still necessary to evaluate whether FUra can alter protein translation by incorporation into other species of RNA, such as snRNA, rRNA, or tRNA.

REFERENCES