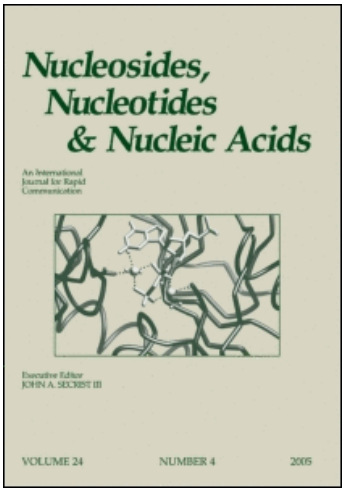

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REVERSE TRANSCRIPTION OF 5-FLUOROURACIL-SUBSTITUTED RNA

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Abstract. The present study examines how FUra substitution alters the ability of an RNA molecule to serve as a template for reverse transcription. We observe that reverse transcription of FUra-substituted RNAs is severely impaired at pH 8.8, but not at pH 7.8, while unsubstituted RNAs serve as efficient templates under both conditions.

Introduction. 5-Fluorouracil (FUra) was discovered 30 years ago, and remains an effective drug used in the treatment of a variety of cancers[1,2]. The structures of uracil (Ura) and 5-fluorouracil (FUra) are compared in FIG. 1. Despite its important antitumor activity, the mechanism of FUra action remains unknown. FUra toxicity was originally thought to result from inhibition of thymidylate synthase (TS) by the FUra metabolite 5-fluorodeoxyuridylic acid[3]. However, it is also known that FUra can be incorporated into cellular RNA. Exogenous thymidine was expected to relieve toxicity due to TS inhibition by providing the required substrate for salvage synthesis of DNA precursors. However, added thymidine was observed to increase both the anti-tumor activity of FUra, and its incorporation into cellular RNA[4]. This unexpected result suggested that FUra might possess one or more RNA-directed cytotoxic mechanisms.

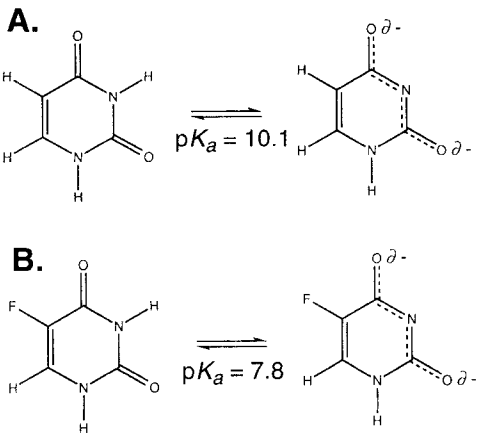

FIG. 1. **Ionization Equilibria.** (A) Ionization of Ura. The pK_a for loss of the H-3 proton of Ura is 10.1. (B) Ionization of FUra. The acidity of the H-3 proton of FUra is increased, corresponding to a pK_a value of 7.8.

Possible RNA-mediated mechanisms of FUra toxicity include translational miscoding[5,6], and inhibition of RNA processing by FUra substitution in the template[7-9] or in the splicing machinery[10-13]. It remains unclear, however, whether clinically-relevant levels of FUra substitution in RNA are responsible for cytotoxicity[14].

When compared with Ura, a notable feature of FUra is the increased acidity of the N-3 proton and subsequent ionization of FUra at physiological pH (FIG. 1). NMR evidence suggests that FUra ionization may be context-dependent[15]. It has been suggested that FUra ionization destabilizes FUra base pairing[16]. Destabilization of RNA secondary and tertiary structure may be responsible for the pH-dependent inhibition of self-splicing observed for FUra-substituted RNAs[13]. FUra also differs from Ura in its polarity, its tendency to adopt the enol tautomer, and the slightly larger size of fluorine relative to hydrogen.

Most experimental data suggest that FUra substitution causes subtle alterations in RNA structure and function[17-20]. For example, complete FUra substitution into globin pre-mRNA did not inhibit in vitro splicing, but resulted only in traces of an additional miss-spliced product[8]. Some evidence for translational miscoding by FUra substitution has been

observed in vivo[5,26], but translational miscoding could not be detected after complete FUra substitution in TS mRNA[6, 21]. Particularly notable is the relatively modest decrease in the rate of self-splicing observed for FUra-substituted Tetrahymena Group I intron sequences[9]. In contrast, unsubstituted snRNAs, but not FUra-substituted snRNAs could restore the activity of depleted yeast splicing extracts[13]. FUra-substituted RNAs have also been shown to assemble into less stable ribonucleoprotein complexes[12], and are poor substrates for pseudouridine synthases[22]. These results demonstrate that certain steps in RNA metabolism may be particularly sensitive to FUra inhibition.

To better understand FUra effects on RNA structure and function it would be desirable to identify RNA sites that are hypersensitive to FUra substitution. Such studies are difficult with clinical samples in which levels of FUra substitution are low. Alternatively, cell-free studies using synthetic RNA substrates may be useful. One approach to assigning sensitive Ura residues within such a target RNA would require partial FUra substitution followed by RNA fractionation on the basis of structure or function. The two resulting RNA populations might then be sequenced to detect sites enriched in FUra residues. Unfortunately, no chemical method is currently available for the differential sequencing of FUra versus Ura in RNA. As an alternative, we are exploring the possibility that sites of FUra substitution in RNA might be detected enzymatically.

The present study explores the ability of a reverse transcriptase (RT) to discriminate between FUra and Ura residues in RNA. In particular, we report conditions where premature termination by the RT occurs preferentially at FUra residues in the RNA template.

Materials and Methods

Materials. Radiochemicals were purchased from Amersham. T4 polynucleotide kinase was purchased from New England Biolabs. FUTP was the generous gift of W. Gmeiner. T7 RNA polymerase and *Tth* DNA polymerase were obtained from Epicentre Technologies (Madison, WI). Moloney Murine Leukemia Virus reverse transcriptase (MMLV-RT) was obtained from Gibco BRL.

Duplex DNA templates for transcription. Duplex DNA templates for RNA transcription were prepared by polymerase chain reaction (PCR) from a DNA sequence[23] cloned into vector pGEM-4Z (Promega). Plasmid DNA was linearized by *Sca*I and PCR was performed in 100 μL reactions using *Tth* DNA polymerase as recommended by the enzyme supplier (Epicentre Technologies, Madison, WI). Oligodeoxyribonucleotides were prepared, purified, and quantitated as previously described[24]. The 5' primer (5'-

TAATACGACTCACTATAGGGAAGCTT introduced a consensus T7 RNA polymerase promoter upstream of the DNA encoding the desired 85-nt RNA product. The 3' primer (5'- GAGCGGAATTCGACCC) was also used for reverse transcription as described below. PCR products were purified by chloroform extraction and ethanol precipitation. Five percent of the resulting DNA template was used for *in vitro* transcription.

RNA. RNA was transcribed from duplex DNA templates using T7 RNA polymerase and an AmpliScribe™ transcription kit (Epicentre Technologies, Madison, WI). Transcription reactions (10 μL) contained DNA template, proprietary transcription buffer, GTP, CTP and ATP (7.5 mM each) and either UTP (7.5 mM), FUTP (7.5 mM) or the appropriate ratio of UTP and FUTP, as indicated. For partial FUTP substitution studies it was assumed that T7 RNA polymerase incorporates UTP and FUTP with similar K_m values. To produce labeled RNA, transcription reactions were supplemented with 5 μCi [α-^{32}P]CTP. Transcription reactions were incubated at 37° C for 2 h, and then treated with DNase I to degrade template DNA. RNAs were purified by electrophoresis through 10% polyacrylamide gels (19:1 acrylamide:bisacrylamide) containing 7.5 M urea in 0.5X TBE buffer. RNAs were identified by UV shadowing, excised, eluted overnight into 2XPK buffer (200 mM Tris-HCl, pH 7.6, 2.5 mM EDTA, 300 mM NaCl, 2% SDS), extracted with phenol:chloroform (1:1), and precipitated from ethanol. RNA concentrations were estimated using the relation 1.0 A_{260} unit = 40 μg RNA/mL.

Reverse Transcription. Reverse transcription reactions (10 μL) contained 1 μM unlabeled template RNA, 0.4 mM deoxyribonucleoside triphosphates, RT buffer [50 mM Tris-HCl, pH 8.3 or 9.3 (at 22° C), 75 mM KCl, 3 mM $MgCl_2$, and 1 mM DTT], and the appropriate radiolabeled DNA primer (5'- GAGCGGAATTCGACCC). Hybridization reactions were incubated at 65° C for 5 min, and at 37° C for 20 min. MMLV-RT (100 units) was then added to each reaction, followed by incubation at 37° C for 30 min. Labeled cDNA was recovered by ethanol precipitation and analyzed by electrophoresis through 8% denaturing polyacrylamide sequencing gels (19:1 acrylamide:bisacrylamide). Gels were dried and analyzed by storage phosphor imaging using a Molecular Dynamics PhosphorImager.

Electrophoretic studies. Labeled RNA was purified by gel electrophoresis as described above. The gel mobilities of RNA samples were studied under native conditions by heating the sample to 65° C for 5 min in RT buffer, followed by a 30-min incubation at 37° C. After the addition of glycerol to 10% (v/v), samples were separated by

electrophoresis at 22° C in 8% polyacrylamide gels (19:1 acrylamide:bisacrylamide) prepared in 0.5X TBE buffer[25] (pH 8.6). The gel mobilities of RNA samples were studied under denaturing conditions by heating the samples in formamide and quick-chilling prior to electrophoresis through 8% polyacrylamide gels (19:1 acrylamide:bisacrylamide) prepared in 0.5X TBE buffer containing 7.5 M urea.

Results and Discussion

Experimental design. We chose to examine the effects of FUra substitution on reverse transcription using an RNA molecule under study in our laboratory[23]. The sequence of this 85-nt RNA is shown in FIG. 2. The sequence is rich in pyrimidines and contains six runs of three or more consecutive uracil (Ura) residues, beginning at positions 26, 41, 49, 56, 62, and 76 (FIG. 2A).

Our experimental approach involved the enzymatic synthesis and purification of two versions of this test RNA. One preparation contained only the four natural ribonucleotides (FIG. 2A). A second RNA preparation contained FUra substituted for all uracil residues of the transcript (FIG. 2B).

A radiolabeled DNA primer was annealed to either the unsubstituted or FUra-substituted RNA template (FIG. 2). The ability of Moloney Murine Leukemia Virus reverse transcriptase (MMLV-RT) to synthesize cDNA from these templates was then monitored by electrophoretic separation and detection of the labeled cDNA products on sequencing gels.

Inhibition of Reverse Transcription of FUra-substituted RNA. Standard reaction conditions for reverse transcription by MMLV-RT include deoxyribonucleoside triphosphates (0.4 mM each) in Tris buffer. The standard buffer pH is 8.3 at 22° C, corresponding to a pH of approximately 7.8 at 37° C. Under these conditions, the expected full-length cDNA was observed as a doublet after reverse transcription of both unsubstituted and FUra-substituted transcripts (FIG. 3, lanes 1-2). Dideoxy sequencing of a cDNA clone of this RNA using the same primer provided reference ladders (FIG. 3, lanes 13-16). The pattern of prematurely-terminated cDNAs were not significantly different for the two template preparations.

When dATP concentrations were reduced to 10 μM to enhance RT pausing at template Ura or FUra residues, the yield of full-length cDNA showed a greater reduction for the FUra-containing templates than for unsubstituted transcripts (FIG. 3, compare lanes 5-6). This result suggests that, relative to unsubstituted RNAs, FUra-substituted RNAs are somewhat more difficult to reverse transcribe at pH 7.8 in the presence of limiting

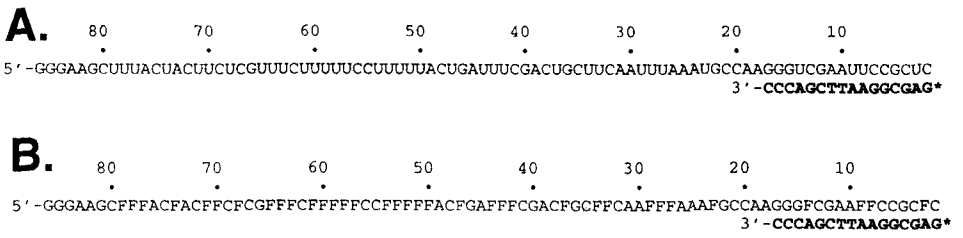

FIG. 2. **Experimental model.** The sequence of the 85-nt RNA transcript utilized in these studies is indicated. The sequence shown in bold depicts the 16-nt DNA primer used for reverse transcription. (*) indicates phosphate labeled with ^{32}P. (A) RNA sequence after transcription in the presence of UTP. (B) RNA sequence after transcription in the presence of FUTP.

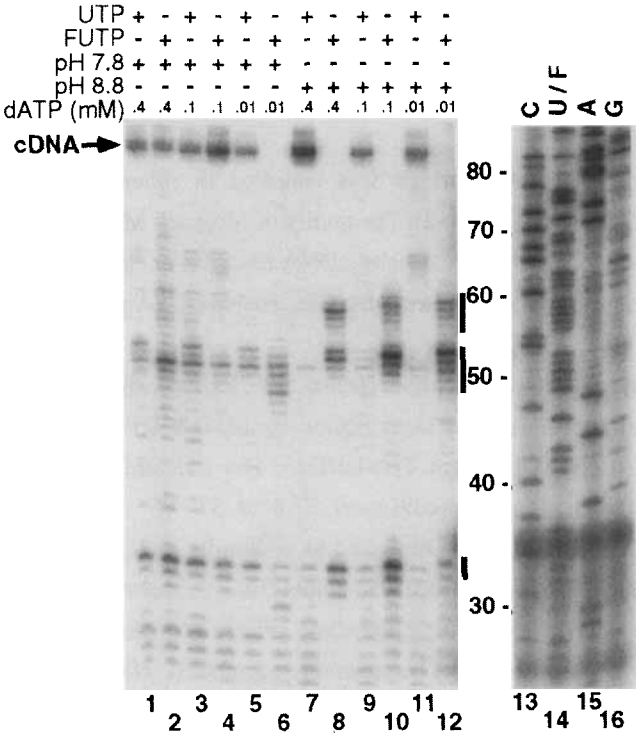

FIG. 3. **Reverse transcription of RNA templates.** Labeled cDNA products of reverse transcription reactions performed under the indicated conditions are shown at left (lanes 1–12). The position of the full-length cDNA (doublet) is indicated, and did not differ between unsubstituted and FUra-substituted templates. The doublet may arise due to heterogeneity in the exact site of T7 RNA polymerase transcription initiation, or could correspond to heterogeneity in the terminating nucleotide during reverse transcription. Sequence markers are shown at right (lanes 13–16). Markers are labeled and numbered to indicate the identity of RNA template residues. Vertical bars to the right of lane 12 indicate premature termination sites observed at pH 9.3.

dATP concentrations. However, the patterns of premature terminations differed only slightly from that observed for the unsubstituted template under the same conditions (FIG. 3, lane 5).

FUra is more easily ionized at moderately alkaline pH than is Ura (FIG. 1). For example, at pH 8.8, 91% of FUra residues are predicted to exist as anions, while only 5% of Ura residues will be ionized. We reasoned that increasing the pH of the RT reaction might allow differential ionization of FUra versus Ura residues. It seemed possible that the presence of an ionized FUra residue in the active site of the RT might increase the probability of premature termination.

To examine this possibility, reverse transcription reactions were performed at elevated pH (FIG. 3, lanes 7-12). A buffer pH of 9.3 (22° C) was chosen, corresponding to a pH value of approximately 8.8 at 37° C. Interestingly, these conditions *increased* the efficiency of full-length cDNA synthesis from the unsubstituted template, relative to that observed at pH 7.8 (FIG. 3, compare lanes 1 and 7). In contrast, *no full-length cDNA was observed using FUra-substituted RNA templates at pH 8.8* (FIG. 3, compare lanes 7 and 8). Rather, several sets of premature termination products were observed. Similar termination patterns were observed at this pH under conditions of limiting dATP concentrations (FIG. 3, lanes 9-12).

Comparison of the premature RT termination sites with sequence markers (FIG. 3, lanes 13-16) demonstrates that the termination occurs preferentially within runs of consecutive template FUra residues (e.g. near positions 32-33, 51 and 58). Although premature termination generally mapped to consecutive FUra residues in the template, the termination pattern is not uniform. For example, enhanced termination can be detected at the pair of FUra residues at positions 32-33 (FIG. 3, lanes 8 and 10), but is less apparent at a nearby triplet of FUra residues (positions 41-43). The basis for this selectivity is unknown. Premature termination is not detected at runs of FUra residues beyond position 60 under these conditions. However, this may simply reflect the low probability of chain elongation through two previous sequences of five consecutive FUra residues.

What is the mechanism of FUra-dependent RT inhibition? We considered the possibility that the FUra-dependent premature terminations observed at elevated pH might be due to FUra-dependent degradation of the RNA template. Radiolabeled transcripts with or without FUra substitution were therefore prepared and subjected to mock reverse transcription at pH 7.8 or pH 8.8 before evaluation by electrophoresis. No evidence of FUra-dependent degradation was observed after treatment at either pH (data not shown).

Less compact structure of FUra-substituted RNA. We wished to rule out the possibility that FUra incorporation inhibits RT activity at elevated pH by stabilizing

elements of RNA secondary structure. Electrophoretic mobility studies were performed to monitor global effects of FUra substitution on the folding of this 85-nt RNA. Radiolabeled RNAs were prepared with different degrees of FUra substitution. The electrophoretic mobilities of these RNAs were then compared under denaturing and native conditions (FIG. 4). Interpretation of these results is based on the reasonable assumption that gel mobility under native conditions is a sensitive measure of molecular shape for molecules of very similar charge and molecular weight (FUra substitution increases the mass of the test RNA by only ~2%).

Purified radiolabeled RNAs with different degrees of FUra substitution comigrate on denaturing gels at pH 8.6 (FIG. 4A). This result demonstrates that the small differences in molecular weight and predicted differences in ionization do not contribute to altered gel mobility under denaturing conditions. Very different results were obtained when the same samples were electrophoresed under native conditions (FIG. 4B). The unmodified RNAs populate an ensemble of structures, seen as two discrete bands of high mobility and a smear of slower mobility species (FIG. 4B, lane 1). The ensemble of folded RNAs changes upon increasing FUra substitution, and the mean mobility of each ensemble decreases (FIG. 4B, compare lanes 2-5). At 100% FUra substitution, a single high mobility species and at least two additional slower species appear. All of these folded RNAs display lower mobilities than the most prominent pair of unsubstituted RNA species (FIG. 4B, compare lanes 5 and 2).

If increased negative charge due to FUra ionization were important for electrophoretic mobility, this should be detected as *increased* mobility of FUra-substituted RNAs. The comigration of unsubstituted and FUra- substituted RNAs under denaturing conditions (FIG. 4A), and the *reduced* mobility of FUra-substituted RNAs under native conditions (FIG. 4B), suggest that electrophoresis is detecting the FUra-dependent destabilization of the various folded structures of this RNA.

This evidence for FUra-dependent RNA unfolding is consistent with previous observations[6, 11, 13]. These results do not support the hypothesis that FUra substitution causes premature RT termination by stabilizing inhibitory secondary structures in the template RNA. The data favor an alternative model wherein RT pausing and premature termination are induced as sequences of consecutive ionized FUra residues appear in the active site of the polymerase.

Implications. Our data suggest that viral reverse transcription might be inhibited by FUra substitution into template RNA if i) the template contains multiple consecutive FUra substitutions, and ii) the effective pH at the site of reverse transcription is slightly alkaline. It is unknown to what extent these criteria might be met in vivo.

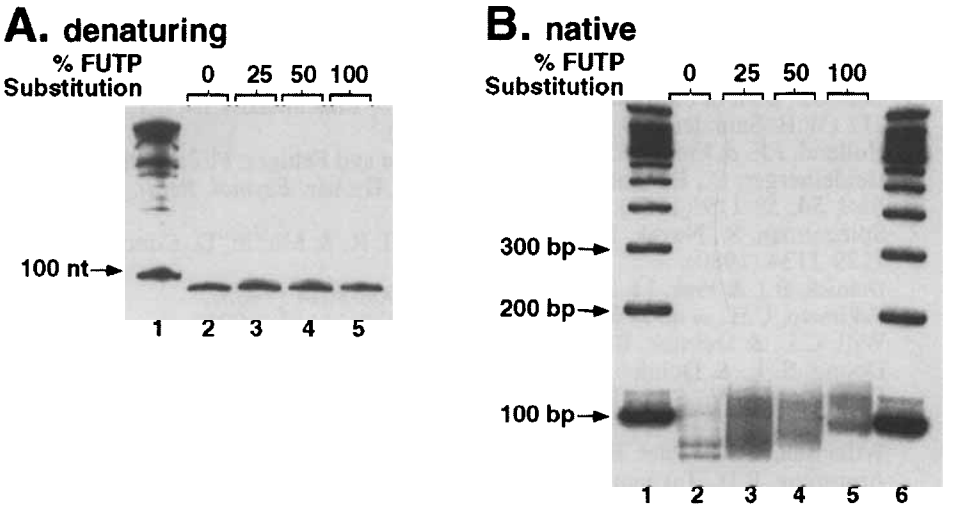

FIG. 4. **Alterations in RNA folding due to FUra substitution.** (A) Gel mobilities under denaturing conditions. Labeled RNA transcripts containing the indicated degrees of FUra substitution were electrophoresed in 0.5X TBE buffer (pH 8.6) in the presence of 7.5 M urea. The position of a 100 nt DNA marker is indicated in lane 1. (B) Gel mobilities under native conditions. RNA transcripts containing the indicated degrees of FUra substitution were electrophoresed in 0.5X TBE buffer (pH 8.6) in the absence of urea. Sizes (bp) of duplex DNA markers (lanes 1 and 6) are indicated.

From an analytical perspective, these results raise the possibility that sites of preferred FUra incorporation might be mapped in partially FUra-substituted RNAs. Such an approach could have value in determining the locations of FUra residues that strongly influence RNA structure or activity. For example, if partially FUra-substituted RNAs can be physically separated on the basis of functional inactivation or differences in structure, the critical FUra residues might be mapped as premature RT termination sites. Additional experiments will be needed to explore this possibility.

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