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SHORT COMMUNICATION

The Effect of 5-Fluorouridine 5′-Triphosphate on RNA Transcribed in Isolated Nuclei *in Vitro*

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SUMMARY

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Using nuclei prepared from rat liver, the effect of 5-fluorouridine 5′-triphosphate (FUTP) was assessed on the nearest neighbor frequency of the incorporation of [α-^{32}P]ATP and [α-^{32}P]GTP into nascent RNA transcribed by RNA polymerase I and II. When UTP was replaced by an equimolar concentration of FUTP, GMP was preferentially incorporated next to AMP in either the presence or the absence of α-amanitin. In addition, the incorporation of AMP, but not GMP, next to UMP and GMP was significantly reduced. The effect of FUTP on GMP incorporation into nearest neighbors was particularly evident in the presence of α-amanitin. These effects were virtually completely reversed by the addition of UTP to the reaction mixture. When endogenous template activity was inhibited by actinomycin D and RNA was transcribed from poly(dA–dT)·poly(dA–dT), misincorporation of AMP by the presence of FUTP was greatly enhanced. These results show that FUTP is capable of producing base-pair transformations with mammalian RNA polymerases, and suggest that this effect may account, in part, for the aberrant effects of 5-fluorouracil on RNA synthesis and processing *in vivo*.

5-Fluorouracil is an effective antimetabolite possessing antitumor activity against a wide variety of experimental and human tumors. Its activity is believed to reside in its inhibitory effect on thymidylate synthetase (1) thereby inhibiting *de novo* synthesis of TMP and hence DNA synthesis (2, 3). In addition, 5-fluorouracil impairs the processing of precursor RNA (4), and is incorporated into many species of nuclear RNA (5), as well as rRNA (6). Interestingly, transcription is virtually unaffected by this antimetabolite (5, 7). This aspect to its action is particularly intriguing since incorporation of 5-fluorouracil into nascent RNA could produce fraudulent RNA via base-pair transformations. Recently, differences in translation *in vitro* were noted between normal hepatic mRNA and 5-fluorouracil-modified mRNA from partially hepatectomized rats treated *in vivo* (8). Point mutations by 5-fluorouracil have been documented in *Escherichia coli* where phenotypic reversion was produced (9). Subsequent studies with *E. coli* RNA polymerase showed that the nucleoside 5′-triphosphate of 5-fluorouracil (FUTP)[2] was utilized only about one-third as effectively as UTP with calf thymus DNA as template, an effect due mainly to an alteration in the V_m of the enzyme at physiological pH (10). However, it was noted that FUTP was more active with dG–dC-rich templates versus dA–dT-rich thymus DNA (10). Later studies with *E. coli* RNA polymerase and synthetic templates of defined sequences demonstrated using nearest neighbor analysis that when UTP and FUTP compete, UMP is preferentially incorporated next to AMP and CMP (11).

Since no comparable studies of the effect of FUTP on mammalian RNA polymerases have been reported, the present investigation was initiated to determine whether FUTP produces changes in the nearest neighbor frequencies of RNA transcribed from endogenous and exogenous templates by rat liver RNA polymerase I and II.

[α-^{32}P]ATP (24 Ci/mmol) and [α-^{32}P]GTP (23 Ci/mmol) were purchased from New England Nuclear, Boston, Massachusetts. The radiochemical purity was greater than 98% as determined by PEI-cellulose thin-layer chromatography with 1.0 M ammonium formate:1.0 N HCl (62:38, v/v). Poly(dA–dT)·poly(dA–dT) was obtained from Miles Laboratories, Elkhart, Indiana. ATP, CTP, UTP, and GTP were purchased from Sigma Chemical Company, St. Louis, Missouri. FUTP was obtained from the Drug Synthesis and Chemistry Branch, Na-

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[2] The abbreviation used is: FUTP, 5-fluorouridine 5′-triphosphate.

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tional Cancer Institute, and migrated as a single band on PEI-cellulose thin-layer plates with 0.8 M LiCl as the solvent (R_f = 0.38). The 250/260 and 260/280 ratios for FUTP were 0.60 and 1.11, respectively, in agreement with previous data (10).

Nuclei were prepared from the livers of male Sprague–Dawley rats (200–250 g) by the procedure of Higashinakagawa *et al.* (12). After the final centrifugation in a discontinuous gradient of 0.34 M sucrose–5 mM $MgCl_2$/0.88 sucrose–5 mM $MgCl_2$, nuclear pellets were resuspended in 25% glycerol–0.25 M sucrose to a concentration of approximately 4 mg DNA/ml.

Assays measuring incorporation of [α-^{32}P]ATP or [α-^{32}P]GTP into RNA were carried out at 37° for 20 min and contained in a final volume of 0.5 ml: 40 mM Tris–HCl (pH 7.9), 1.6 mM dithiothreitol, 1.6 mM $MnCl_2$, 50 mM $(NH_4)_2SO_4$, 100 μM each of ATP, CTP, GTP, either 10 μCi [α-^{32}P]ATP or 10 μCi [α-^{32}P]GTP as indicated, 2 μg α-amanitin where indicated, 100 μl of a suspension of nuclei (equivalent to approximately 400 μg of DNA) and either (1) 100 μM UTP, (2) 100 μM FUTP or (3) 50 μM UTP and 50 μM FUTP. α-Amanitin produced 45% inhibition of activity under these assay conditions.

Assays measuring RNA polymerase activities in the presence of exogenous template contained: 40 mM Tris–HCl (pH 7.9), 1.6 mM dithiothreitol, 1.6 mM $MnCl_2$, 50 mM $(NH_4)_2SO_4$, 100 μM ATP, 100 μg actinomycin D, 10 μCi [α-^{32}P]ATP, 5 μg poly(dA–dT)·poly(dA–dT), 100 μl of nuclei and either (1) 100 μM UTP, (2) 100 μM FUTP or (3) 50 μM UTP and 50 μM FUTP.

Analysis of ^{32}P incorporated into adjacent nucleotides was carried out as previously described (13, 14), except that separation of nucleoside 3′-monophosphates was performed by high-voltage electrophoresis with an MRA apparatus (Clearwater, Fla.). Alkaline hydrolysates were neutralized with perchloric acid and lyophilized. Samples were dissolved in 100 μl of water and 20-μl aliquots were spotted on flexible cellulose thin-layer sheets (Eastman Kodak). Analyses contained 20,000–50,000 dpm per 20-μl aliquot.

Initial experiments were carried out with varying concentrations of UTP or FUTP or both in the presence or absence of α-amanitin, a specific inhibitor of RNA polymerase II at the concentration used (Fig. 1). FUTP was utilized only about 25 to 35% as efficiently as UTP when the net incorporation of [α-^{32}P]GTP was measured. The addition of equimolar concentrations of UTP and FUTP produced a small but consistent stimulation of activity in the absence (Fig. 1A) or presence of α-amanitin (Fig. 1B). This stimulatory effect also was present when [α-^{32}P]-ATP served as precursor, although the stimulation was not as great in the presence of α-amanitin. It should be noted that since RNA polymerase III was not detected previously in rat liver nuclei after QAE-Sephadex chromatography (14, 15), and since 100 μg/ml of α-amanitin did not further reduce nuclear polymerase activity, assays in the absence of α-amanitin represent RNA polymerase I and II activities while those in its presence measure only RNA polymerase I activity.

In order to test if FUTP alters the frequency of nucleotide incorporation into RNA, nearest neighbor analyses were carried out with [α-^{32}P]GTP and [α-^{32}P]ATP

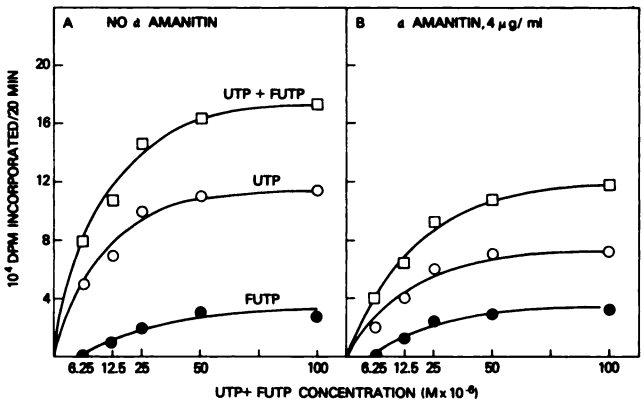

FIG. 1. *Incorporation of [α-^{32}P]GTP into RNA transcribed in isolated nuclei in the presence and absence of FUTP*

Assays were carried out as described in the text, in the absence (A) or presence (B) of 4 μg/ml of α-amanitin. The concentrations on the abscissa refer to the amount of either UTP (○), FUTP (●) or equimolar amounts of UTP and FUTP (□) in the assay mixture containing: 40 mM Tris–HCl (pH 7.9), 1.6 mM dithiothreitol, 1.6 mM $MnCl_2$, 50 mM $(NH_4)_2SO_4$, 10 μCi [α-^{32}P]GTP, and 100 μM each of ATP, GTP and CTP. Background incorporation in the absence of UTP was approximately 20,000 dpm, and has been subtracted from the values to reflect net incorporation of [α-^{32}P]GTP.

in the absence and presence of α-amanitin (Tables 1 and 2). Replacement of UTP by FUTP resulted in an elevation in the incorporation of [^{32}P]GMP next to AMP in either the presence or absence of α-amanitin (Table 1). When both UTP and FUTP were present in the equivalent final concentrations as either UTP or FUTP, increased incorporation of [^{32}P]GMP next to AMP and UMP were present in the absence of α-amanitin, whereas only marginal reduction of [^{32}P]GMP incorporation next to UMP was apparent in the presence of the polymerase inhibitor.

More striking changes in nearest neighbor frequencies were evident in the presence of [α-^{32}P]ATP (Table 2). When polymerase activity was assayed with FUTP alone, elevated incorporation of [^{32}P]AMP next to AMP and reduced incorporation next to GMP and UMP were found regardless of whether or not α-amanitin was present in the assay. More importantly, when UTP and FUTP were both present, no significant changes in nearest neighbor frequencies persisted in the absence of α-amanitin, while a diminished, yet not a significant, decrease in the incorporation of [^{32}P]AMP next to UMP remained in the presence of α-amanitin.

These experiments suggest that UTP is preferentially utilized by RNA polymerase I and II and that the aberrant nearest neighbor frequencies produced by FUTP can be largely reversed by UTP.

To test whether these effects were dependent on endogenous template, assays were carried out in the presence of high concentrations of actinomycin D and the synthetic template, poly(dA–dT)·poly(dA–dT), which is not affected by the dG–dC base-pair specific inhibitor (Table 3). Assays with FUTP alone produced an enormous reduction in the nearest neighbor frequency of the incorporation of [^{32}P]AMP next to UMP, but was markedly reversed by the addition of UTP to the reaction. The large increase in the misincorporation of [^{32}P]AMP

TABLE 1

Nearest neighbor analysis of GMP incorporation into RNA transcribed in isolated nuclei in the presence of FUTP

Assay of RNA polymerase activities were carried out as described in the text. Each value is the mean ± SE of five separate experiments. Assays either contained the complete mixture described under Materials and Methods (+UTP), the replacement of UTP with 100 μM FUTP (+FUTP), or 50 μM UTP and 50 μM FUTP (UTP + FUTP). RNA polymerase activity with FUTP replacing UTP or with UTP + FUTP was 35 and 138%, respectively, of activity with UTP in the absence of α-amanitin, and 42 and 137%, respectively, of activity with UTP in the presence of α-amanitin. Numbers in parentheses represent the percentages of control values from assays with UTP = 100%.

Nearest neighbor	Assay conditions					
	−α-Amanitin			+α-Amanitin		
	+UTP	+FUTP	UTP + FUTP	+UTP	+FUTP	UTP + FUTP
	(% distribution of radioactivity)					
Cp	25.2 ± 0.5 (100)	23.9 ± 0.6 (95)	21.1 ± 0.4 (84)***	29.0 ± 0.4 (100)	28.3 ± 0.8 (98)	29.7 ± 0.6 (102)
Ap	19.3 ± 0.1 (100)	23.1 ± 0.7 (120)***	21.8 ± 0.3 (113)***	17.5 ± 0.4 (100)	20.0 ± 0.9 (114)*	16.2 ± 0.8 (93)
Gp	33.5 ± 0.6 (100)	30.7 ± 0.6 (92)*	32.5 ± 0.2 (98)	33.4 ± 0.7 (100)	31.7 ± 0.6 (95)	34.6 ± 0.7 (104)
Up	22.0 ± 0.3 (100)	22.4 ± 0.2 (102)	24.4 ± 0.2 (111)**	20.1 ± 0.3 (100)	20.1 ± 0.7 (100)	19.2 ± 0.2 (96)*

* $P < 0.05$ vs control assay containing UTP. ** $P < 0.01$ vs control assay containing UTP. *** $P < 0.001$ vs control assay containing UTP.

next to AMP produced by FUTP was also counteracted by the presence of UTP, although not completely. No incorporation of [^{32}P]AMP was present in the region of GMP and CMP in the electrophoretograms, indicating that actinomycin D completely suppressed endogenous template activity. Incorporation of [α-^{32}P]AMP in the presence of only ATP resulted in the incorporation of 5% of the radioactivity compared to assays containing both ATP and UTP (Table 3). These data indicate that nuclear poly(A) polymerase contributed a minor proportion of the activity determined under the assay conditions. In addition, the latter enzyme neither is inhibited by actinomycin D (16), nor is active with poly(dA–dT)·poly(dA–dT) as primer (17).

The present study is apparently the first to document that FUTP can produce base-pair transformations in nascent RNA transcribed by mammalian RNA polymerases. One similarity in the effect of FUTP on nuclear RNA polymerases with previous results using bacterial RNA polymerase (9, 10) was that FUTP was utilized only about 30–50% as efficiently as UTP. However, the presence of FUTP in the complete assay mixture stimulated the incorporation [α-^{32}P]GTP into RNA (Fig. 1), even at apparently saturating levels of UTP. It is not known at present whether this is a direct or indirect effect on eukaryotic RNA polymerases present in isolated nuclei, but the fact that this effect did not occur with exogenous poly(dA–dT) as template suggests that this phenomenon may be associated with dC–rG base pairing.

Our studies have shown an increased frequency of incorporation of GMP and particularly AMP next to AMP, and a decreased preference for AMP to incorporate next to GMP and UMP, using the endogenous DNA template in isolated nuclei. These results are not directly comparable with those of Slapikoff and Berg (11) who noted an increased frequency of UMP incorporation next to AMP and CMP with little or no preference for incorporation of UMP next to GMP.

TABLE 2

Nearest neighbor analysis of AMP incorporation into RNA transcribed in isolated nuclei in the presence of FUTP

Assays of RNA polymerase activities were carried out as described in Table 1. Each value is the mean ± SE of three separate experiments. RNA polymerase activity with FUTP replacing UTP and with UTP + FUTP was 55 and 143%, respectively, of activity with UTP in the absence of α-amanitin, and 63 and 113%, respectively, of activity with UTP in the presence of α-amanitin. Numbers in parentheses represent percentages of control values from assays with UTP = 100%.

Nearest neighbor	Assay conditions					
	−α-Amanitin			+α-Amanitin		
	+UTP	+FUTP	UTP + FUTP	+UTP	+FUTP	UTP + FUTP
	(% distribution of radioactivity)					
Cp	22.3 ± 0.6 (100)	25.0 ± 0.5 (112)*	25.8 ± 1.8 (116)	21.6 ± 1.9 (100)	17.9 ± 0.1 (83)	20.7 ± 1.2 (96)
Ap	33.1 ± 0.4 (100)	40.2 ± 0.9 (121)**	31.4 ± 1.5 (95)	33.5 ± 2.1 (100)	50.4 ± 0.7 (150)**	37.7 ± 0.4 (113)
Gp	27.6 ± 0.7 (100)	22.0 ± 1.1 (79)*	27.0 ± 0.3 (97)	27.2 ± 0.7 (100)	20.9 ± 0.2 (77)***	25.9 ± 0.9 (95)
Up	16.9 ± 0.4 (100)	12.7 ± 0.2 (75)***	15.9 ± 0.2 (94)	17.7 ± 0.3 (100)	10.7 ± 0.6 (60)***	15.7 ± 0.2 (89)**

* $P < 0.05$ vs control assay containing UTP. ** $P < 0.01$ vs control assay containing UTP. *** $P < 0.001$ vs control assay containing UTP.

TABLE 3

Nearest neighbor analysis of AMP incorporation into RNA transcribed in isolated nuclei with poly(dA–dT)·poly(dA–dT) as template in the presence of FUTP and actinomycin D

Assays of RNA polymerase activities were assayed as described in the text. Each value is the mean ± SE of four separate experiments. RNA polymerase activity with FUTP replacing UTP and with UTP + FUTP was 35 and 82% of the activity with UTP. Total incorporation of [α-^{32}P]AMP in the absence of UTP (ATP) was 5, 20 and 6% of the activity with ATP + UTP, ATP + FUTP and ATP + UTP + FUTP, respectively. Incorporation under the latter three assay conditions has been corrected for background incorporation next to AMP in the absence of UTP. Numbers in parentheses represent percentages of control values from assays with ATP + UTP = 100%.

Nearest neighbor	Assay conditions			
	ATP + UTP	ATP	ATP + FUTP	ATP + UTP + FUTP
	(% distribution of radioactivity)			
Ap	12.8 ± 0.5 (100)	100 (5)	61.7 ± 2.3 (482)**	18.1 ± 1.4 (141)*
Up	87.2 ± 0.5 (100)	0 (0)	38.3 ± 3.3 (44)**	81.9 ± 1.4 (94)*

* $P < 0.05$ vs control assay containing ATP + UTP. ** $P < 0.001$ vs control assay containing ATP + UTP.

The effects of FUTP on endogenous RNA polymerase activities were confirmed, in part, by utilizing the synthetic template, poly(dA–dT)·poly(dA–dT) in the presence of actinomycin D to suppress endogenous template activity (18, 19). These experiments demonstrated that reduced incorporation of AMP next to UMP occurred as a result of the base-pair transversion, dA–rU to dA–rA.

It is not too speculative to surmise that the parent drug, 5-fluorouracil, after its metabolism to 5FUTP (20, 21), may produce aberrant synthesis and processing of rRNA (4–6) via base-pair transformations. This is strongly supported by the findings that the nearest neighbor frequencies for the incorporation of AMP were enhanced in the presence of α-amanitin, and therefore, under assay conditions that reflect mainly the activity of RNA polymerase I, the nucleolar enzyme responsible for the synthesis of precursor rRNA.

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REFERENCES

1. Hartman, K. U., and C. Heidelberger. Studies on fluorinated pyrimidines XIII. Inhibition of thymidylate synthetase. *J. Biol. Chem.* **236:** 3006–3013 (1961).
2. Danneberg, P. B., B. J. Montag, and C. Heidelberger. Studies on fluorinated pyrimidines. IV. Effects on nucleic acid metabolism *in vivo*. *Cancer Res.* **18:** 329–334 (1958).
3. Bosch, L., E. Harbers and C. Heidelberger. Studies on fluorinated pyrimidines. V. Effects on nucleic acid metabolism *in vitro*. *Cancer Res.* **18:** 335–343 (1958).
4. Wilkinson, D. S., A. Cihak and H. C. Pitot. Inhibition of ribosomal ribonucleic acid maturation in rat liver by 5-fluoroorotic acid resulting in the selective labeling of cytoplasmic messenger ribonucleic acid. *J. Biol. Chem.* **246:** 6418–6427 (1971).
5. Glazer, R. I. and A. L. Peale. The effect of 5-fluorouracil on the synthesis of nuclear RNA in L1210 cells *in vitro*. *Mol. Pharmacol.* **16:** 270–277 (1979).
6. Carrico, C. K. and R. I. Glazer. Augmentation by thymidine of the incorporation and distribution of 5-fluorouracil into ribosomal RNA. *Biochem. Biophys. Res. Commun.* **87:** 664–670 (1979).
7. Wilkinson, D. S., T. D. Tisty and R. J. Hanas. The inhibition of ribosomal RNA synthesis and maturation in Novikoff hepatoma cells by 5-fluorouridine. *Cancer Res.* **35:** 3014–3020 (1975).
8. Carrico, C. K. and R. I. Glazer. The effect of 5-fluorouracil on the synthesis and translation of poly(A)RNA from regenerating liver. *Cancer Res.* **39:** 3694–3701 (1979).
9. Champe, S. P. and S. Benzer. Reversal of mutant phenotypes by 5-fluorouracil: an approach to nucleotide sequences in messenger RNA. *Proc. Natl. Acad. Sci. USA* **48:** 532–546 (1962).
10. Kahan, F. M. and J. Hurwitz. The role of deoxyribonucleic acid in ribonucleic acid synthesis. IV. The incorporation of pyrimidine and purine analogues into ribonucleic acid. *J. Biol. Chem.* **237:** 3778–3785 (1962).
11. Slapikoff, S. and P. Berg. Mechanism of ribonucleic acid polymerase action. Effect of nearest neighbors on competition between uridine triphosphate and uridine triphosphate analogs for incorporation into ribonucleic acid. *Biochemistry* **6:** 3654–3658 (1967).
12. Higashinakagawa, T., M. Muramatsu and H. Sugano. Isolation of nucleoli from rat liver in the presence of magnesium ions. *Exp. Cell Res.* **71:** 65–74 (1972).
13. Glazer, R. I. Comparison of the fidelity of transcription of RNA polymerase I and II following *N*-hydroxy-2-acetylaminofluorene treatment. *Nucleic Acids Res.* **5:** 2607–2616 (1978).
14. Glazer, R. I. Alterations in the transcriptional capacity of hepatic DNA-dependent RNA polymerase I and II by *N*-hydroxy-2-acetylaminofluorene. *Cancer Res.* **36:** 2282–2289 (1976).
15. Legraverend, M. and R. I. Glazer. Inhibition of the phosphorylation of non-histone chromosomal proteins of rat liver by cordycepin and cordycepin triphosphate. *Cancer Res.* **38:** 1142–1146 (1978).
16. Hadidi, A. and S. V. Sethi. Polyadenylate polymerase from cytoplasm and nuclei of NIH Swiss mouse embryos. *Biochim. Biophys. Acta* **425:** 95–109 (1976).
17. Rose, K. M. and S. T. Jacob. Nuclear poly(A) polymerase from rat liver and a hepatoma. *Eur. J. Biochem.* **67:** 11–21 (1976).
18. Yu, F. L. Two functional states of the RNA polymerases in the rat hepatic nuclear and nucleolar fraction. *Nature* **251:** 344–346 (1974).
19. Lindell, T. J. Inhibitors of mammalian RNA polymerases. *Pharmacol. Ther. A.* **2:** 195–225 (1977).
20. Chaudhiri, N. K., B. J. Montag and C. Heidelberger. Studies on fluorinated pyrimidines. III. The metabolism of 5-fluorouracil-2-^{14}C and 5-fluoroorotic acid-2-^{14}C *in vivo*. *Cancer Res.* **18:** 318–328 (1958).
21. Cory, J. G., J. Crumley and D. S. Wilkinson. Evidence for role of purine nucleoside phosphorylase in sensitivity of Novikoff hepatoma cells to 5-fluorouracil. *Advan. Enzyme Regul.* **15:** 153–166 (1977).

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