Proc. Natl. Acad. Sci. USA
Vol. 73, No. 5, pp. 1528–1531, May 1976
Biochemistry

Biological consequences of incorporation of 5-fluorocytidine in the RNA of 5-fluorouracil-treated eukaryotic cells

(mutagenesis/tobacco mosaic virus/HeLa cell/pyrimidine analogs)

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Contributed by H. Fraenkel-Conrat, February 23, 1976

ABSTRACT **Treatment of HeLa cells with 5-fluoro-[^{3}H]uracil leads to the incorporation into cellular RNA of 5-fluorocytidine to the extent of about 0.2% of the 5-fluorouridine incorporated. In tobacco mosaic virus RNA produced in tobacco leaves this ratio is one order of magnitude lower.**

Copolymers of cytidylic with 5-fluorocytidylic acids show unchanged template activity with *E. coli* RNA polymerase, but slightly altered messenger activity in the wheat germ system, compared to poly(C), and it is suggested that some of the biological consequences of 5-fluorouracil treatment of living cells and organisms may be attributed to this mechanism.

It was demonstrated many years ago that 5-fluorouracil can be incorporated in viral RNA to the extent of replacing up to half of its uracil (1–3). Similarly, 5-bromouracil was found to replace thymine in bacterial and phage DNA (4). Neither of these replacements appears to alter the tautomeric state of the pyrimidine (5), and thus neither would be expected to represent a mutagenic event. In line with this, poly(5-fluorouridylic acid) was found to behave like poly(U) when tested for messenger activity (6). In the case of tobacco mosaic virus (TMV) and poliovirus, very few or no mutations were observed to result from extensive 5-fluorouracil incorporation, which also caused no significant change in infectivity (2, 7–9). In bacteria mutagenesis was frequently observed upon replacing uracil or thymine by the 5-fluoro- or 5-bromo-derivatives, although at levels suggesting that this was a rare consequence of that modification (3, 10, 11).

As a possible explanation for this paradox it was suggested by one of us in 1969 (9) that some 5-fluorouracil residues might be metabolically converted to 5-fluorocytosine and that incorporation of the latter, in place of uracil or cytosine, might result in mutations. Recently the postulated conversion of 5-fluorouracil to 5-fluorocytosine was shown to occur in *Escherichia coli* (12). The present study demonstrates that this is the case also in mammalian cells, and to a lesser extent in plant cells. Furthermore, the *in vitro* study of the messenger activity of copolymers of cytidylic with 5-fluorocytidylic acid in the wheat germ system showed that phenotypic mutations might result from this metabolic event.

MATERIALS AND METHODS

Growth of HeLa Cells. HeLa cells were maintained on 100 mm plastic tissue culture dishes in Dulbecco's modification of Eagle's minimal medium (13), supplemented with 2% calf serum. Cells were kept in a Wedco incubator at 37° with 5% CO_2 atmosphere.

Labeling of Cells. [^{3}H]Fluorouracil was administered in 6 ml of growth medium for 18–24 hr. One millicurie of label was distributed over four to eight dishes of cells, each containing a nearly confluent monolayer (approximately 10^7 cells per dish).

Isolation of the RNA. Medium was removed, and the cells were washed once with Dulbecco's phosphate-buffered saline (14). Cells were removed from the tissue culture dishes by treating them with crystalline trypsin (20 μg/ml in Dulbecco's phosphate-buffered saline) for 10–15 min. The cell suspension was centrifuged in a clinical centrifuge at approximately ⅔ speed. The pellet was suspended in TMK-2 buffer (Tris·HCl, 10 mM; $MgCl_2$, 3 mM; KCl, 15 mM; heparin 0.1 mg/ml; pH 7.4), centrifuged again, and then resuspended in the buffer. Triton X-100 was added to 1%, and the suspension was vortexed vigorously for 1–2 min to lyse the cells and then centrifuged again in the clinical centrifuge. The supernatant solution was saved as the cytoplasmic fraction, whereas the pellet represented the nuclear fraction. Pellets were checked under a microscope for the presence of nuclei and absence of intact cells. In some experiments it was necessary to retreat the pellet with Triton in TMK-2 to obtain complete cell lysis. All these operations were performed in the cold.

Nuclei were resuspended in TMK-2 buffer containing 1% sodium dodecyl sulfate, vortexed vigorously, and then centrifuged to remove membrane debris. The two supernatant solutions (cytoplasmic and nuclear) were each phenol extracted three times, ether extracted twice, and finally precipitated with three volumes of ethanol (−20°). In the case of the nuclear fraction it was found helpful to pass the supernate several times through a syringe equipped with a 23 gauge needle before phenol extraction. This step shears DNA without damaging the RNA. The poly(A)-containing mRNA was isolated by passing the cytoplasmic RNA over an oligo(dT)-cellulose column as previously described (15, 16), except that the diethylpyrocarbonate was eliminated from both high-and low-salt eluting buffers.

Digestion to Nucleosides. RNA fractions were digested with pancreatic ribonuclease A (EC 2.7.7.16; 20 μg/mg of RNA) in 75 mM Tris·HCl at pH 8.3 for 30 min to 1 hr, 37°. Then bacterial alkaline phosphatase (EC 3.1.3.1; 10 μg/mg of RNA) and snake venom phosphodiesterase (EC 3.1.4.1; 5 μg/mg of RNA) were added and incubation at 37° was continued for approximately 4 hr. Additional quantities of these enzymes were then added and digestion was allowed to proceed overnight.

Chromatography and Electrophoresis. The digested RNA was spotted on Whatman 1 paper and chromatographed in Solvent A (butanol:ethanol:water, 80:10:25) for 24–48 hr. Nucleoside marker and blank areas were cut out and eluted with water to 1 ml then dried in an air stream at 37°. Samples were redissolved in 0.1 ml water and the radioactivity of an aliquot was determined. The remainder of each fraction was chro-

Abbreviation: TMV, tobacco mosaic virus.

matographed separately (again on Whatman 1 paper) in Solvent B (isopropanol:hydrochloric acid:water, 130:37:32). Chromatograms were then cut into 2 cm strips and the strips were counted in 5 ml of Bray's scintillation fluid. Thin-layer chromatography was on cellulose 6065 precoated plastic sheets (Eastman Kodak). Flatbed electrophoresis was on Whatman 3 MM paper with 0.05 M sodium citrate at pH 3, 1000 V, 4 hr.

Preparation of Labeled TMV RNA. About 12 large tobacco leaves infected with 0.1 mg/ml of TMV were cut off after 3 days and their stems were quickly immersed in 0.5 ml of water containing 1 mCi of chromatographically purified 5-fluoro-[^{3}H]uracil. When this had been taken up, more water was added. The leaves were then floated on water in glass-covered baking dishes for 7 days with 16 hr illumination. Isolation of TMV by differential centrifugation, and of its RNA by phenol treatment, was by standard procedures.

Materials. 5-Fluoro[6-^{3}H]uracil was obtained from Amersham Searle (specific activity 1 Ci/mmol). In the first experiment the compound was used without purification. Later, however, we found that approximately 5% of the radioactivity was not coincident with 5-fluorouracil when chromatographed in Solvent A. Therefore, in subsequent experiments the base was purified by paper chromatography in Solvent A just before administering to the cells. The 5-fluorocytidine was a gift of Dr. W. E. Scott of Hoffmann-La Roche, Nutley, N.J. All enzymes were purchased from Worthington, except polynucleotide phosphorylase (polyribonucleotide nucleotidyltransferase, EC 2.7.7.8) (*Micrococcus lysodeikticus*), which was from P. L. Biochemicals, Inc., and RNA polymerase (RNA nucleotidyltransferase, EC 2.7.7.6) (*M. luteus*) from Miles Laboratories. Poly(C) and poly(U) were also obtained from Miles Laboratories; oligo(dT)-cellulose was from Collaborative Research, Inc. [^{3}H]ATP (17 Ci/mmol), [^{3}H]GTP (8 Ci/mmol), L-[^{3}H]leucine (53 Ci/mmol), L-[^{14}C]serine (156 mCi/mmol), and L-[^{14}C]phenylalanine (465 mCi/mmol) were purchased from Schwarz/Mann, and L-[^{3}H]proline was purchased from ICN Pharmaceuticals, Inc.

Preparation of Poly(C,FC) and Tests of the Activity of Such Polymers to Direct Nucleoside Triphosphate and Amino Acid Incorporation. 5-Fluorocytidine was treated with crude wheat shoot phosphotransferase, and the 5'-5-fluorocytidylic acid was purified according to Giziewicz and Shugar (17). The preparation of the diphosphate was according to Moffatt and Khorana (18).

Cytidine 5'-diphosphate mixed with various amounts of 5-fluorocytidine 5'-diphosphate was polymerized with polynucleotide phosphorylase under the enzyme assay conditions of Thanassi and Singer (19), allowing the reaction to proceed about 16 hr. The reaction mixture was extracted twice with phenol:chloroform (1:1), dialyzed with several changes of water, and finally precipitated with three volumes of ethanol at −20°. The polymers behaved on sucrose gradients similarly to commercial polynucleotides (molecular weight > 150,000). They were analyzed for C/FC ratios by the same methods of digestion and chromatography in Solvent A described for RNA above. The composition of the products closely reflected that of the input diphosphates.

Triphosphate incorporation tests (0.2 ml) contained Tris·HCl at pH 7.4 (0.125 M), $MnCl_2$ (6 mM), GTP (0.5 mM), ATP (0.05 mM), 1 μCi of the labeled triphosphate, 100 μg of polynucleotide, and 4 μg of RNA polymerase. The reaction mixtures were incubated at 37° for 1–1.5 hr, then three aliquots of 50 μl were removed from each and spotted onto DEAE-paper disks. The disks were washed eight to ten times with 50–100 ml of 0.4 M Na_2HPO_4, then once briefly with water and twice with ethanol, dried, and counted in toluene Omnifluor scintillation fluid.

Table 1. 5-Fluorocytidine in RNA after fluoro[^{3}H]uracil treatment of cells*

	[^{3}H]cpm	Percent of total
I. HeLa total cytoplasmic RNA	520	0.29
II. HeLa total cytoplasmic RNA†	316	0.18
HeLa polysomal rRNA	158	0.11
HeLa polysomal mRNA	237	0.11
HeLa nuclear RNA	481	0.28
I. TMV RNA	66	0.02
II. TMV RNA†	230	0.03

* These data represent the cpm (corrected for mechanical losses) in the 5-fluorocytidine area that were recovered from Solvent B chromatography, with the fraction of the total radioactivity (almost all 5-fluorouridine) given as percent. Roman numerals indicate separate experiments.

† In these experiments the sample was divided into two aliquots and each aliquot was separately digested and chromatographed; the data are averages of the two results.

Wheat germ S30 was prepared and in principle used according to Marcu and Dudock (20) starting with 6 g of dry wheat germ and 9 ml of grinding buffer, and applying the entire centrifugation supernatant to a Sephadex G-25 column. The high UV absorbancy fractions were frozen and used for amino acid incorporation as follows: the 25 μl sample contained 10 μl purified wheat extract, pH 7.6 *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) buffer (20 mM), dithiothreitol (2 mM), magnesium acetate (12 mM), potassium chloride (0.12 M), ATP (1 mM), GTP (0.02 mM), creatine phosphate (8 mM), 1 μg of creatine kinase (EC 2.7.3.2), 19 unlabeled amino acids (0.04 mM), 0.5 μCi ^{3}H-labeled or 0.1 μCi of ^{14}C-labeled amino acid to be tested, 1.4 μg of wheat tRNA, and 16 μg of polymer. After 2 hr at 39°, 1 ml of ice-cold 5% trichloroacetic acid was added. The mixture was then heated 15 min at 90°, cooled on ice 15 min, and filtered on glass fiber filters. Filters were rinsed four times with 3 ml of 5% trichloroacetic acid, dried, and counted in toluene Omnifluor scintillation fluid.

RESULTS

Detection of 5-Fluorocytidine in RNA. Analyses of the RNA of HeLa cells treated with 5-fluoro[^{3}H]uracil show a high level of incorporation of 5-fluoro[^{3}H]uridine, with 1000–4000 cpm/μg being found in the cytoplasmic RNA, and about 20,000 cpm/μg in the nuclear and in the cytoplasmic mRNA fractions. Upon chromatography of the digests of the RNAs, some radioactivity trailed behind the large far-moving peak of 5-fluorouridine in Solvent A, with a slight maximum being at times observed in the 5-fluorocytidine area. When the radioactivity chromatographing with 5-fluorocytidine was eluted and rechromatographed in Solvent B, it yielded two well-separated radioactive products coinciding with 5-fluorouridine and 5-fluorocytidine, respectively. No significant amounts of radioactive material were found associated with the other nucleosides (0.05% or less) upon rechromatography when purified 5-fluorouridine was used for incorporation, but some unidentified radioactivity was found at times in other areas, mostly far-moving (beyond all nucleosides, including 5-fluorouridine). The 5-fluorocytidine content ranged from 0.1 to 0.3% of the total 5-fluorouridine incorporated in different experiments and different HeLa cell RNA fractions (Table 1).

The authenticity of the radioactivity cochromatographing

Table 2. Amino acid incorporation with homo- and copolymers of nucleotides as messengers

Messenger	Net incorporation of amino acids (cpm)*			
	[^{3}H]Proline	[^{14}C]Phenylalanine	[^{3}H]Leucine	[^{14}C]Serine
Poly(C)	13,900	90	60	80
Poly(C_4,FC)	17,800	270	20	None
Poly(C,FC)	10,700	460	750	None
Poly(C,FC_2)	6,200	380	460	30
Poly(C,U)	14,200	3,630	7,290	2,200
Poly(U)	None	34,430	23,300	1,100

* See *Materials and Methods* for reaction conditions; other amino acids (not listed) showed no stimulation of incorporation with the polynucleotide listed. Data are given after subtracting blank incorporation, obtained without messenger polynucleotide (370, 2070, 800, and 900 cpm, respectively, for the four amino acids listed).

with 5-fluorocytidine in Solvent B was further substantiated by thin-layer chromatography with Solvent D of Kaiser and Kwong (12), followed by paper electrophoresis. With an allowance made for mechanical losses, all the radioactivity was found associated with the 5-fluorocytidine marker under these conditions.

When TMV RNA, from virus synthesized in the presence of purified 5-fluoro[^{3}H]uracil, was analyzed in the same manner, it was found to have incorporated 600 and 1000 cpm/μg in two experiments. The 5-fluorocytidine, however, represented only about 0.025% of the fluorouridine in this RNA.

Template and Messenger Activities of Copolymers of 5-Fluorocytidylic and Cytidylic Acid. When the three copolymers studied, with C:FC ratios of 4, 1, and 0.3, were used as templates for [^{3}H]GTP incorporation, no diminishment was observed: 19,100, 17,600, and 24,100 cpm compared to 17,900 with poly(C). There was also no evidence for mispairing, since the incorporation of [^{3}H]ATP averaged 282, 289, and 230 cpm, for the three copolymers, compared to 300 for poly(C), 809 for poly(C_{13},U), and 94 and 133 for blanks lacking enzyme or template.

The data concerning the messenger activity of these copolymers in the wheat germ system are summarized in Table 2. It appears that proline incorporation is diminished with the copolymers containing half or more 5-fluorocytidylic acid, and that phenylalanine and leucine, but not serine, incorporation is somewhat increased, compared to poly(C); the unexpected amino acids incorporated by poly (5-bromocytidylate), threonine and histidine (21, 22), are not incorporated by the fluorine-containing polymers, nor are glutamine, glutamic acid, or asparagine.

DISCUSSION

The suggestion has been made that the mutagenic effect of 5-fluorouracil treatment of cells might be due to mispairing or miscoding by 5-fluorocytidine residues formed from it and incorporated into cellular mRNA (9). That 5-fluorocytidine can be detected in the RNA of 5-fluorouracil-treated cells has previously been reported for *E. coli* (12), and this is now demonstrated to be the case also in animal cells. Since 5-fluorouracil treatment has been found to be much more mutagenic in bacterial cells than in plant cells, the proposed working hypothesis predicts that the incorporation of 5-fluorocytidine should be much lower in plant than in bacterial cells. This seems to be borne out by the present data, as compared to the levels found by Kaiser and Kwong (12). On the basis of the different levels found in animal and plant cells one would predict that 5-fluorouracil should be more mutagenic in the former. No search for low-level mutagenesis in animal cells has, to our knowledge, been reported, and quantitative comparative data concerning plant and animal cells would be difficult to obtain.

Since *in vitro* systems have become available for transcription and translation of RNA, these have been successfully applied to identify the mechanism of action of point-mutagenic agents. Thus, in the presence of Mn in lieu of Mg, RNA as well as polyribonucleotides can be used as templates for *E. coli* DNA-dependent RNA polymerase. The mutagenicity of reagents modifying cytidine was demonstrated in this manner *in vitro*, nitrous acid, hydroxylamine, and methoxylamine treatment of poly(C) causing it to incorporate some adenosine into the resultant poly(G), whereas methylation of the N3 position caused nonspecific incorporation (23), and the presence of 5-bromocytidine caused no significant incorporation of triphosphates other than GTP (21). The activity of all modified templates in terms of GTP incorporation was lessened. It now appears that 5-fluorocytidine is the only known analog that is indistinguishable from cytidine in qualitative, as well as in quantitative, terms in template function, as tested by this method.

In contrast, when the same polynucleotides were tested for their messenger activity in the wheat germ protein synthesizing system, the increased incorporation of phenylalanine and leucine with the 5-fluorocytidine-containing polymers, particularly when related to the decreasing proline incorporation, strongly suggests that 5-fluorocytidine can simulate uridine during translation. The lack of stimulation of serine incorporation by the 5-fluorocytidine-containing polynucleotides remains as yet unexplained. It appears possible that this may be due to a structural peculiarity of the wheat serine tRNA anticodon, which might render it incompatible with 5-fluorocytidine.

Thus we tentatively conclude that 5-fluorouracil treatment of cells and organisms has biological consequences correlated with its varying level of metabolic amination. The resultant 5-fluorocytidine apparently becomes incorporated into nucleic acids and/or transcribed only in lieu of cytidine, but it can occasionally be misread in mRNAs as uridine, thus producing "phenotypic mutation". 5-Fluorouracil and 5-fluorocytosine are now in widespread therapeutic, largely topical, use as anticancerous agents. It would be beyond the scope of this paper to discuss the possibility that the efficacy of these base analogs to preferentially affect the metabolism of the (pre)cancerous cell is at least in part due to their preferential action on translation rather than transcription.

We are indebted to Dr. I. Pietrzykowska, Warsaw, for collaborating with us in the initial experiments, and for her, as well as Prof. D. Shugar's, continued interest and suggestions in regard to this research project. This investigation was supported by National Science Foundation Grant BMS 75-07854.

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