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The Effect of 5-Fluorouracil on the Synthesis of Nuclear RNA in L1210 Cells In Vitro

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SUMMARY

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The effect of 5-fluorouracil on nuclear RNA synthesis and its incorporation into nuclear RNA were studied in L1210 cells *in vitro*. Inhibition of the incorporation of [^{3}H]uridine into total RNA by 5-fluorouracil was proportional to the incorporation of drug into RNA. The antimetabolite inhibited the labeling of total nuclear RNA by [^{3}H]uridine but not its methylation. Fractionation of nuclear RNA into ribosomal RNA, nonpolyadenylic acid-heterogeneous RNA and polyadenylic acid-heterogeneous RNA revealed that although incorporation of [^{3}H]uridine was impaired by 5-fluorouracil, the incorporation of [^{3}H]-adenosine was not; however, polyadenylic acid synthesis was significantly impaired by 5-fluorouracil. The incorporation of [^{3}H]5-fluorouracil into the three species of nuclear RNA revealed that the concentration of drug per unit of RNA was greatest in polyadenylic acid-heterogeneous RNA, while the non-poly(A)heterogeneous RNA fraction contained the greatest total amount of 5-fluorouracil. These results show that although transcription is not impaired by 5-fluorouracil, posttranscriptional polyadenylation is inhibited. Moreover, incorporation of 5-fluorouracil into nuclear RNA, and particularly polyadenylic acid-containing RNA, appears to be the predominant feature of this drug with respect to transcription in L1210 cells.

INTRODUCTION

5-Fluorouracil is an efficacious anticancer drug against a variety of transplanted tumors (1). One mode of action by which this drug is believed to act is via inhibition of thymidylate synthesis by the intracellularly generated metabolite, 5-FdUMP (2). This effect accounts for the extensive inhibition of formate incorporation into DNA by this drug *in vivo* (3) and *in vitro* (4). A second aspect to the mechanism of action of 5-FU[1] involves its predominant conversion to ribonucleotides and hence its incorporation into RNA (4, 5). That the latter action may be equally as important as inhibition of DNA synthesis has been shown by the correlation between chemotherapeutic response and the incorporation of 5-FU into ribonucleotides and RNA in several murine tumors (6). Moreover, the incorporation of 5-FU into RNA and inhibition of RNA synthesis by the antimetabolite showed a positive correlation with the sensitivity and resistance of L1210 cells towards this drug (7, 8). Inhibition of rRNA maturation by 5-FU in Novikoff hepatoma cells also directly correlated with the degree

[1] The abbreviations used are: 5-FU, 5-fluorouracil; 5-FdUMP, 5-fluoro-2′-deoxyuridylic acid; hnRNA, nuclear heterogeneous RNA; poly(A), polyadenylic acid; nRNA, nuclear RNA; rRNA, ribosomal RNA; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid.

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of drug sensitivity and resistance of the cells (9).

An interesting aspect to the incorporation of 5-FU into RNA is the markedly higher incorporation of drug into nuclear RNA vs cytoplasmic RNA in Ehrlich ascites tumor cells (5). Similar results have been found with 5-F-orotic acid (10) and 5-F-uridine (7). The consequences of such incorporation have been characterized as impaired processing or maturation of nuclear rRNA without impaired transcription (11). However, inhibition of transcription of nuclear ribosomal precursor 45S RNA has been reported when hepatoma cells were preincubated with 5-FU (12).

To assess the action of 5-FU on nRNA, *in vitro* studies were carried out with L1210 cells. Methylation and synthesis of nRNA was measured, as was the incorporation of 5-FU into and its effect on the synthesis of non-poly(A) and poly(A) nRNA.

MATERIALS AND METHODS

Materials. 5-FU was kindly provided by Dr. Harry B. Wood Jr., National Cancer Institute. [^{3}H-Methyl]-L-methionine (80 Ci/mmole), [2,8-^{3}H]adenosine (31 Ci/mmole) [5,6-^{3}H]uridine (48 Ci/mmole), [6-^{3}H]5-FU (2 Ci/mmole) and [^{14}C (U)]uridine (462 mCi/mmole) were purchased from New England Nuclear Corp., Boston, MA.

Animals. L1210 cells were inoculated into BALB/c × DBA/2 F_1 mice at an inoculum of 10^5 cells/0.1 ml Hanks' balanced salt solution. Cells were harvested 6 days after inoculation, washed once in Dulbecco's medium and were further diluted with the same medium to 2×10^7 cells/ml.

Incubations. Incubations of L1210 cells were carried out at 37° in a shaking water bath at 100 rpm that consisted of: 25 ml Dulbecco's medium, 0.25% glucose, 5×10^7 cells and either 50 μCi [^{3}H-methyl]methionine (80 Ci/mmole) and 5 μCi [^{14}C]uridine (462 mCi/mmole) or 50 μCi [^{3}H]uridine (100 mCi/mmole) or 250 μCi [^{3}H]adenosine (31 Ci/mmole) as indicated. 5-FU was added at the indicated concentrations and incubated with the cells for 30 min before addition of isotope. Labeling with the various precursors was carried out for 30 min to 1 hr as indicated. Labeling with 25 μCi [^{3}H]5-FU (5-1428 mCi/mmole) was carried out under similar incubation conditions except that the total time of incubation with drug was 1 hr.

RNA extraction. After incubation cells were centrifuged at 400 × *g* for 5 min and washed once with Dulbecco's medium. Nuclei were prepared according to the method of Daskal *et al.* (13) with Triton X-100 as the detergent. Nuclear rRNA, non-poly(A) hnRNA and poly(A) hnRNA were extracted using the sodium dodecyl sulfate: phenol procedure previously described (14). Poly(A) hnRNA was isolated on polyuridylic acid-Sepharose as described (15).

Poly(A) isolation. Poly(A) hnRNA was digested with 20 μg of RNase and 20 units of RNase T_1, according to the method of Eiden and Nichols (16). Incubations were similar to those described above except that 250 μCi of [^{3}H]adenosine (31 Ci/mmole) was the labeled precursor.

Electrophoresis. Disc gel electrophoresis of nRNA or poly(A) was carried out by the method of Dudov *et al.* (17) with 1.75% or 2.5% agarose, respectively, in 6 M urea:25 mM Na_2HPO_4:0.63 mM citric acid (pH 3.0) with 25 mM Na_2HPO_4:0.63 mM citric acid (pH 8.0) as the running buffer. Gels were dissolved in 0.3 ml of 60% perchloric acid, mixed with 10 ml of Aquasol and counted in a Searle Mark III liquid scintillation spectrometer.

DEAE Sephadex chromatography. Alkaline hydrolysates of poly(A) hnRNA were prepared and chromatographed on DEAE Sephadex equilibrated with 7 M urea:20 mM Tris-HCl (pH 7.6) as previously described (18).

Concentration and Specific Radioactivity of UTP. L1210 cells (5×10^7 cells) were incubated in 25 ml of Dulbecco's medium containing 0.25% glucose and varying concentrations of 5-FU. After 30 min, 50 μCi [^{3}H]uridine (100 mCi/mmole) was added and incubation was continued for 30 min. After incubation, cells were centrifuged at 200 × *g* for 5 min at 4° and washed once in Dulbecco's medium. Cells were then vortexed with 1 ml of 1.0 N perchloric acid and the levels and specific radioactivity of UTP was determined as previously described (19).

RESULTS

Relationship between the incorporation of 5-FU and [^{3}H]uridine into total RNA. Initial experiments were designed to see if a correlation existed between the incorporation of 5-FU and [^{3}H]uridine into total cellular RNA. Incubation of L1210 cells with varying concentrations of 5-FU showed that the median inhibitory concentration was approximately 3×10^{-5}M (Fig. 1). At this concentration, 260 pmoles of 5-FU was incorporated into total RNA and represented approximately 0.17% of the total drug concentration. When [^{3}H]uridine incorporation into total RNA was inhibited by 90%, 0.13% of the 5-FU was incorporated into RNA.

Methylation of nRNA. Studies of the methylation and synthesis of total nRNA were next carried out using 5×10^{-5}M 5-FU (Fig. 2). In these experiments, nRNA was labeled with [^{3}H-*methyl*]methionine and [^{14}C]uridine and was digested with KOH. Alkaline hydrolysates were then chromatographed on DEAE Sephadex to separate mono-, di- and oligonucleotide fractions of −2, −3, and −4.5 to −6.5 charge, respectively (18). Although methylation of the nRNA hydrolysate fractions was not affected by 5-FU, [^{14}C]uridine incorporation was inhibited (Table 1). [^{14}C]Uridine incorporation into the oligonucleotide (−4.5 to −6.5) fraction which represents the 5′ terminus of nuclear mRNA was inhibited to the greatest extent. Radiolabeling by [^{14}C]uridine of the dinucleotide (−3) fraction which contains one 2′-O-methylated residue was inhibited by 60%, and labeling of the mononucleotide (−2) fraction, which represents mainly base methylated residues was inhibited by 40%.

Fractionation of nRNA. The effect of 5-FU was then assessed on nRNA, which was fractionated into three components: rRNA, non-poly(A)hnRNA and poly(A)hnRNA by the use of differential sodium dodecyl sulfate:phenol extraction procedures (14) (Fig. 3). Agarose-urea gel electrophoresis of these three species of nRNA indicated that

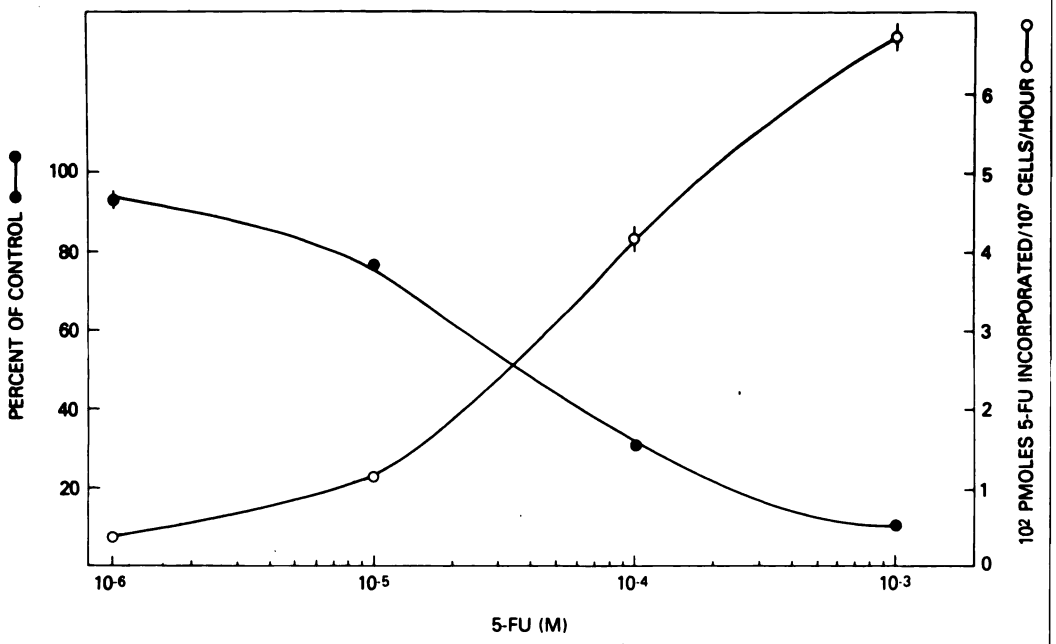

FIG. 1. *Relationship between the incorporation of 5-FU and [^{3}H]uridine into total RNA*

L1210 cells were incubated with varying concentrations of either [^{3}H]5-FU for 1 hr or with unlabeled 5-FU for 30 min followed by [^{3}H]uridine for 30 min. Acid-insoluble radioactivity was determined as described under MATERIALS AND METHODS. (○), incorporation of [^{3}H]5-FU into TCA precipitable radioactivity; (●), incorporation of [^{3}H]uridine into TCA precipitable radioactivity expressed as % of control cells without 5-FU. Each value represents the mean ± S.E. of 3 assays.

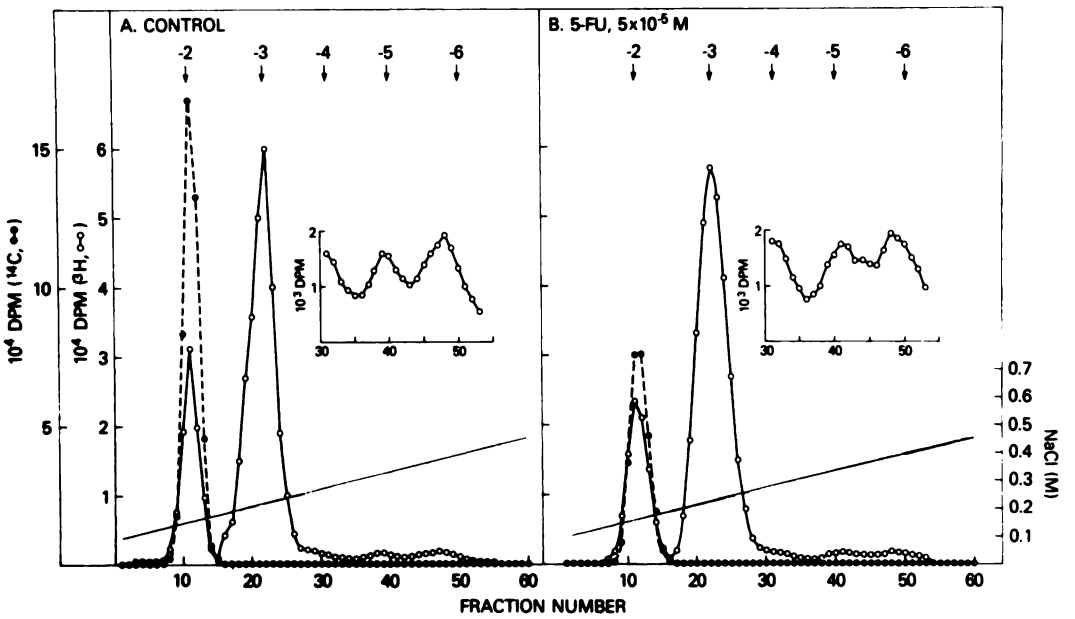

FIG. 2. *DEAE Sephadex-urea chromatography of alkaline hydrolysates of nRNA*

L1210 cells were incubated with 5-FU for 30 min followed by labeling with [^{3}H-methyl]methionine (○) and [^{14}C]uridine (●) for 1 hr. nRNA was extracted and hydrolyzed as described under MATERIALS AND METHODS. The *inset* represents an expansion in scale of the radioactivity present in the −4.5 to −6.5 region.

TABLE 1

DEAE Sephadex-urea chromatography of alkaline hydrolysates of total nRNA labeled with [^{3}H-methyl]-methionine and [^{14}C]uridine

L1210 cells were incubated with 5×10^{-5}M 5-FU for 30 min followed by labeling with 50 μCi [^{3}H]methionine (80 Ci/mmole) and 5 μCi [^{14}C]uridine (462 mCi/mmole) for 1 hr. Total nuclear RNA was extracted, hydrolyzed with KOH and chromatographed on DEAE Sephadex, as described under MATERIALS AND METHODS and in Fig. 2.

Addition	RNA Fraction											
	−2				−3				−4.5 to −6.5			
	^{3}H	%	^{14}C	%	^{3}H	%	^{14}C	%	^{3}H	%	^{14}C	%
	(Total 10^3 dpm)											
Control	93.5	100	457.5	100	276.3	100	5.7	100	21.7	100	1.6	100
5-FU, 50×10^{-5}M	92.7	99	267.5	58	318.0	115	2.3	40	24.9	115	0.2	13

5-FU: 1) impaired the labeling by [^{3}H]uridine of 18S and 4–5S RNA to a greater extent than 28S RNA (Fig. 3A), 2) inhibited the incorporation of [^{3}H]uridine into heterodisperse non-poly(A)hnRNA throughout the gel (Fig. 3B), and 3) reduced the labeling of poly(A)hnRNA in the 12–17S and 6–9S regions (Fig. 3C).

Analysis of the effect of different concentrations of 5-FU on these three species of nRNA labeled with either [^{3}H]uridine or [^{3}H]adenosine, as well as poly(A) derived from poly(A)hnRNA is shown in Fig. 4. These results show that although 5-FU inhibits the incorporation of [^{3}H]uridine into the three classes of nRNA, it does not impair the utilization of [^{3}H]adenosine, except for poly(A) synthesis. These results suggest that 5-FU impairs the uptake or utilization of uridine for nucleotide synthesis or both, but not that of adenosine, and thus the impression that RNA synthesis is impaired is artifactual. To rule out the possibility that 5-FU impaired the utilization of uridine for RNA synthesis by inhibiting its uptake or conversion to UTP, the concen-

tration and specific radioactivity of UTP was measured (Table 2). 5-FU at concentrations up to 1×10^{-4}M did not significantly affect either parameter. Therefore, it appears that 5-FU, after its conversion to F-UTP, effectively substitutes for UTP during transcription and does not impair either uridine uptake, the synthesis of UTP or transcription *per se*. Moreover, it appears that the posttranscriptional process of polyadenylylation is significantly inhibited by the antimetabolite.

Incorporation of 5-FU into nRNA. Since the effects of 5-FU did not truly represent a reduction in transcription, the utilization of this drug in the synthesis of nRNA was monitored. Incorporation of [^{3}H]5-FU into the three classes of nRNA increased pro-

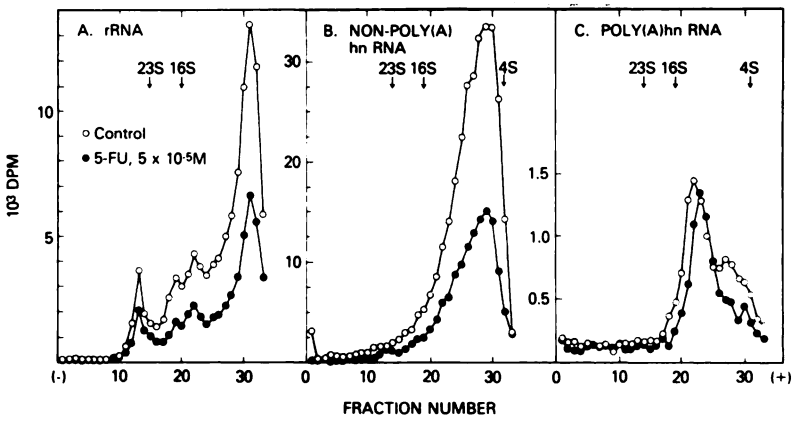

FIG. 3. *Agarose-urea gel electrophoresis of rRNA*

L1210 cells were incubated without (○) or with 5×10^{-5}M 5-FU (●) for 30 min followed by labeling with [^{3}H]uridine for 30 min. rRNA, non-poly(A) hnRNA and poly(A) hnRNA were isolated as described under MATERIALS AND METHODS.

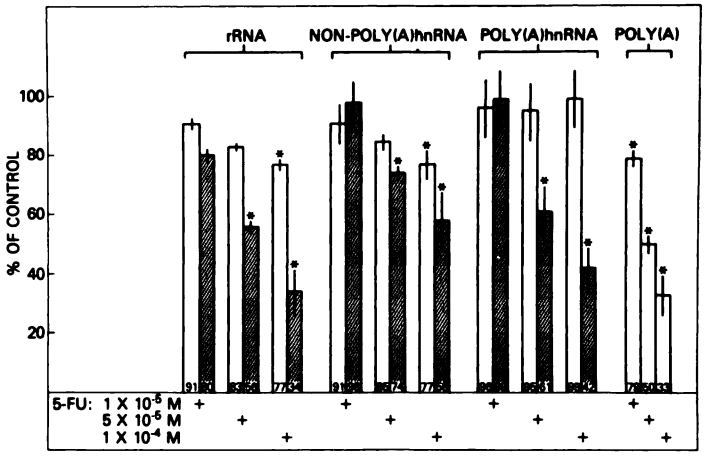

FIG. 4. *Dose-response studies of the effect of 5-FU on nRNA synthesis*

L1210 cells were incubated with 5×10^{-5}M 5-FU for 30 min followed by labeling with either [^{3}H]uridine (*hatched bars*) or [^{3}H]adenosine (*open bars*) for 30 min. rRNA, non-poly(A) hnRNA, poly(A) hnRNA and poly(A) were isolated as described under MATERIALS AND METHODS. Each value is the mean ± S.E. of 4–6 assays. *Numbers* in each *bar* indicate percentage of control. *, statistically significant difference ($p < 0.05$) vs. controls. Control values (10^3 dpm/5×10^7 cells/30 min) for the incorporation of [^{3}H]uridine into nRNA are: rRNA, 173.3 ± 10.4; non-poly(A)hnRNA, 188.6 ± 16.0; poly(A)-hnRNA, 0.065 ± 0.006. Control values (10^6 dpm/5×10^7 cells/30 min) for the incorporation of [^{3}H]adenosine into nRNA are: rRNA, 3.60 ± 0.03; non-poly(A)hnRNA, 4.06 ± 0.03; poly(A)hnRNA, 0.111 ± 0.0004; poly(A), 0.007 ± 0.0006.

TABLE 2

The effect of 5-FU on the concentration and specific radioactivity of UTP

L1210 cells were incubated with 5-FU for 30 min followed by labeling with 50 μCi [^{3}H]uridine (100 mCi/mmole) for 30 min. UTP was isolated as described under MATERIALS AND METHODS.

Addition	nmoles UTP/5 × 10^7 cells	%	10^6 dpm/mmole UTP	%
Control	89.1 ± 4.0	100	53.8 ± 2.6	100
5-FU, 1×10^{-5}M	89.2 ± 1.9	100	60.1 ± 2.7	112
5×10^{-5}M	77.1 ± 4.3	87	52.6 ± 0.5	98
1×10^{-4}M	78.9 ± 4.1	89	45.4 ± 2.3	84

portionally with time (Fig. 5). It was also found that upon agarose-urea electrophoresis of 5-FU-substituted nRNA (Fig. 6), molecular weight distributions of these three RNA species were similar to those obtained using [^{3}H]uridine as precursor (Fig. 3). Quantitation of the incorporation of 5-FU into total non-poly(A)hnRNA was twice that of rRNA and 20 times that of poly(A)hnRNA, but the specific radioactivity of poly(A)hnRNA was more than 6 times greater than rRNA and non-poly(A)hnRNA (Table 3). The percent substitution by 5-FU of rRNA, non-poly(A)hnRNA and poly(A)hnRNA was 0.4%, 0.5% and 2.5%, respectively.

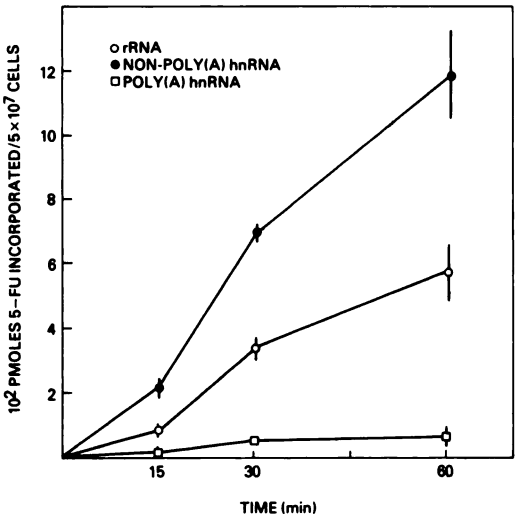

FIG. 5. *Time course of incorporation of [^{3}H]5-FU into nRNA*

L1210 cells were incubated for 15, 30 and 60 min with 1×10^{-4}M [^{3}H]5-FU (50 mCi/mmole) and rRNA, non-poly(A)hnRNA and poly(A)hnRNA were isolated as described under MATERIALS AND METHODS. Each value represents the mean ± S.E. of 3 experiments.

DISCUSSION

The conversion of 5-FU to ribonucleotide metabolites and their subsequent incorporation into RNA may be an important cytostatic event in many sensitive transplanted tumors (4–8). Previous studies have documented the action of 5-FU on the processing of nucleolar RNA to mature rRNA and concluded that 5-FU primarily impaired the processing of the 45S precursor to 28S and 18S rRNA and not transcription (9, 11). Inhibition of transcription was evident only at high concentrations of 5-FU (12). The present study has elucidated the effects of 5-FU on different species of nRNA. 5-FU impaired in a dose-dependent manner the incorporation of uridine into rRNA, non-poly(A)hnRNA and poly(A)-hnRNA, as well as inhibited the synthesis of poly(A). Transcription *per se* may not be involved directly in the effect of 5-FU on nRNA as suggested by the data showing that [^{3}H]adenosine incorporation into various species of nRNA was not impaired, despite inhibition of poly(A) synthesis. Inhibition of uridine incorporation into RNA was due primarily to substitution of UMP by 5-F-UMP in RNA, since the concentration and specific radioactivity of UTP was unchanged by the antimetabolite. These results are the first to suggest that 5-FU primarily impairs a posttranscriptional process that may be required for the stability of many species of mRNA. Interestingly, methylation of nRNA was not inhibited by 5-FU at drug concentrations which reduced poly(A) synthesis by 50%.

Although the consequences of 5-FU incorporation into RNA are not known, recent studies on the translation of 5-FU-modified poly(A) RNA may give a clue (20). 5-FU-modified poly(A) RNA prepared

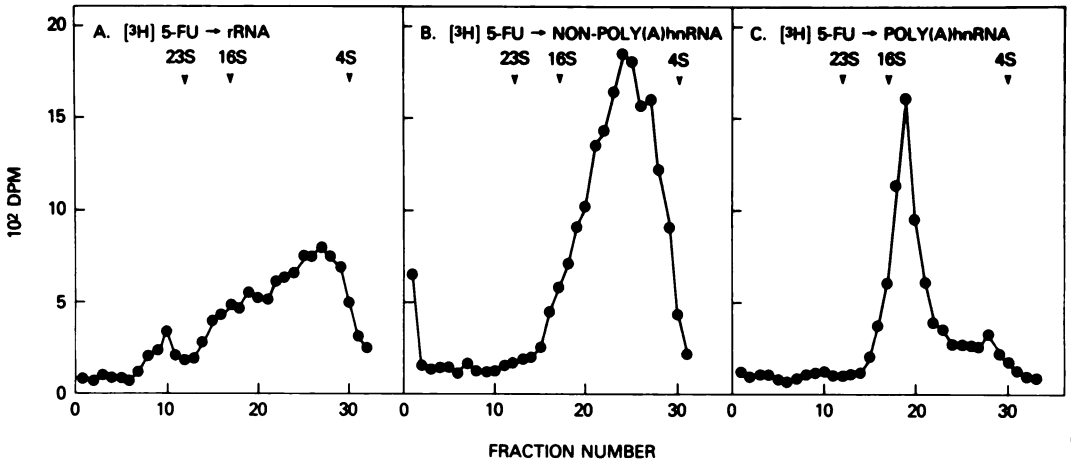

FIG. 6. *Agarose-urea gel electrophoresis of nRNA labeled with [³H]5-FU*

L1210 cells were incubated with 1×10^{-4}M [³H]5-FU (50 mCi/mmole) for 1 hr. rRNA, non-poly(A)hnRNA and poly(A)hnRNA were isolated as described under MATERIALS AND METHODS.

TABLE 3

Incorporation of [³H]5-FU into nRNA in vitro

L1210 cells were incubated with 100 μM [³H]5-FU (50 mCi/mmole) for 1 hr and nRNA was isolated and fractionated as described under MATERIALS AND METHODS. Each value is the mean ± S.E. of 4 determinations.

RNA Fraction	pmoles 5-FU incorporated				% Substitution[a]
	per A_{260}	Ratio[b]	Total per 5×10^7 cells	Ratio	
rRNA	449 ± 37	1.0	610 ± 69	1.0	0.38 ± 0.03
Non-poly(A)hnRNA	575 ± 22	1.3	1151 ± 80	1.9	0.49 ± 0.02
Poly(A)hnRNA	3,000 ± 85	6.7	111 ± 1	0.2	2.55 ± 0.07

[a] $\text{pmoles 5-FU}/\frac{\text{pg RNA}}{340} \times 100 = \%$ substitution.

[b] Ratio refers to the relative incorporation of 5-FU into the RNA fractions with rRNA = 1.0.

from hepatic polysomes of partially hepatectomized rats treated with 5-FU during the early and late G_1 phases of liver growth showed a greater specific radioactivity of [³H]5-FU than rRNA, and was translated at a greater rate than control poly(A) RNA (20). In the present study with L1210 cells, poly(A)hnRNA contained the highest concentration of 5-FU. In view of these data, it would not seem too speculative to suggest that perhaps 5-FU-modified poly(A) RNA can produce aberrant protein synthesis via miscoding or interference with initiation or structural sequences in mRNA.

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