MOLECULAR PHARMACOLOGY, 19:117-121



# Analysis of the Effect of 5-Fluorouracil on the Synthesis and Translation of Polysomal Poly(A)RNA from Ehrlich Ascites Cells

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Received July 15, 1980; Accepted September 9, 1980

SUMMARY

GLAZER, R. I., AND K. D. HARTMAN. Analysis of the effect of 5-fluorouracil on the synthesis and translation of polysomal poly(A)RNA from Ehrlich ascites cells. *Mol. Pharmacol.* **19**:117-121 (1981).

The effects of 5-fluorouracil on the synthesis and translation *in vitro* of polyadenylic acid-containing polysomal RNA [poly(A)RNA] were studied in Ehrlich ascites cells incubated *in vitro*. Exposure of cells for 2 hr to concentrations of $5 \times 10^{-6}$–$5 \times 10^{-4}$ M 5-fluorouracil did not significantly alter the size distribution of poly(A)RNA labeled with either [$^3$H]adenosine or [$^3$H]5-fluorouracil. Incorporation of [$^3$H]adenosine into poly(A)RNA was reduced by 28% and 57% at concentrations of $5 \times 10^{-5}$ M and $5 \times 10^{-4}$ M 5-fluorouracil, respectively. The latter effect correlated with increased drug substitution which ranged from approximately 0.1% to 2% at concentrations of $5 \times 10^{-6}$–$5 \times 10^{-4}$ M 5-fluorouracil. Translation of 5-fluorouracil-substituted poly(A)RNA *in vitro* in either a rabbit reticulocyte lysate or wheat germ extract system did not indicate a quantitative alteration in messenger RNA activity. Autoradiography of labeled translation products did not indicate major qualitative alterations between control and 5-fluorouracil-modified poly(A)RNA. These results suggest that impairment in the synthesis of messenger RNA occurs only at high concentrations of 5-fluorouracil, but that the integrity of the translational activity of the RNA is not significantly impaired.

## INTRODUCTION

Investigations of the mechanism of action of 5-fluorouracil on RNA synthesis have shown that the degree of chemotherapeutic sensitivity and resistance of several animal tumors to the drug is related to its incorporation into RNA (1–5). The relevance of this phenomenon to the toxicity of the drug is undoubtedly related to which particular species of 5-FU[1]-modified RNA perturbs the reproductive function of the tumor cell to the greatest extent. Thus far various studies have shown that rRNA is particularly sensitive to 5-FU, and that interference with the processing of rRNA is proportional to the sensitivity of the tumor to 5-FU (6) and to the extent of incorporation of the drug into rRNA (7). Impaired transcription of rRNA occurs only at high drug concentrations (6, 8), and the synthesis of tRNA or poly(A)-containing mRNA is relatively insensitive to 5-FU (7–11). Recent studies measuring transcriptional activity in rat liver nuclei *in vitro* indicated that 5-FUTP in the presence of UTP produced only minor alterations in nearest neighbor analyses of the nascent RNA (12).

One mechanism thought to play a significant role in the cytotoxicity produced by 5-FU as a result of its incorporation into RNA is miscoding of mRNA during translation (13). Studies of the translational activity of 5-FU-modified poly(A)RNA from regenerating liver in a wheat germ extract system did not show impaired mRNA activity, and in fact enhancement of translation occurred at a certain degree of 5-FU substitution (9).

The present study was initiated to determine whether or not impaired translation via 5-FU-modified mRNA is a significant factor in the cytotoxicity produced by the drug on tumor cells. Evaluation of the activity of polysomal poly(A)RNA from control and drug-treated cells was determined with two cell-free translation systems *in vitro* and forms the basis for this report.

## MATERIALS AND METHODS

*Materials.* 5-FU was obtained from the Drug Synthesis and Chemistry Branch, National Cancer Institute. 5-[6-$^3$H]FU (2.8 Ci/mmole) was obtained from Amersham/Searle Corporation, Arlington Heights, Ill. and [2,8-$^3$H]adenosine (34.4 Ci/mmole) and L-[3,4,5-$^3$H]leucine (111.2 Ci/mmole) were purchased from New England

[1] The abbreviations used are: 5-FU, 5-fluorouracil; 5-FUTP, 5-fluorouridine 5′-triphosphate; poly(A), polyadenylic acid; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N,N*′-tetraacetic acid.

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Nuclear Corporation, Boston, Mass. Rabbit reticulocyte lysate and wheat germ extract translation kits were obtained from Bethesda Research Laboratories, Rockville, Md. Heat-inactivated newborn calf serum was purchased from Flow Laboratories, Mclean, Va. Low molecular weight protein standards were obtained from Pharmacia Fine Chemicals, Piscataway, N. J.

*Animals.* Ehrlich ascites tumor cells were maintained in NIH swiss mice at an i.p. inoculum of $10^5$ cells/0.1 ml of Hanks' balanced salt solution. Cells were harvested 1 week after inoculation and washed once with RPMI 1630.

*Incubations.* Incubations of Ehrlich cells were carried out at 37° in a shaking water bath at 100 rpm and consisted of 250 ml of RPMI 1630 medium containing 10% newborn calf serum, $5 \times 10^8$ cells, and either 250 $\mu$Ci of [$^3$H]adenosine (34.4 Ci/mmole) or 500 $\mu$Ci of 5-[$^3$H]FU (4, 40, or 400 mCi/mmole).

*RNA extractions.* After incubation, cells were centrifuged at 400 × *g* for 20 min and washed once with incubation medium. Polyribosomes were prepared as previously described (9) except that the concentration of heparin in the homogenization medium was 0.05%. Total polysomal RNA was extracted by continuous agitation with 1 volume of 0.1% SDS: 0.1 M Tris-HCl (pH 9.0) and 0.5 volume of phenol mixture (phenol:*m*-cresol:$H_2O$, 7:2:2, v/v containing 0.1% 8-hydroxyquinoline) followed by 0.5 volume of chloroform. The emulsion was clarified by centrifugation at 12,000 × *g* for 10 min, and the upper phase was removed and precipitated with 3 volumes of 95% ethanol at −20° for 3 hr. Poly(A)RNA was isolated from total polysomal RNA by poly(U)Sepharose chromatography as previously described (9), except that the poly(A)RNA was eluted with 3 ml of 50% formamide in 0.01 M Tris-HCl (pH 7.5).

*Electrophoresis.* Poly(A)RNA was electrophoresed in 2% agarose gels containing 15 mM iodoacetate, 6 M urea, and 0.4 M Tris-0.02 M sodium acetate-0.033 M acetic acid-0.001 M EDTA (pH 7.4) as described by Locker (14). Gels were sliced into 2-mm sections, dissolved in 70% perchloric acid, and mixed with 10 ml of Aquasol (New England Nuclear Corporation). Radioactivity was determined in a Searle Mark III liquid scintillation spectrometer.

Translation products labeled with [$^3$H]leucine were electrophoresed in 3-mm thick SDS-10% polyacrylamide gels with the acrylamide to bisacrylamide ratio equal to 100:1 (15). After electrophoresis, gels were fixed in 10% acetic acid-30% methanol-10% TCA for 1 hr and then soaked in Enhance autoradiography scintillant solution (New England Nuclear Corporation) for 2 hr. After rinsing the gel in deionized water, it was dried and exposed to Kodak BB-1 X-ray film for 1 week at −80°. Autoradiograms were scanned at 650 nm with a Gilford spectrometer equipped with a linear transport accessory. Protein standards used as molecular weight markers were phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,100), and $\alpha$-lactalbumin (14,400).

*Translation assays.* The translation *in vitro* of poly(A)RNA was performed with either the rabbit reticulocyte lysate or wheat germ extract system. The reticulocyte lysate assay contained, in a volume of 30 $\mu$l, 25 mM Hepes (pH 7.2), 40 mM KCl, 10 mM creatine phosphate, 50 $\mu$M each of 19 amino acids, 87 mM potassium acetate, 5 $\mu$Ci of [$^3$H]leucine (111.2 Ci/mmole), 10 $\mu$l of lysate (contributing a final concentration of: 1.2 mM $MgCl_2$, 0.017 mM EDTA, 8.3 mM KCl, 0.17 mM dithiothreitol, 8.3 $\mu$M hemin, 0.5 $\mu$g of creatine kinase, 0.33 mM $CaCl_2$, 0.67 mM EGTA, and 23.3 mM NaCl) and 0.25 to 2.0 $\mu$g of poly(A)RNA. Assays were carried out at 30° for 1 hr. The wheat germ extract assays contained, in a final volume of 30 $\mu$l, 20 mM Hepes (pH 7.5), 63.3 mM potassium acetate, 0.7 mM magnesium acetate, 1.2 mM ATP, 0.1 mM GTP, 5.5 mM creatine phosphate, 0.2 mg of creatine kinase, 80 $\mu$M spermidine phosphate, 50 $\mu$M each of 19 amino acids, 5 $\mu$Ci of [$^3$H]leucine (111.2 Ci/mmole), 10 $\mu$l of wheat germ extract (contributing a final concentration of 6.7 mM Hepes (pH 7.5), 1.67 mM magnesium acetate, 33.3 mM KCl, and 1.67 mM mercaptoethanol), and 0.25–2.0 $\mu$g of poly(A)RNA. Assays were carried out at 30° for 1 hr.

Following incubation of the translation assays, 30 $\mu$l of 1 M Tris (pH 10.7) was added to each assay tube and further incubated at 37° for 10 min. Aliquots of 3 $\mu$l were then spotted on glass fiber filter paper strips (1 × 5 cm, Reeve Angel 934AH) and chromatographed in scintillation vials containing 0.5 ml of a solution of 10% (w/v) TCA containing 10% (v/v) acetic acid and 30% (v/v) methanol. The area within 1 cm from the origin was removed and incubated in a capped scintillation vial with 0.5 ml of 1 N NaOH for 30 min at 60°. Samples were cooled, neutralized with 50 $\mu$l of glacial acetic acid, mixed with 10 ml of Aquasol, and the radioactivity was determined.

## RESULTS

*Poly(A)RNA synthesis.* The electrophoretic profiles of poly(A)RNA isolated from polysomes of Ehrlich ascites cells incubated *in vitro* with 5-FU and labeled with [$^3$H]adenosine are presented in Fig. 1. The modal distribution of poly(A)RNA from control cells of approximately 10 S was similar to that for cells treated with $5 \times 10^{-6}$–$5 \times 10^{-4}$ M 5-FU, although approximately 60% inhibition of [$^3$H]adenosine incorporation occurred at the highest concentration.

When the incorporation of [$^3$H]5-FU into poly(A)RNA was determined during the same total incubation period of 2 hr, a distribution pattern similar to that seen with [$^3$H]adenosine as precursor was observed (Fig. 2).

The degree of incorporation of [$^3$H]5-FU into poly(A)RNA correlated with the extent of inhibition of labeling with [$^3$H]adenosine, and approximately 1%–2% substitution was required for significant inhibition of synthesis to occur (Fig. 3).

*Translation studies.* Previous experiments from this laboratory indicated that 5-FU-substituted poly(A)RNA from regenerating liver either did not affect or stimulated *in vitro* translation (9). To determine whether or not this phenomenon pertained to 5-FU-sensitive tumor cells, *in vitro* translation assays were conducted with a rabbit reticulocyte lysate system and, in some instances, with a wheat germ extract system.

The time course for incorporation of [$^3$H]leucine into

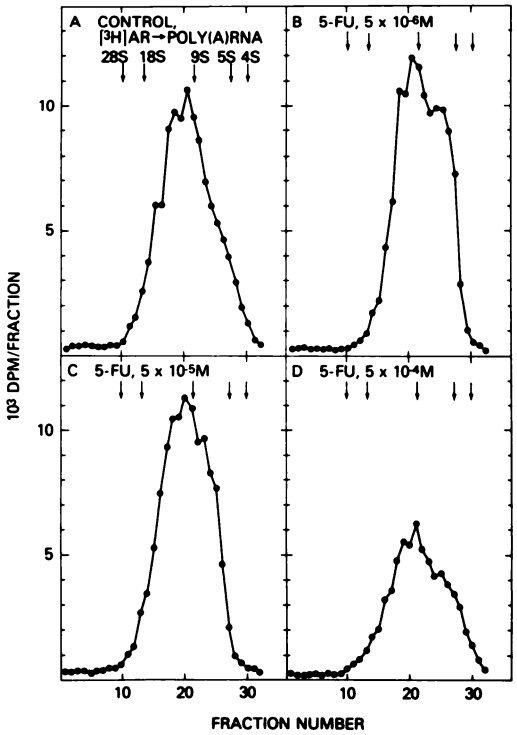


FIG. 1. *Agarose-urea gel electrophoreses of poly(A)RNA labeled with [$^3$H]adenosine*

Ehrlich ascites cells were incubated with varying concentrations of 5-FU for 1 hr followed by incubation with [$^3$H]adenosine for 1 hr in the presence of the drug. Poly(A)RNA was isolated and electrophoresed as described under Materials and Methods.

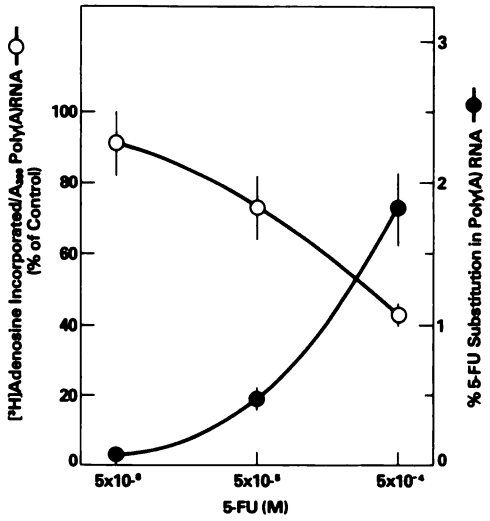


FIG. 3. *Substitution of 5-FU and its effect on [$^3$H]adenosine incorporation into poly(A)RNA*

Ehrlich ascites cells were either incubated for 1 hr with unlabeled 5-FU followed by incubation with [$^3$H]adenosine for 1 hr or incubated for 2 hr with [$^3$H]5-FU. Poly(A)RNA was isolated as described under Materials and Methods. The control specific radioactivity [disintegrations per minute of [$^3$H]adenosine incorporated per $A_{260}$] of poly(A)RNA was 324,200 ± 31,200 for six determinations. The specific radioactivities [picomoles of [$^3$H]5-FU incorporated per $A_{260}$] of poly(A)RNA isolated from cells incubated in the presence of $5 \times 10^{-6}$, $5 \times 10^{-5}$, and $5 \times 10^{-4}$ M [$^3$H]5-FU were 90 ± 3, 560 ± 80, and 2150 ± 300, respectively, for three determinations. The percentage substitution of poly(A)RNA by 5-FU was determined by the picomoles of 5-FU incorporated/pmole of poly(A)RNA × 100.

total translation products in a reticulocyte lysate system directed by poly(A)RNA from control and 5-FU-treated cells is shown in Fig. 4. No significant differences in the translational activities between the control and drug-modified mRNA were observed. Incorporation of [$^3$H]leucine was dependent on the concentration of mRNA (Fig. 5), and significantly different quantitative effects were not observed among 6 to 10 experiments between control and 5-FU-modified poly(A)RNA with either the reticulocyte lysate system (Figs. 4 and 5) or the wheat germ extract system (results not shown).

To assess qualitative differences in the *in vitro* translation products, polyacrylamide gel electrophoresis was performed. The densitometric tracing of autoradiograms

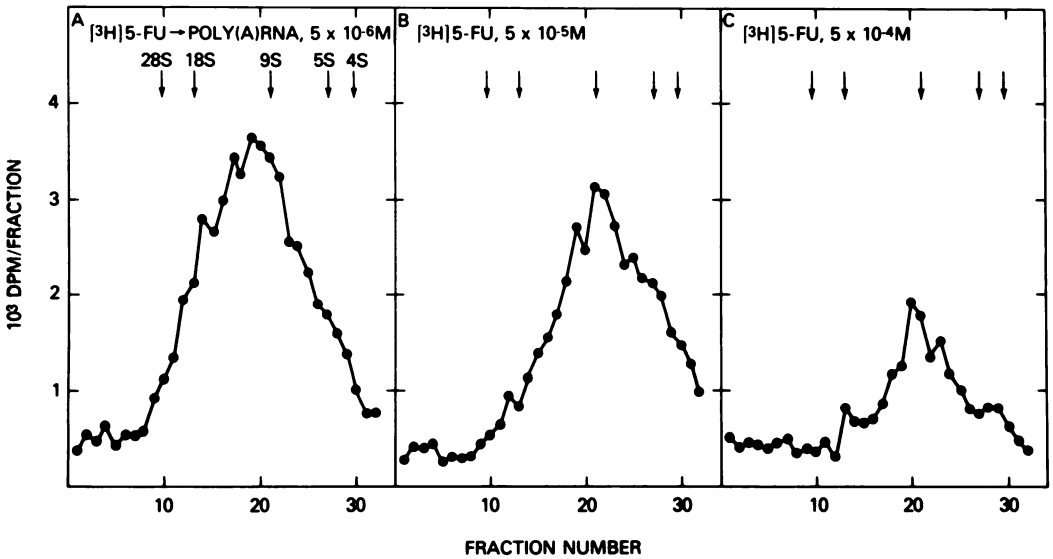


FIG. 2. *Agarose-urea gel electrophoreses of poly(A)RNA labeled with [$^3$H]5-FU*

Ehrlich ascites cells were incubated for 2 hr with varying concentrations of [$^3$H]5-FU, and poly(A)RNA was isolated and electrophoresed as described under Materials and Methods.

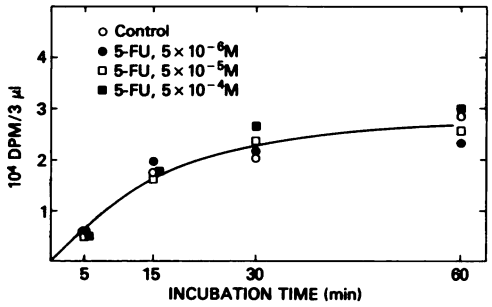


FIG. 4. *Translational activity of 5-FU-modified poly(A)RNA in a rabbit reticulocyte lysate system versus incubation time of translation assay*

Ehrlich ascites cells were incubated for 2 hr with varying concentrations of 5-FU, poly(A)RNA was isolated, and 1 μg was translated *in vitro* as described under Materials and Methods. Each value is the mean of duplicate determinations from a typical experiment. Assays were carried out for 1 hr. Globin mRNA (0.5 μg) produced the incorporation of 190,000 ± 13,000 dpm per 3-μl aliquot per hr for 11 determinations.

of the labeled proteins translated in the reticulocyte lysate system is shown in Fig. 6. The major products translated in this system were of 80,000, 53,000, 47,000, 36,000, 26,000, and 18,000 mol wt with control and 5-FU-modified poly(A)RNA (Fig. 6, *B–E, right*). There was a complete absence of globin-heme products as evidenced by comparison with the products translated from globin mRNA (Fig. 6, *A, right*). A similar analysis using a wheat germ extract system revealed major products of 66,000, 60,000, 47,000, 42,000, 33,000, 30,000, 27,000, 24,000, 17,000 and 13,000 mol wt with control and 5-FU-modified poly(A)RNA (Fig. 6, *left*). No major differences in the size of the translation products occurred except for some minor, non-dose-related differences in the 66,000 and 60,000 mol wt peptides. Two-dimensional electrophoresis of the translation products directed by control and 5-FU-substituted poly(A)RNA did not reveal any differences in the synthesized peptides of pI 3 to 9 and 12,000 to 200,000 mol wt (results not shown).

## DISCUSSION

The mechanism of action of 5-FU on the synthesis,

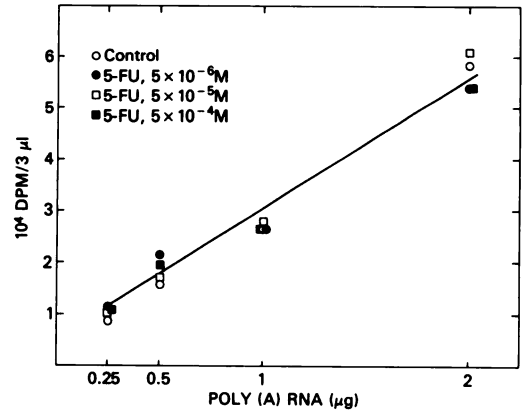


FIG. 5. *Translational activity of 5-FU-modified poly(A)RNA in a rabbit reticulocyte lysate system versus concentration of poly(A)RNA*

Ehrlich ascites cells were incubated for 2 hr with varying concentrations of 5-FU, and poly(A)RNA was isolated and translated as described in legend to Fig. 4. Translation assays were incubated for 30 min.

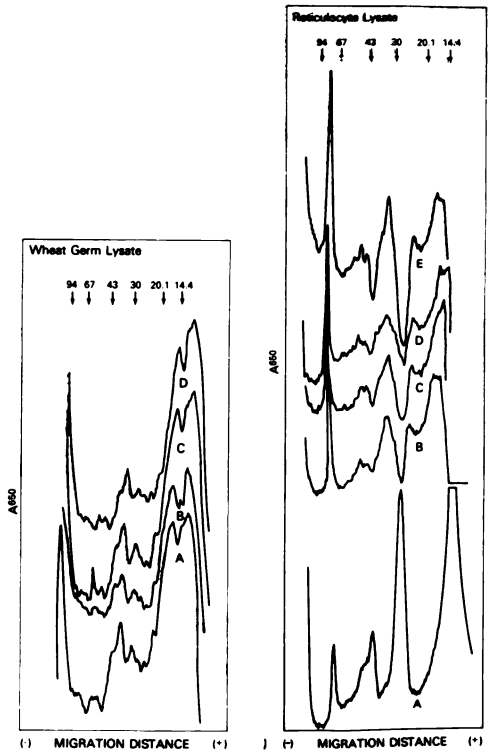


FIG. 6. *Densitometric tracings of the autoradiograms of the translation products from the poly(A)RNA-directed rabbit reticulocyte lysate and wheat germ extract translation systems*

Ehrlich ascites cells were incubated for 2 hr with 5-FU, and poly(A)RNA was isolated and translated as described in legend to Fig. 4.

*Right,* the densitometric tracings represent the translation products resolved by autoradiography of the electrophoretograms from reticulocyte lysate assays directed by (*A*) 0.5 μg of globin mRNA or assays directed by 1 μg of poly(A)RNA obtained from cells incubated for 2 hr with (*B*) 0, (*C*) $5 \times 10^{-6}$ M, (*D*) $5 \times 10^{-5}$ M, and (*E*) $5 \times 10^{-4}$ M 5-FU.

*Left,* the densitometric tracings represent the translation products resolved by autoradiography of the electrophoretograms from wheat germ extract assays directed by 1 μg of poly(A)RNA obtained from cells incubated for 2 hr with (*A*) 0, (*B*) $5 \times 10^{-6}$ M, (*C*) $5 \times 10^{-5}$ M, and (*D*) $5 \times 10^{-4}$ M 5-FU.

processing, and biological activity of various species of RNA has received considerable attention (5–9, 11, 12, 16). It is generally conceded that 5-FU is not a potent inhibitor of transcription unless cells are exposed to a high concentration of the drug (8, 12, 16). This occurs at concentrations of 5-FU equivalent to that required to inhibit the methylation of tRNA (11) but greater than that required to impair the processing of 45 S precursor rRNA (6, 16, 17). Of the species of nuclear RNA whose synthesis is inhibited by 5-FU, rRNA appears to be more sensitive than poly(A)RNA (5, 7–9, 13) whereas tRNA and non-poly(A)-heterogeneous nuclear RNA are more resistant.

The fidelity of translation by 5-FU-modified poly(A)RNA does not appear to be particularly affected by drug substitution. No quantitative effects were noted in either of the two cell-free translation systems, although minor, but inconsistent qualitative changes were noted in some instances. These data suggest that 5-FU does not exert a major influence on translation at the level of

mRNA activity under our experimental conditions. Although we have not examined the cell cycle dependence of 5-FU substitution on the many populations of mRNA, both polyadenylylated and non-polyadenylylated (18), previous studies exposing the rapidly proliferating liver to 5-FU during the early and late $G_1$ phases of cell growth found a similar negative result employing a wheat germ extract *in vitro* translation system. Thus, it appears that under conditions where there is a high degree of drug substitution, i.e., approximately 2%, the precision of translation in two cell-free systems is unaffected by drug-substituted poly(A)-containing mRNA.

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