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In Vitro Translation of Messenger RNA following Exposure of Human Colon Carcinoma Cells in Culture to 5-Fluorouracil and 5-Fluorouridine

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SUMMARY

Total mRNA from human colon carcinoma cell line HT-29 treated with either 5-fluorouracil or 5-fluorouridine was assessed *in vitro* in a reticulocyte lysate translation system. Under conditions of known drug-induced cell lethality, fluoropyrimidine-modified mRNA did not show major quantitative or qualitative changes in translational activity. These results suggest that drug-modified mRNA is probably not associated with the cytotoxicity manifested by these drugs.

INTRODUCTION

The mechanism of cytotoxicity produced by 5-FU[1] is associated with both RNA- and DNA-directed effects depending on the tumor cell line (1, 2). While inhibition of dTMP dynthetase by FdUMP is believed to be growth-limiting in some instances, several recent investigations of the antitumor activity of fluoropyrimidines have focused on the RNA-associated effects of these drugs as a basis for their cytotoxicity. Synergistic combinations of *N*-(phosphonacetyl)-L-aspartate (3–6) or methotrexate (7) with 5-FU or 5-FUR have shown a correlation between the antiproliferative effects of these drug regimens and the incorporation of 5-FU and 5-FUR into RNA. The natural variation in sensitivity of several mouse and human tumor cell lines to 5-FU (8), as well as the association of cell lethality produced by 5-FU and 5-FUR with the incorporation of drug into RNA (9) have also implicated the involvement of RNA-directed cytotoxicity.

The functional basis for producing cell lethality via an RNA-dependent mechanism has not been established (10). Earlier studies of the *in vitro* translational activity of mRNA from 5-FU-treated partially hepatectomized rats (11), as well as Ehrlich ascites cells (12), revealed little or no effects of drug substitution. However, the major drawback of these studies was the inability to correlate the cytocidal activity of 5-FU with its substitution into mRNA. To rectify this problem, the present study was undertaken with a human colon carcinoma cell line which was previously characterized with respect to the cell lethality produced by 5-FU and 5-FUR (9). In this investigation, we utilized an extraction procedure yielding translationally active mRNA from human tumor cells in monolayer culture under strictly defined conditions of cell viability.

EXPERIMENTAL PROCEDURES

Materials. L-[^{35}S]Methionine (1053 Ci/mmole), [2,8-^{3}H]AR (31 Ci/mmole), and reticulocyte lysate translation kits were purchased from New England Nuclear Corporation (Boston, Mass.). [6-^{3}H]5-FU (18 Ci/mmole) and [6-^{3}H]5-FUR (18 Ci/mmole) were purchased from Moravek Biochemicals (Brea, Calif.). 5-FU and 5-FUR were obtained from the Drug Synthesis and Chemistry Branch, National Cancer Institute.

Tissue culture. HT-29 cells were initially plated at a density of 8.3×10^5 cells/100 ml of medium and grown in 150 cm^2 plastic flasks (Costar) at 37° under an atmosphere of 5% CO_2:air in RPMI medium 1640 supplemented with 10% dialyzed, heat-inactivated fetal calf serum and gentamicin (50 μg/ml). Logarithmically growing (3-day) cells were incubated with 1×10^{-3} M 5-FU or 1×10^{-4} M 5-FUR for 2 hr or 1×10^{-4} M 5-FU or 1×10^{-5} M 5-FUR for 24 hr. RNA was pulse-labeled with 100 μCi (0.03 μM) of [^{3}H]AR per 100 ml of medium for 1 hr before the cells were harvested. RNA was labeled with 1×10^{-4} M [^{3}H]5-FU (5 mCi/mmole) or 1×10^{-5} M [^{3}H]5-FUR (5 mCi/mmole) for 24 hr.

Total RNA isolation. A modification of the procedure of Strohman *et al.* (13) and Patterson and Bishop (14) was used. Cells were rinsed with 10 ml of ice-cold Hanks' balanced salt solution and immediately scraped into 6 ml of −20° 8 M guanidine (pH 7)/2 M potassium acetate (pH 5) (19:1, v/v). Two 150-cm^2 flasks were used per 6 ml of guanidine/acetate buffer. The cell lysate, maintained at 4°, was homogenized with 15 strokes of a motor-driven Teflon-glass homogenizer driven at full speed. The homogenate was mixed with 0.5 volume of 95% ethanol and precipitated for 2 hr at −20°. The samples were centrifuged at 8,000 × *g* for 10 min at 4° and drained, and the

[1] The abbreviations used are: 5-FU, 5-fluorouracil; 5-FUR, 5-fluorouridine; poly(A), polyriboadenylic acid; PBS, phosphate-buffered saline (6.3 mM Na_2HPO_4, 0.8 mM KH_2PO_4 0.154 M NaCl, pH 7.4); SDS, sodium dodecyl sulfate; AR, adenosine.

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pellet was dissolved in 4.75 ml of 8 M guanidine (pH 7) and 0.25 ml of 0.5 M EDTA (pH 7). RNA was precipitated with 0.5 volume of 2% potassium acetate in 95% ethanol for 2–4 hr at −20°. Samples were centrifuged at 8,000 × *g* for 10 min at 4°, dissolved in 1 ml of 0.02 M EDTA (pH 7), and mixed with 3 ml of chloroform/1-butanol (4:1). Phases were separated at 3000 × *g* for 1 min; the upper phase was saved and the interphase was re-extracted with 2 ml of 0.02 M EDTA. The two aqueous phases were combined and precipitated with 2 volumes of 4.5 M sodium acetate (pH 6) overnight at −20°. Samples were centrifuged at 12,000 × *g* for 30 min at 4° and drained, and the RNA precipitate was dissolved in 1 ml of water and reprecipitated with 3 volumes of 2% potassium acetate in 95% ethanol for 2 hr at −20°. The RNA was centrifuged at 8,000 × *g* for 30 min, washed once with 95% ethanol, centrifuged at 8,000 × *g* for 15 min, drained, and dissolved in water. The concentration of RNA was measured at 260 nm (25 A_{260} units = 1 mg).

In vitro translation assay. RNA was translated in a reticulocyte lysate translation system. The assay contained in a final volume of 25 μl: 10 μCi of [^{35}S]methionine, 10 μl of lysate, 0.7 mM magnesium acetate, 80 mM potassium acetate, 220 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (pH 7.4), 0.33 mM GTP, 0.22 mM spermidine, 1.1 mM creatine phosphate, 2.2 mM dithiothreitol, and 3–6 μg of RNA. Assay mixtures were incubated at 37° for 1 hr, reactions were stopped by the addition of 25 μl of 1 M Tris (pH 10.9), and the mixtures were further incubated at 37° for 30 min to hydrolyze labeled tRNA. Aliquots (5 μl) were spotted on a strip of glass fiber filter paper and chromatographed as previously described (12).

Electrophoresis. Duplicate translation assay samples (total volume of 100 μl) were dialyzed in Spectrapor tubing (6000–8000 molecular weight cutoff) against 1 liter of electrophoresis sample buffer (2% SDS/0.063 M Tris-HCl (pH 6.8)/10% glycerol/5 mM dithiothreitol) overnight at 4°. Five microliter of 1% bromphenol blue tracking dye were added after dialysis and the samples were electrophoresed in polyacrylamide slab gels (14 × 14 × 0.3 cm) containing a 5%, 1.5-cm high acrylamide stacking gel above a 12.5-cm high 10% acrylamide separating gel (15).

Two-dimensional gel electrophoresis was performed by first dialyzing the duplicate translation samples against 1 liter of 8 M urea. Samples were then polymerized in plastic tubes (0.5 × 12 cm) with pH 3.5–10 Ampholine (LKB) according to the procedure of Gronow and Griffiths (16). Isoelectric focusing was carried out overnight (16 hr) at room temperature at 200 V. After focusing, gels were removed from the tubes and placed atop a 2.5-cm high 5% acrylamide stacking gel above a 12.5-cm high 10% acrylamide separating gel as described above for one-dimensional slab gel electrophoresis. Electrophoresis was first carried out at 100 V with running buffer (15) containing 2% SDS in the upper chamber and standard running buffer containing 0.1% SDS in the lower chamber until the tracking dye reached the separating gel. Then the running buffer in the upper chamber was replaced with the standard running buffer and electrophoresis continued until the tracking dye reached 1 cm from the bottom of the gel. Gels were then dried and autoradiographed using Kodak XAR-2 X-Omat film.

Agarose gel electrophoresis. RNA was characterized by electrophoresis in 1.5% agarose/urea gels according to the method of Locker (17).

Cs_2SO_4 density gradient centrifugation. [^{3}H]AR-labeled RNA (10 μg) was heated at 65° for 10 min in 0.1 ml of 50% formamide, diluted to 5.1 ml with 10 mM EDTA, and mixed with 5.1 ml of saturated Cs_2SO_4. Samples were centrifuged at 45,000 rpm in a Beckman 50Ti rotor for 60 hr. Fractions (0.5 ml) were collected from the bottom of the tubes and assayed directly for radioactivity.

RESULTS

In our initial study of the cell lethality produced in HT-29 cells by 5-FU and 5-FUR, it was observed that a 2-hr exposure to 10^{-3} M 5-FU or 10^{-4} M 5-FUR caused 98.6% and 99.6% reductions in colony formation, respectively (9). Similarly, 24-hr exposure to 10^{-4} M 5-FU or

TABLE 1

Guanidine-extracted RNA from control, 5-FU-, and 5-FUR-treated cells

HT-29 cells were treated for 2 hr or 24 hr with 5-FU or 5-FUR, and RNA was isolated as described under Experimental Procedures. Each value is the mean ± standard error of three duplicate experiments.

Treatment	Exposure time	A_{260}/A_{280}	$A_{260}/10^6$ cells
	hr		
Control	2	1.89 ± 0.004	0.74 ± 0.10
5-FU, 10^{-3} M	2	1.91 ± 0.04	0.72 ± 0.12
5-FUR, 10^{-4} M	2	1.85 ± 0.04	0.71 ± 0.09
Control	24	1.90 ± 0.03	0.80 ± 0.05
5-FU, 10^{-4} M	24	1.87 ± 0.02	0.49 ± 0.09
5-FUR, 10^{-5} M	24	1.83 ± 0.03	0.55 ± 0.07

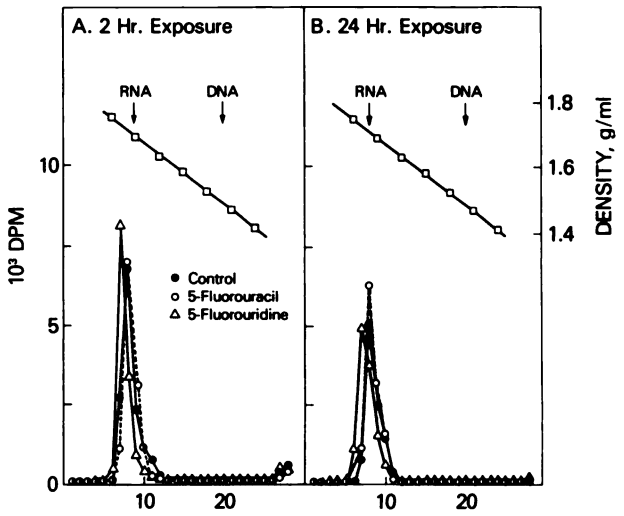

FIG. 1. *Cs_2SO_4 density gradient centrifugation of guanidine-extracted RNA from control, 5-FU-, and 5-FUR-treated cells*

HT-29 cells were treated for either 2 hr with 10^{-3} M 5-FU or 10^{-4} M 5-FUR or for 24 hr with 10^{-4} M 5-FU or 10^{-5} M 5-FUR, and RNA was labeled with [^{3}H]AR during the last hour. RNA was extracted and analyzed by density gradient centrifugation as described under Experimental Procedures.

TABLE 2

RNA synthesis, drug incorporation, and in vitro translational activity of RNA from control, 5-FU-, and 5-FUR-treated cells

HT-29 cells were treated for 2 or 24 hr with unlabeled or ^{3}H-labeled 5-FU or 5-FUR. Cells treated with unlabeled drug were pulse-labeled with 100 μCi of [^{3}H]AR during the last hour. RNA was extracted and translated as described under Experimental Procedures. Each value is the mean ± standard error of four to eight duplicate experiments.

Treatment	RNA synthesis		Drug incorporation	*In vitro* translational activity	
	[^{3}H]AR incorporated	%		[^{35}S]Methionine incorporated	%
	dpm/A$_{260}$		*pmoles/A*$_{260}$	*dpm/hr/μg RNA*	
2 hr					
Control	298,800 ± 14,000	100	—	112,100 ± 20,000	100
5-FU, 10^{-3} M	236,100 ± 17,900	79	930 ± 100	116,700 ± 4,500	104
5-FUR, 10^{-4} M	227,100 ± 17,900	76	1940 ± 30	107,600 ± 5,600	96
24 hr					
Control	199,000 ± 37,500	100	—	134,000 ± 18,000	100
5-FU, 10^{-4} M	163,200 ± 17,900	82	1310 ± 200	124,600 ± 8,000	93
5-FUR, 10^{-5} M	121,400 ± 13,900	61	2600 ± 290	115,200 ± 10,700	86

10^{-5} M 5-FUR produced 99.92% and 99.94% reductions in colony formation. Using these sets of conditions, total cellular RNA was extracted by the guanidine procedure (13, 14), since this method gave yields of translatable mRNA [both poly(A)- and non-poly(A)-containing mRNA] and the *in vitro* translational activity of the mRNA contained in these preparations was determined in a reticulocyte lysate system.

RNA isolation. RNA from control and drug-treated cells was characterized by their A_{260}/A_{280} ratios and Cs_2SO_4 density gradient centrifugation. The absorbance ratios of all RNA preparations were similar for control and drug-treated cells following 2- and 24-hr treatment intervals (Table 1). The recovery of RNA was similar after 2-hr treatment but was reduced by 30–40% following 24-hr treatment with 5-FU and 5-FUR. Isopyknic centrifugation of all RNA preparations revealed a sharp band at a density of 1.70 g/ml (Fig. 1). The small change in density of 5-FUR-modified RNA after 2- and 24-hr treatment may be a reflection of the high degree of substitution by this analogue (Table 2).

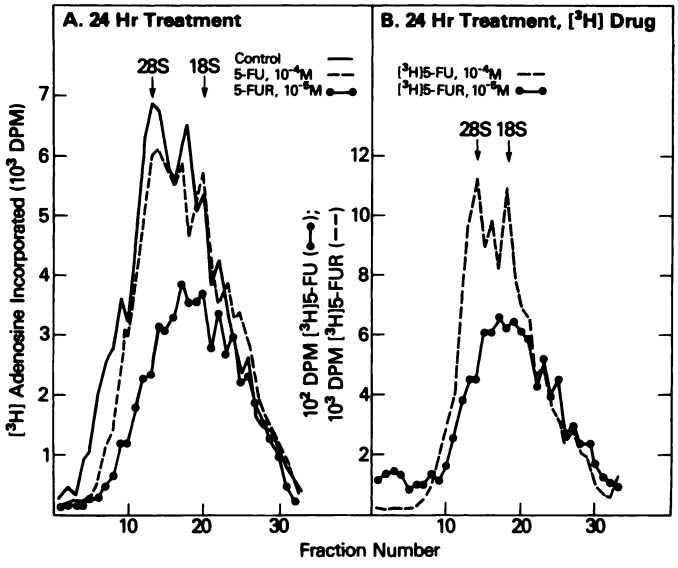

FIG. 2. *Agarose-urea gel electrophoresis of [^{3}H]AR or [^{3}H]drug-labeled RNA after 24 hr exposure to 5-FU or 5-FUR*

HT-29 cells were treated for 24 hr with unlabeled or ^{3}H-labeled 5-FU or 5-FUR. Cells treated with unlabeled drug were pulse-labeled with [^{3}H]AR during the last hour. RNA was extracted and electrophoresed as described under Experimental Procedures.

Exposure of cells for 2 hr to 10^{-3} M 5-FU or 10^{-4} M 5-FUR inhibited the incorporation of [^{3}H]AR by 21–24%, while 24 hr treatment with 10-fold lower drug concentrations reduced [^{3}H]AR incorporation into RNA by 18 and 39%, respectively (Table 2). Analyses of ATP levels under these experimental conditions indicated that a 10–25% increase was produced by drug treatment; the only nucleoside triphosphate to be significantly affected by either 5-FU or 5-FUR was UTP, where 2- and 3-fold elevations occurred after 2 and 24 hr, respectively (results not shown). Hence, RNA synthesis was essentially unchanged following drug treatment after correction for the reduced specific radioactivity of [^{3}H]ATP. Measurements of drug incorporation into RNA revealed that about 2-fold more 5-FUR than 5-FU was utilized for RNA synthesis (Table 2). Characterization of [^{3}H]AR- and [^{3}H]drug-labeled RNA by agarose gel electrophoresis is shown in Fig. 2. Most of the inhibition of labeling occurred in the higher molecular weight species of RNA coincident with rRNA (Fig. 2A). Labeling of RNA for 24 hr with [^{3}H]5-FU or [^{3}H]5-FUR revealed a size distribution similar to that found with [^{3}H]AR as precursor (Fig. 2B).

In vitro translation. Data of the analyses of the translational activity of drug-modified RNA using a reticulocyte lysate system is presented in Table 2. Drug treatment using either exposure interval did not significantly alter the translational activity of mRNA, although there was a tendency for the mRNA from cells treated for 24 hr with 5-FUR to be of slightly lower activity than control mRNA.

Since drug-modified mRNA could conceivably produce an alteration in the translation of discrete peptides without affecting other translation products, one- and two-dimensional electrophoretic analyses of [^{35}S]methionine-labeled translation products were performed. One-dimensional gel electrophoresis at two mRNA concentrations did not reveal any alteration in the amounts of translation products encoded by control and drug-modified mRNA (Fig. 3). Major proteins of 33,000, 35,000, 51,000,

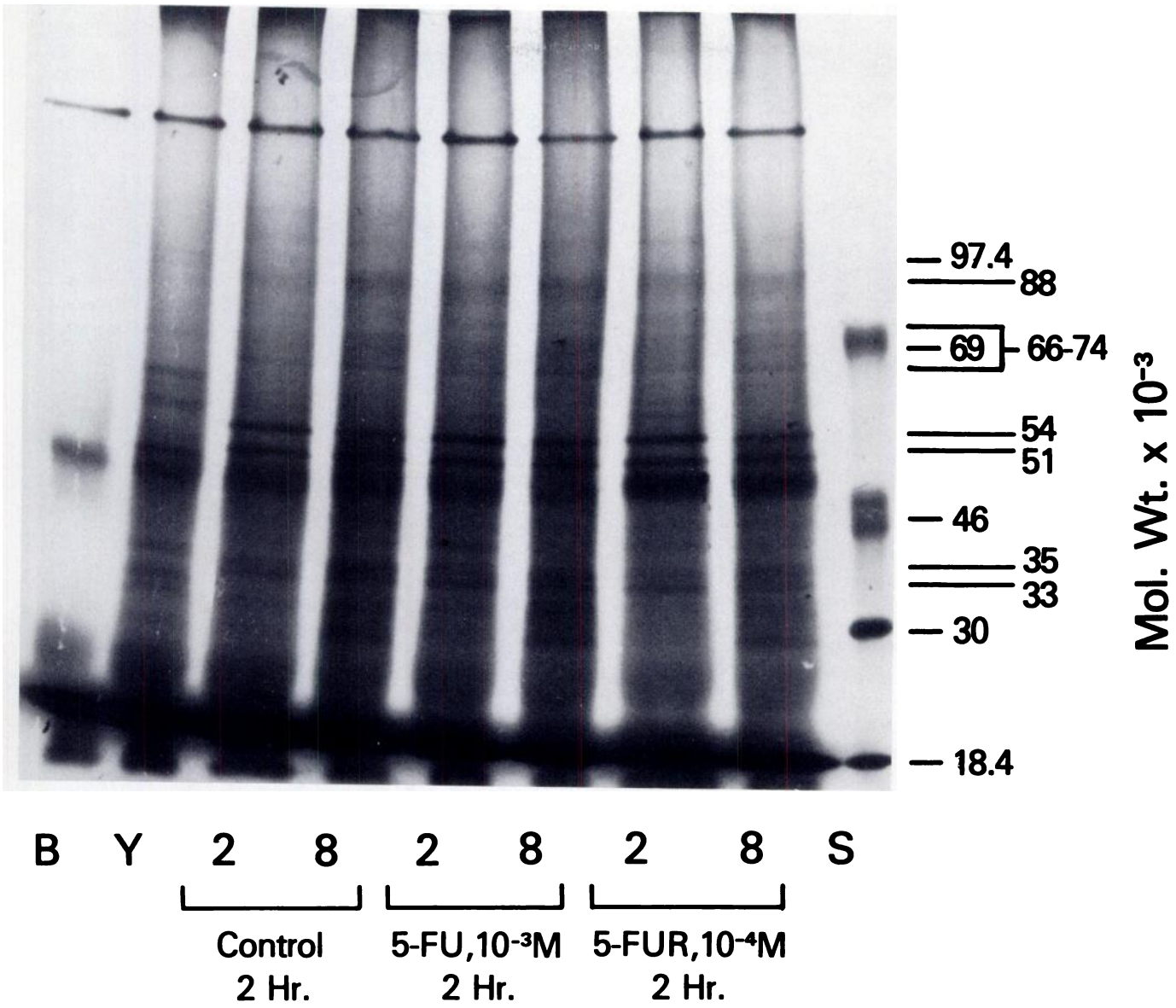

FIG. 3. *Polyacrylamide gel electrophoresis of [^{35}S]methionine-labeled products translated by RNA from control, 5-FU-, and 5-FUR-treated cells*

HT-29 cells were treated for 2 hr with 5-FU or 5-FUR as indicated, and 2 and 8 μg of RNA were translated *in vitro* and electrophoresed as described under Experimental Procedures. An autoradiograph of [^{35}S]methionine-labeled products is shown. *B*, blank; *Y*, yeast RNA; *2* and *8* denote 2 and 8 μg of HT-29 RNA; *S*, ^{14}C-labeled protein standards.

54,000, and 88,000 daltons and a minor triplet of 66,000–74,000 daltons were translated by all mRNA preparations from HT-29 cells. Proteins of 18,000, 46,000, and 140,000 were attributable to endogenous mRNA activity in the reticulocyte lysate. There was a proportionality between mRNA input and [^{35}S]methionine incorporation over 60 min at 37°.

Autoradiographs of the two-dimensional electrophoretograms of the [^{35}S]methionine-labeled translation products directed by control and drug-modified mRNA is shown in Figs. 4 and 5. 5-FU- or 5-FUR-modified mRNA did not produce any qualitative alterations in labeled translational products following a 2- or 24-hr exposure interval; however, 5-FUR-modified mRNA appeared to have a somewhat diminished ability to translate peptides of 46,000–69,000 daltons (Fig. 4), and longer drug exposure intervals appeared to sustain the slightly reduced translational capacity of mRNA from drug-treated cells (Fig. 5).

DISCUSSION

The present study has assessed the *in vitro* translational activity of total 5-FU-modified mRNA under growth conditions wherein both 5-FU and 5-FUR produce a known degree of cell lethality. Previous results with cell line HT-29 indicated that DNA-directed effects did not correlate with the cytocidal activity of 5-FU or 5-FUR, but that the incorporation of drug into RNA did exhibit such an interrelationship. The results of the present study indicate that both fluoropyrimidines did not

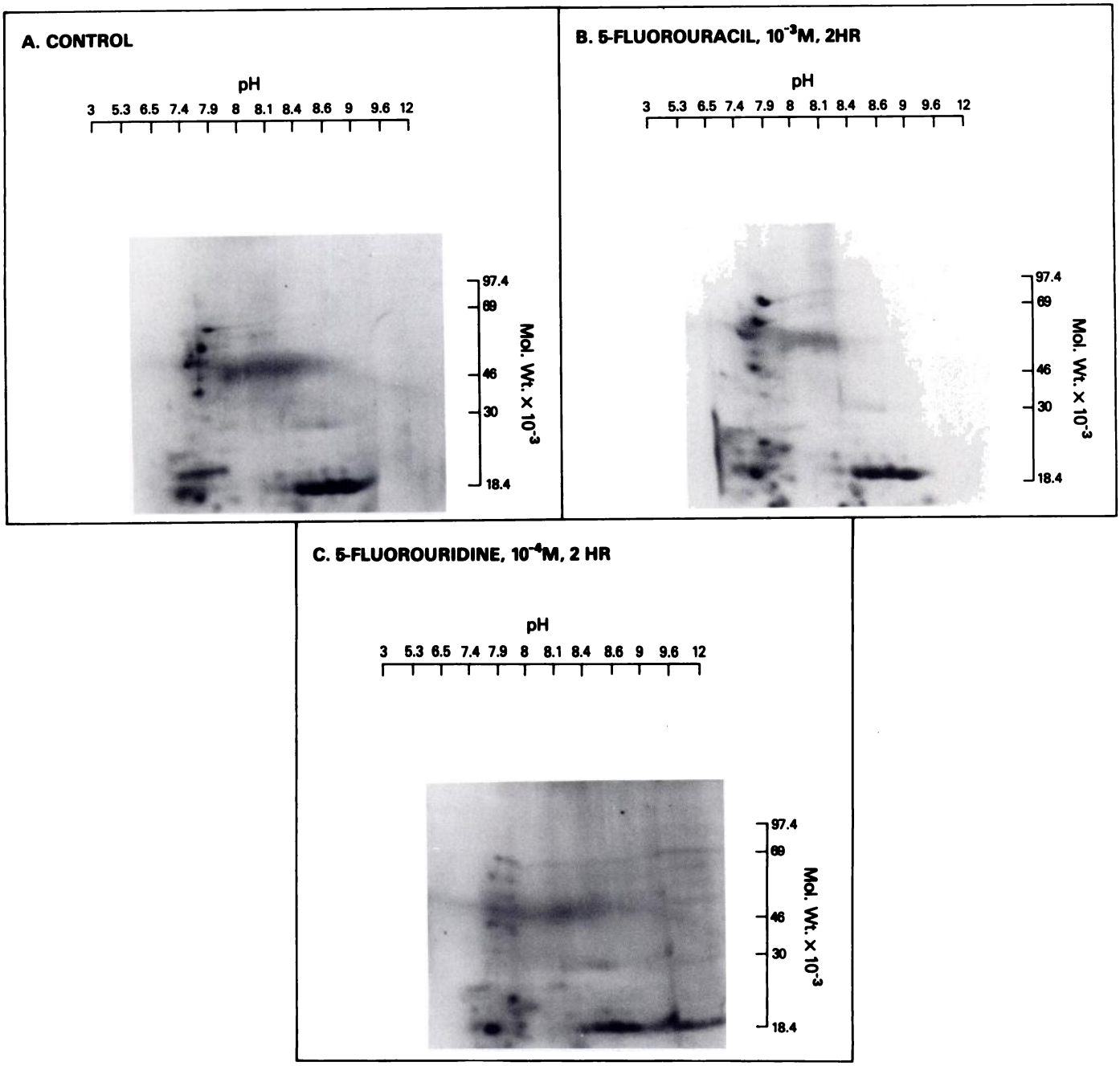

FIG. 4. *Two-dimensional electrophoresis of [^{35}S]methionine-labeled products translated by RNA from control, 5-FU-, and 5-FUR-treated cells*

HT-29 cells were treated for 2 hr with 5-FU or 5-FUR, and RNA was extracted, translated, and electrophoresed as described under Experimental Procedures. Autoradiographs of the [^{35}S]methionine-labeled products are shown.

markedly impair the fidelity of translation of mRNA *in vitro*. Similar results were previously found with poly(A)RNA from 5-FU-treated Ehrlich ascites cells (12) and regenerating liver (11), but the latter experiments were not carried out under defined conditions of drug cytotoxicity. The analyses of radiolabeled translation products by one-dimensional gel electrophoresis did not reveal any major changes in the size distribution of peptides translated *in vitro*, and confirm our previous results (12). However, two-dimensional analyses did reveal some minor quantitative changes in several translation products as early as 2 hr following treatment with 5-FUR, and these changes were apparent following a 24-hr exposure interval to both drugs.

Whether there is a parallelism between impaired RNA function and drug-induced cell death remains to be unequivocally proven. The evidence, thus far, remains circumstantial except for the impaired processing of rRNA precursor reported by Wilkinson *et al.* (18–20). Impaired transcription in general does not appear to be a signifi-

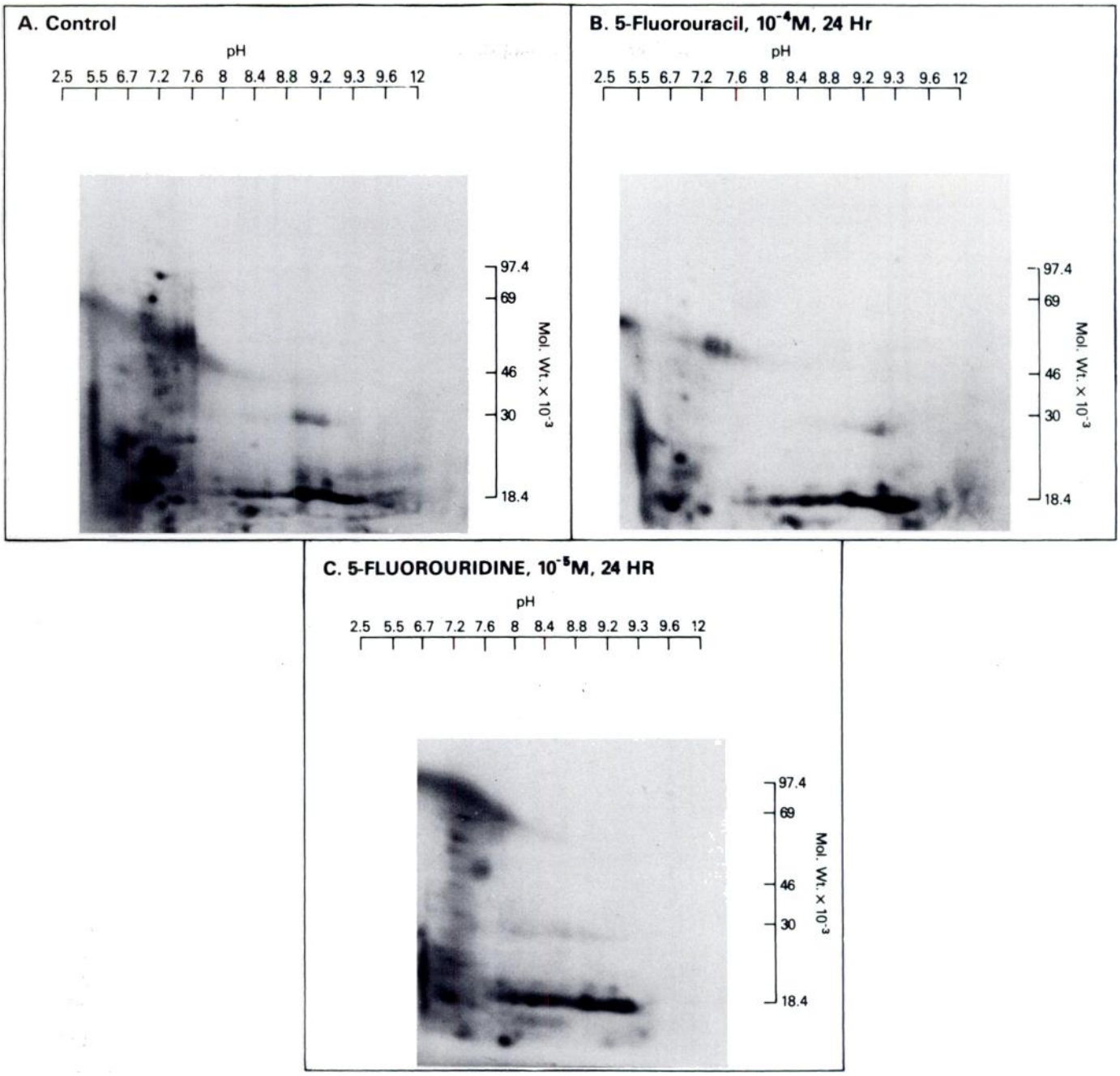

FIG. 5. *Two-dimensional electrophoresis of [^{35}S]methionine-labeled products translated by RNA from control, 5-FU-, and 5-FUR-treated cells*

HT-29 cells were treated for 24 hr with 5-FU or 5-FUR, and RNA was extracted, translated, and electrophoresed as described under Experimental Procedures. Autoradiographs of [^{35}S]methionine-labeled products are shown.

cant feature of 5-FU activity (10), and synthesis of mRNA, in particular, has not been shown to be inhibited (9, 11, 12). Translation studies thus far have also not yielded any evidence that 5-FU or 5-FUR may act via impairment of translation via miscoding (11, 12).

The present evidence of a virtually unchanged capacity of 5-FU-substituted mRNA to translate certain polypeptide deems it unlikely that this effect is related to the cell lethality produced by the drugs unless a discrete population of growth-related peptides is affected. We have not ruled out, however, whether an RNA-directed effect requires several cell doublings for its expression as would be the case in the soft agar clonogenic assay used to assess cell lethality or whether defective nuclear processing of mRNA precursors is involved in the antitumor effects of these drugs. In terms of the latter possibility, we have yet to address the role of small nuclear RNA species which are rich in uridylic acid and may be involved in exon recognition during mRNA splicing (21–23). Perhaps the use of complementary or cloned DNA

probes for specific mRNA sequences will yield an answer as to whether fluoropyrimidine-modified mRNA is involved in the expression of its antitumor effects.

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