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Association of Cell Lethality with Incorporation of 5-Fluorouracil and 5-Fluorouridine into Nuclear RNA in Human Colon Carcinoma Cells in Culture

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SUMMARY

The cytokinetic and biochemical effects of 5-fluorouracil and 5-fluorouridine were examined in a human colon carcinoma cell line (HT-29) in culture. Logarithmically growing cells were approximately 100 times more sensitive to the lethal effects of 5-fluorouridine than 5-fluorouracil as measured by colony formation in soft agar medium. A 2-hr exposure of cells to 10^{-3} M 5-fluorouracil or 10^{-5} M 5-fluorouridine produced a 2-log reduction in colony formation, a 31–33% inhibition of [^{14}C]deoxyguanosine incorporation into DNA, and 30–40% inhibition of [^{3}H]adenosine incorporation into total RNA. Increasing the duration of drug exposure to 24 hr produced a proportional reduction in the drug concentration required to produce similar biochemical and cytocidal effects. However, cell lethality produced by either drug did not correlate quantitatively with inhibition of DNA or RNA synthesis. Examination of nuclear rRNA and 4 S RNA synthesis by agarose gel electrophoresis following 2-hr and 24-hr exposure to 5-fluorouracil or 5-fluorouridine indicated that processing of rRNA was not impaired, rRNA synthesis was inhibited by 10–40%, and 4 S RNA synthesis was unaffected. In contrast to these results, measurements of the incorporation of [^{3}H]5-fluorouracil or [^{3}H]5-fluorouridine into nuclear RNA showed that a significant correspondence existed between the amount of drug incorporated into nuclear RNA and cell lethality. These results indicate that the primary determinant of cell lethality in HT-29 cells is the degree of fluoropyrimidine substitution in nuclear RNA and not inhibition of either DNA or RNA synthesis.

INTRODUCTION

Many investigations of the mechanism of action of the fluoropyrimidines, 5-FU[1] and 5-FUR, have focused on RNA-dependent events as a basis for the expression of their antitumor activities. One basis for ascribing an RNA-dependent action to 5-FU and 5-FUR is the ineffectiveness of thymidine supplementation to reverse their cytotoxicity (1–7). This effect also depends on the metabolic characteristics of a particular tumor, since thymidine only partially protects some cell lines from the lethal effects of 5-FU (2, 3, 8–10). Nevertheless, there appears to be a good correlation between the ability of a cell to anabolize 5-FU into RNA and its sensitivity to the drug. This has recently been documented in a variety of mouse and human tumor cell lines (5, 11, 12). This phenomenon has also been confirmed pharmacologically, where it was found that the incorporation of 5-FU or 5-FUR into RNA was enhanced in proportion to the growth-inhibitory effects produced by synergistic combination with *N*-(phosphonacetyl)-L-aspartate (5, 13–15) or methotrexate (16).

The molecular basis for the RNA-dependent toxicity produced by 5-FU and 5-FUR has not been unequivocally established. Analysis of the ability of 5-FU-modified poly(A)RNA to function in an *in vitro* translation system has shown enhanced activity for mRNA isolated from partially hepatectomized rats treated with 5-FU (17) or no change in activity for mRNA isolated from Ehrlich ascites cells treated *in vitro* with the drug (18), despite the fact that poly(A)RNA contained the highest level of drug substitution of any polysomal or nuclear RNA species (17, 19). 5-FU and 5-FUR are also incorporated into tRNA, where a decrease in lysine aminoacylation of yeast (20) and bacterial (21) tRNA has been observed. Methylation of tRNA is impaired in L1210 (22) and mammary (23) tumors without any impairment in transcription (22). Thus, it is possible that interference with tRNA "charging" may be ascribable to the action of 5-FU or 5-FUR. Interference with the maturation of rRNA by 5-FU and 5-FUR has also been implicated in their antitumor activities (24–26), but this effect has not been correlated with cell lethality or drug substitution.

[1] The abbreviations used are: 5-FU, 5-fluorouracil; 5-FUR, 5-fluorouridine; GdR, 2′-deoxyguanosine; AR, adenosine; SDS, sodium dodecyl sulfate.

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Thus, the RNA-dependent effects of 5-FU and 5-FUR appear to be essential for producing cytotoxicity in some tumor systems, but there is a paucity of data relating these effects to a particular species of RNA, as well as to cell lethality. In the present study, we have examined the cytocidal activity of 5-FU and 5-FUR by soft agar cloning, and have attempted to relate these effects to nucleic acid synthesis, as well as drug incorporation into nuclear RNA.

MATERIALS AND METHODS

Materials. [8-^{14}C]GdR (54.6 mCi/mmole) was purchased from Schwarz/Mann (Orangeburg, N. Y.), [2,8-^{3}H]AR (31.2 Ci/mmole) was purchased from New England Nuclear Corporation (Boston, Mass.), and [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. RPMI medium 1640 was purchased from Hem Research, Inc. (Rockville, Md.), trypsin and heat-inactivated fetal calf serum was purchased from GIBCO Laboratories (Grand Island, N. Y.), and gentamycin was purchased from Flow Laboratories (McLean, Va.).

Tissue culture. HT-29 cells originally derived from a human colon carcinoma (27) were obtained from Dr. L. Erickson, National Cancer Institute. Cells were grown under 5% CO_2-air in RPMI 1640 supplemented with 10% fetal calf serum and gentamycin (50 μg/ml). Cell inocula were 0.83×10^5 cells/10 ml of medium in 25 cm^2 plastic flasks (Costar, Cambridge, Mass.) or increased 10-fold in 150 cm^2 flasks for nRNA analyses.

Drug treatment. Before addition of drugs, the medium of log phase (3-day) cells was replaced with RPMI 1640 supplemented with 10% dialyzed fetal calf serum and gentamycin (50 μg/ml). Cells were treated with the indicated concentrations of 5-FU or 5-FUR for either 2 hr or 24 hr. After drug treatment, cells were harvested by first decanting the growth medium, rinsing the cell monolayer with 3 ml of 0.05% trypsin in Hanks' balanced salt solution without Ca^{2+} and Mg^{2+} and containing 0.02 M EDTA, and by incubation with 0.4 ml of trypsin solution for 10 min at 37°. Treatment with trypsin was terminated by the addition of 10 ml of RPMI 1640 containing 10% fetal calf serum and gentamycin (50 μg/ml).

Cell viability determinations. Soft agar cloning was performed as described by Vistica *et al.* (28) except that RPMI 1640 was used. Duplicate 6-cm plastic Petrie dishes were plated with 200, 2,000, or 20,000 cells for control and drug-treated flasks. After 14 days, colonies were fixed with 95% ethanol and stained with 0.01% gentian violet in 1% acetic acid. Cell viability is expressed as the number of colonies of drug-treated cells ÷ the number of colonies of control cells (corrected for cloning efficiency) × 100. Cloning efficiency ranged from 50% to 90%.

DNA and RNA determinations. Following addition of 5-FU or 5-FUR, cells were pulse-labeled during the last hour of drug treatment with 0.5 μCi of [5-^{3}H]UR or 0.5 μCi [^{3}H]AR, and 0.5 μCi [^{14}C]GdR. After treatment with trypsin, the cells were centrifuged at 400 × *g* for 10 min at 4° and washed once with 15 ml of ice-cold phosphate-buffered saline (0.154 M NaCl, 6.6 mM NaH_2PO_4, 0.8 mM K_2HPO_4, pH 7.4). DNA and RNA were co-extracted by the addition of 2 ml of 1% SDS, 0.1 M Tris-HCl (pH 8.0), 0.01 M EDTA followed by 1.0 ml of phenol mixture [phenol, *m*-cresol, and water, 7:2:2 (v/v) containing 0.1% 8-hydroxyquinoline], and 1.0 ml of chloroform. After vigorously vortexing for 5 min, the emulsion was clarified by centrifugation at 10,000 × *g* for 10 min. Total nucleic acids were precipitated with 2 volumes of 2% potassium acetate in 95% ethanol at −20° overnight. After centrifugation at 10,000 × *g* for 20 min, DNA and RNA were dissolved in 1.0 ml of water and radioactivity was determined in a Searle Mark III liquid scintillation spectrometer. Incorporation of radioactive precursors into total RNA and DNA is expressed per 10^6 cells.

Agarose electrophoresis of rRNA. Cells were grown at 10-fold the number used in the cell viability experiments (8.3×10^5 cells/100 ml of medium in 150-cm^2 flasks) and pulse-labeled for 1 hr with 100 μCi of [^{3}H]AR, for 15 min with 200 μCi of [^{3}H]AR, or for 2 or 24 hr with 50 μCi of [^{3}H]5-FU (1, 10, 100, or 1,000 dpm/pmole) or 50 μCi of [^{3}H]5-FUR (10, 100, 1,000, or 10,000 dpm/pmole).

Cells were washed once with 200 ml of phosphate-buffered saline, and nuclei were isolated as previously described (23). The rRNA was extracted with 3 ml of 0.1% SDS, 0.14 M NaCl, 0.02 M sodium acetate (pH 5.1), and 3 ml of phenol mixture by vigorously vortexing for 5 min. The aqueous phase was removed after centrifugation at 10,000 × *g* for 10 min and RNA was precipitated with 3 volumes of 95% ethanol at −20° overnight. RNA was separated electrophoretically in 2% agarose, urea, and iodoacetate gels as described by Locker (29). Gels were sliced into 2-mm sections, dissolved in 0.2 ml of 70% perchloric acid, and mixed with 10 ml of Aquasol and the radioactivity was determined. Approximately 0.5 A_{260} unit of RNA was applied per gel.

RESULTS

Cell viability. Initial experiments were designed to establish the concentration and time dependence of cell toxicity produced by 5-FU and 5-FUR. Cell viability was determined by soft agar cloning and measurement of the surviving colonies after drug treatment (Fig. 1). After 2 hr of drug exposure, 5-FU was toxic only at 10^{-3} M whereas almost a 2-log reduction in colony formation was achieved by 10^{-5} M 5-FUR. Following a 24-hr duration of drug exposure, cell viability was disproportionally affected so that a 3-log reduction in colony formation was produced by 10^{-4} M 5-FU or 10^{-5} M 5-FUR and more than a 2-log reduction in cloning was achieved by 10^{-6} M 5-FUR. The pattern of cell survival in all instances was of the threshold exponential type; i.e., at low concentrations no significant cell killing was achieved, but higher drug concentrations produced an exponential reduction in cell survival. In addition, cell lethality reached a plateau at the highest concentrations used where no further augmentation of the cytocidal effects of the drug was produced.

DNA and RNA synthesis. To examine the effect of 5-FU or 5-FUR on DNA and total RNA synthesis, HT-29 cells were pulse-labeled with [^{14}C]GdR and [^{3}H]AR, respectively, since the metabolism of these precursors is

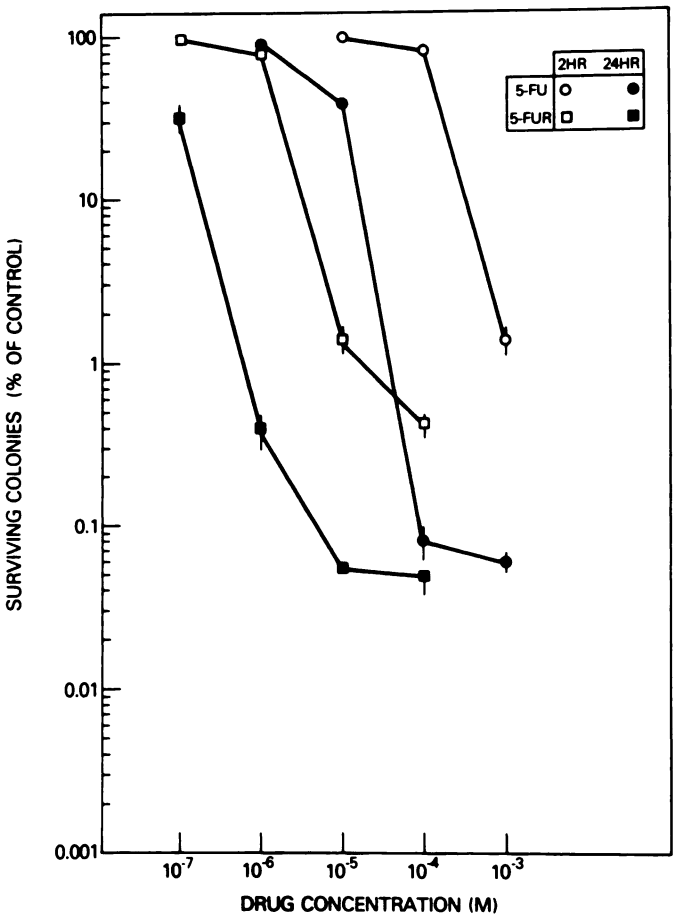

FIG. 1. *Viability of HT-29 cells following exposure to 5-FU or 5-FUR*

HT-29 cells were exposed for 2 or 24 hr to the indicated concentrations of drug, and cell viability was determined by soft agar cloning as described under Materials and Methods. Results are expressed as the percentage of surviving cell colonies corrected for cloning efficiency versus control colonies taken as 100%. Each value is the mean ± standard error of six to nine determinations.

not directly affected by the two drugs (23, 30). Following either a 2-hr (Fig. 2A) or 24-hr (Fig. 2B) exposure to 5-FU or 5-FUR, RNA and DNA syntheses were inhibited in a concentration-dependent manner. After a 24-hr interval of treatment, DNA and RNA syntheses were disproportionally inhibited by 5-FUR, but not by 5-FU; i.e., there appeared to be a 100-fold increase in inhibition by a 10-fold increase in drug concentration for a 24-hr versus a 2-hr exposure interval. A similar phenomenon was noted for cell lethality under identical assay conditions.

To examine more fully the effect of 5-FU and 5-FUR on RNA synthesis, nuclear RNA was labeled with [^{3}H]AR and separated by agarose gel electrophoresis (Fig. 3). Although nuclear rRNA was inhibited 10–40% by 5-FU and 5-FUR depending on the duration of drug exposure, the degree of inhibition was not highly dose-dependent (Fig. 3A–F). The synthesis of low molecular weight (4 S) nuclear RNA was not significantly affected by treatment with either drug. In addition, no significant interference with the processing of rRNA was found by long- and short-term exposure to 5-FU and 5-FUR as assessed by lack of accumulation of rRNA > 28 S and by inhibition of formation of 28 S and 18 S rRNA. A short duration (15 min) of pulse labeling also failed to exhibit interference with rRNA processing in these cells, and, in fact, less inhibition of rRNA was observed (Fig. 3G–I) than after a 1-hr labeling period (Fig. 3A–C).

Incorporation of 5-FU and 5-FUR into nuclear RNA. To derive a measure of drug substitution in RNA, the labeling of nuclear RNA with [^{3}H]5-FU and [^{3}H]5-FUR was measured under conditions identical with those used for cell viability studies (Fig. 4). Incorporation of either drug into nuclear RNA was proportional to exposure time and concentration; however, the incorporation of 5-FUR, in contrast to 5-FU, into nRNA reached a plateau at high concentrations and paralleled its similar effect on cell viability (Fig. 1).

Measurements of the incorporation of [^{3}H]5-FU and [^{3}H]5-FUR into nuclear RNA by agarose gel electrophoresis indicated that 28 S and 18 S rRNA, as well as 4 S RNA, were the main RNA species labeled at noncyto-

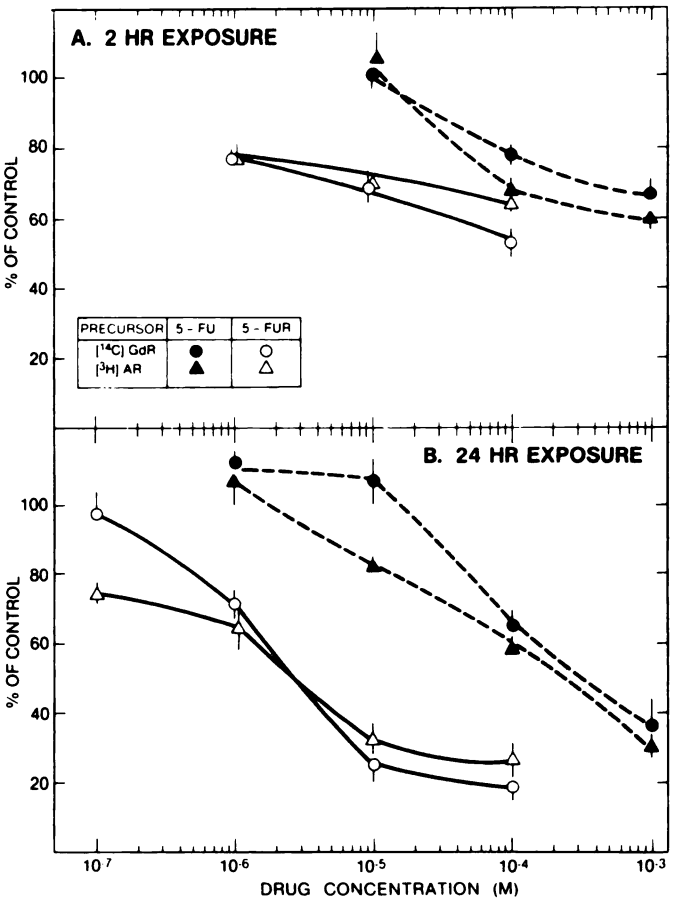

FIG. 2. *Total RNA and DNA synthesis following exposure to 5-FU and 5-FUR*

HT-29 cells were treated with 5-FU or 5-FUR for 2 hr (A) or 24 hr (B) with the indicated drug concentrations, and were then pulse-labeled for 1 hr with 0.5 μCi each of [^{14}C]GdR and [^{3}H]AR. The incorporation of precursors into DNA ([^{14}C]GdR) or RNA ([^{3}H]AR) was measured as described under Materials and Methods. Incorporation of radiolabeled precursors was expressed as disintegrations per minute per 10^6 cells, and results are presented as a percentage of control values (60,000–90,000 dpm/10^6 cells). Each value is the mean ± standard error of five or six determinations.

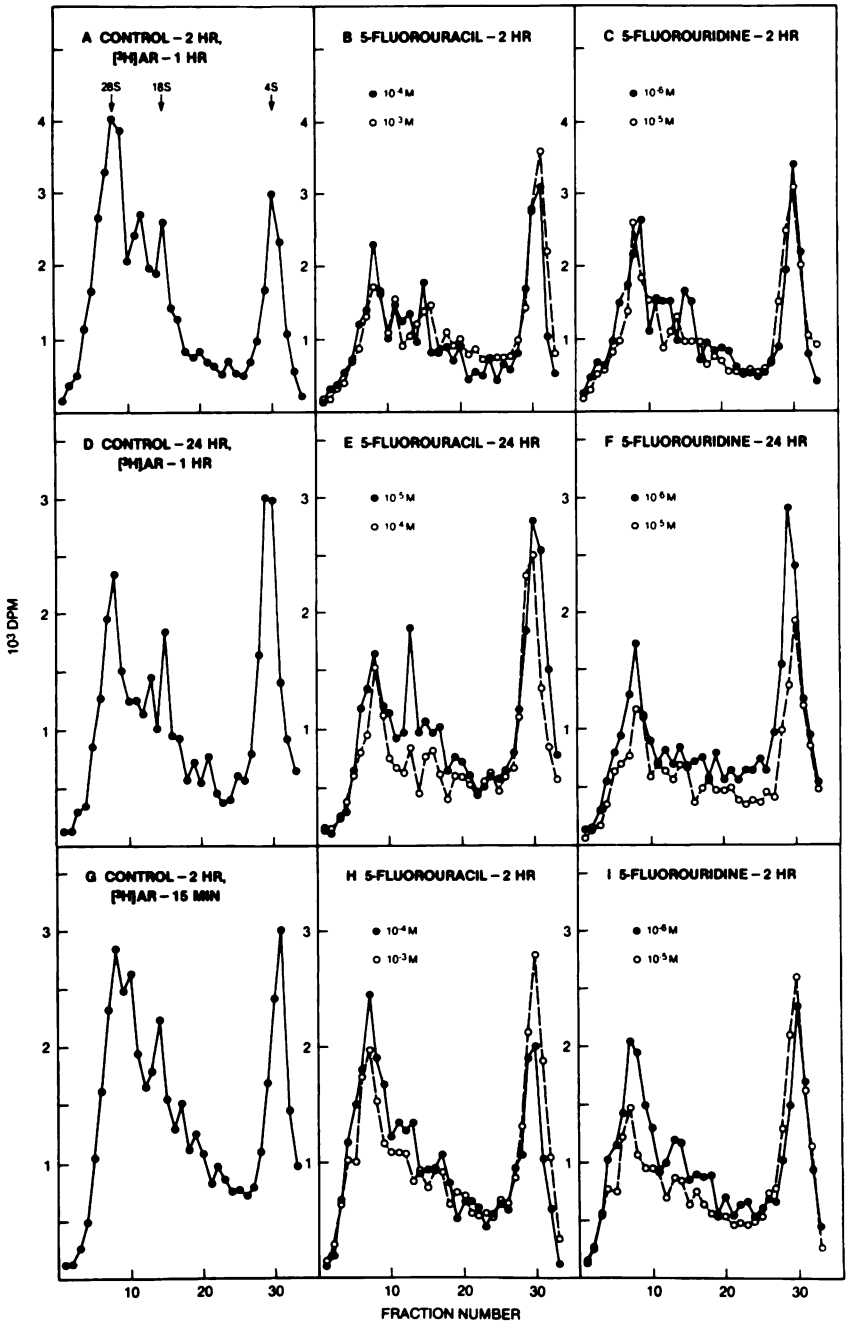

FIG. 3. *Agarose gel electrophoresis of nuclear RNA following exposure to 5-FU or 5-FUR*

HT-29 cells were treated for 2 hr or 24 hr with 5-FU or 5-FUR, and were then pulse-labeled for 1 hr with 50 μCi of [^{3}H]AR or for 15 min with 200 μCi of [^{3}H]AR. Nuclear RNA was extracted from isolated nuclei, and 0.5 A_{260} unit was electrophoresed as described under Materials and Methods.

toxic concentrations of drug (Fig. 5A, B, D, and G). At cytocidal drug concentrations (Fig. 5C, E, F, H, and I), 5- to 10-fold more [^{3}H]5-FU or [^{3}H]5-FUR was incorporated into nuclear 4 S RNA than rRNA. The specific activity of [^{3}H]5-FU in nRNA was too low to assess by electrophoresis after a 2-hr exposure interval.

DISCUSSION

The present investigation has attempted to relate cell lethality produced by 5-FU and 5-FUR with a specific metabolic process related to nucleic acid synthesis. It is apparent from a comparison of the dose-response curves of cell viability with that of total RNA and DNA synthesis that neither of the latter processes correlated quantitatively with cell death. For example, exposure for 2 hr to 10^{-4} or 10^{-3} M 5-FU produced a 60-fold difference in cytotoxicity but comparable inhibition of DNA and RNA synthesis. Similarly, a 24-hr exposure to 10^{-7} or 10^{-6} M 5-FUR produced an 80-fold difference in cell viability but only a small difference in the synthesis of nucleic acids; however, long exposure intervals did show a qualitative relationship to cytotoxicity. Electrophoretic analysis of nuclear RNA also indicated that, although some impairment in the transcription of rRNA occurred, it was not

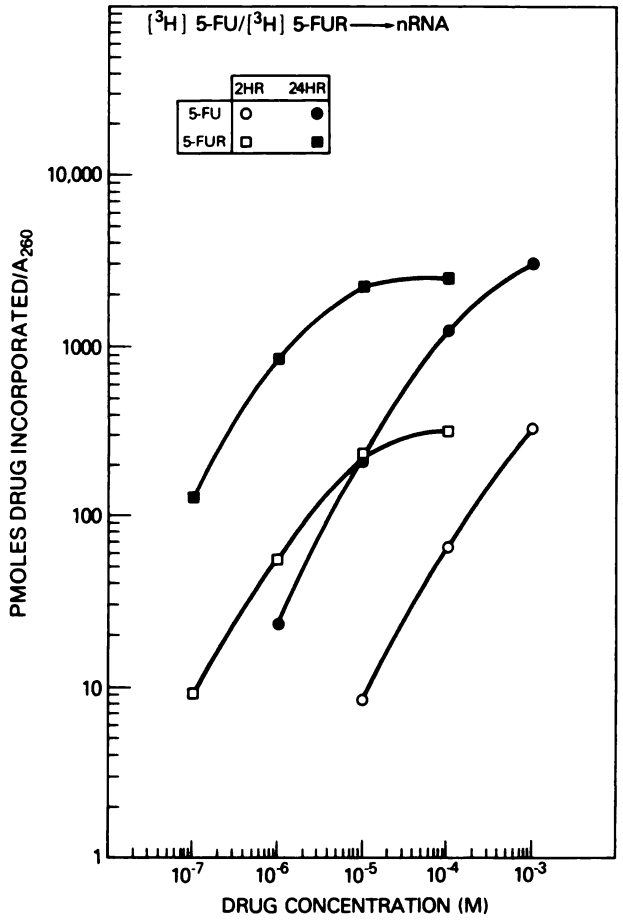

FIG. 4. *Incorporation of [^{3}H]5-FU or [^{3}H]5-FUR into nuclear RNA*

HT-29 cells were treated for 2 hr or 24 hr with 50 μCi of [^{3}H]5-FU (1, 10, 100, or 1,000 dpm/pmole) or 50 μCi of [^{3}H]5-FUR (10, 100, 1,000 or 10,000 dpm/pmole), and the incorporation of radioactivity into nuclear RNA was determined as described under Materials and Methods. Each value is the mean ± standard error of four determinations. The standard error bars are within each point and are thus not visible on a log scale.

completely dose- or time-related to cell lethality. On the other hand, the parameter that correlated well with the cytocidal activity of both drugs was their incorporation into nuclear RNA (Fig. 6). The relationship between the absolute amount of drug incorporated and cell lethality not only showed the same threshold exponential shape as the cell lethality versus drug concentration curve, but also produced a complete continuum for the incorporation of both 5-FU and 5-FUR at both intervals of drug exposure. Of particular note was the precipitous decrease in the cell survival rate by small changes in drug substitution once a threshold level of drug substitution was achieved. For example, increasing the incorporation of 5-FU or 5-FUR from 100 to 200 pmoles per A_{260} unit resulted in a decrease in the cell survival rate from 60% to only 6% of control. Therefore, these data provide strong evidence that the cytocidal action of 5-FU and 5-FUR in HT-29 cells is RNA directed.

Also of interest was the relative incorporation of 5-FU and 5-FUR into nuclear rRNA and 4 S RNA at long exposure times. At drug concentrations which were lethal, the amount of 5-FU or 5-FUR incorporated into 4 S RNA was 5- to 10-fold greater than that incorporated into rRNA. At nontoxic drug concentrations, the incorporation of drug into rRNA and 4 S RNA was equivalent. These data may not only reflect the cytotoxicity of the drugs but also their rates of incorporation over the exposure times that were examined. Since cloning experiments are carried out over a 2-week period following drug treatment, the relative incorporation of 5-FU or 5-FUR into various species of nRNA over long drug exposure intervals may give a more accurate assessment of the relationship of this parameter to cell lethality.

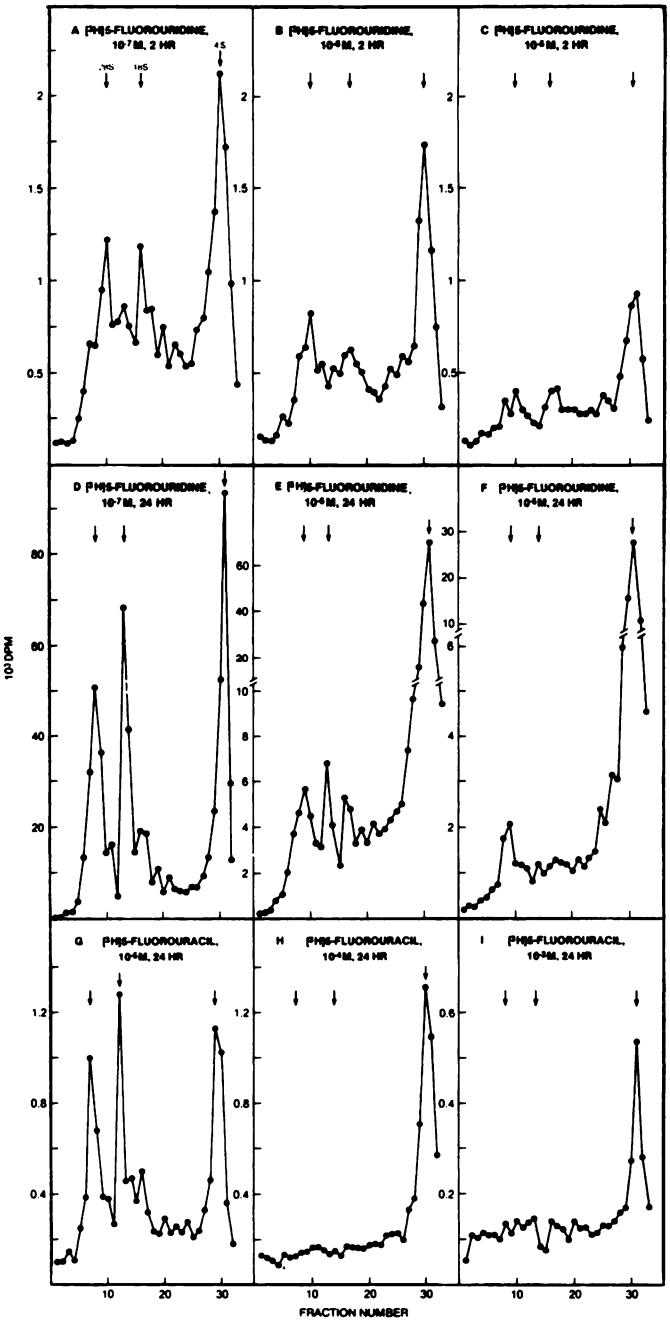

FIG. 5. *Agarose gel electrophoresis of nuclear RNA labeled with [^{3}H]5-FU or [^{3}H]5-FUR*

HT-29 cells were treated as described in Fig. 4.

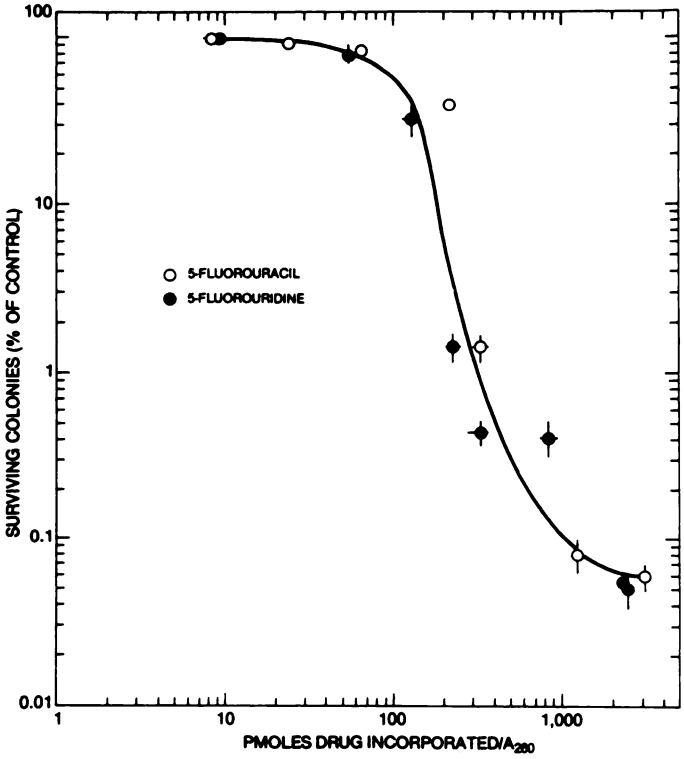

FIG. 6. *Correlation of cell viability with incorporation of [^{3}H]5-FU or [^{3}H]5-FUR into nuclear RNA*

Cell viability as determined by surviving colonies in soft agar (Fig. 1) is plotted as a function of the amount of [^{3}H]5-FU or [^{3}H]5-FUR incorporated into nuclear RNA (Fig. 4).

Whether colon carcinoma *in vivo* is also chemotherapeutically responsive because of its ability to anabolize 5-FU and 5-FUR into RNA remains to be established. It is noteworthy that, in studies *in vitro*, human colon carcinoma LoVo displayed an unusual sensitivity to the RNA-dependent (irreversible by thymidine) effects of 5-FU (7). In addition, the synergistic effect of methotrexate on 5-FU cytotoxicity in a human colorectal carcinoma (HCT-8) correlated with the increased accumulation of 5-FUTP (31). Therefore, it appears that in several human colon carcinomas tested *in vitro* thus far, RNA-dependent toxicity by 5-FU and 5-FUR is the determining feature of their antitumor activity.

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