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Lethality Associated with Incorporation of 5-Fluorouracil into Preribosomal RNA

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SUMMARY

We have previously demonstrated a highly significant relationship between incorporation of 5-fluorouracil (FUra) into total cellular RNA and loss of clonogenic survival. The present study extends these findings by demonstrating a similar relationship between incorporation of FUra into preribosomal nuclear RNA (45 S and 32 S) and lethal cellular events. The results also demonstrate that the extent of FUra incorporation into preribosomal RNA (prRNA) correlates significantly with inhibition of maturation to cytoplasmic 28 S and 18 S rRNA. These findings suggest that the incorporation of FUra into prRNA alters recognition sites involved in the processing of 45 S and 32 S RNA. These data are further supported by our finding of an enhanced degradation of (FUra)prRNA by RNase III, an enzyme implicated in the maturation of *Escherichia coli* prRNA and T7 mRNA. These observations suggest that the incorporation of FUra into prRNA is responsible for altered processing to cytoplasmic rRNA and cell lethality.

INTRODUCTION

Several mechanisms of action have been proposed for FUra.[3] This drug is converted to 5-fluorodeoxyuridine monophosphate, which binds irreversibly to thymidylate synthetase and inhibits DNA synthesis (1). FUra also incorporates into RNA and disrupts RNA processing (2–5). More recently, FUra residues have been detected in eukaryotic DNA (6–9), and excision of these residues may contribute to the cytotoxicity and mutagenicity of this agent (10, 11).

Although the relative importance of each cytotoxic mechanism may be dependent upon varying patterns of intracellular FUra metabolism, the incorporation of FUra into total cellular RNA has been correlated with loss of clonogenic survival (12). This relationship is also maintained when the DNA effects of this agent are reversed by thymidine (13). These observations suggest that the formation of (FUra)RNA is a major cytotoxic mechanism. FUra incorporates to a significant extent in all species of RNA and interferes with their function (2–5). The precise mechanism by which the formation of (FUra)RNA results in lethal cellular events remains unclear.

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[3] The abbreviations used are: FUra, 5-fluorouracil; prRNA, preribosomal RNA; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate.

FUra inhibits 28 S and 18 S rRNA synthesis in bacteria (14), yeast (13), and mammalian cells (15) by a post-transcriptional effect on the processing mechanism. FUra has also been shown to inhibit the transcription of the 45 S rRNA precursor (5, 16). Although exposure to 10^{-7} M FUra had no significant effect on rRNA maturation, complete inhibition of 28 S and 18 S RNA formation has occurred during exposure to 10^{-4} M FUra (3). Although this concentration-dependent drug effect suggests that the extent of FUra substitution is related to inhibition of processing, previous studies have not addressed this issue. Furthermore, FUra does not appear to affect methylation of the 45 S prRNA and does not result in accumulation of this species (15). These findings suggest a structural alteration of the 45 S prRNA molecule that results in misreading by processing enzymes.

We have monitored the formation of (FUra)RNA, the effects of FUra incorporation on RNA processing, and loss of clonogenic survival. The results demonstrate a highly significant relationship between incorporation of FUra into prRNA and lethal cellular events. The results also demonstrate that the inhibition of 28 S and 18 S RNA synthesis is significantly related to the extent of FUra misincorporation into prRNA. Furthermore, we have found an enhanced degradation of (FUra)prRNA by RNase III, an enzyme implicated in the maturation of *Escherichia coli* preribosomal RNA and T7 mRNA (17, 18). These findings suggest that the incorporation of FUra into prRNA is responsible for altered processing to rRNA and cell lethality.

MATERIALS AND METHODS

Cell culture. Friend erythroleukemia (MEL) cells, clone PC-4, were grown in suspension in α-minimal essential medium (GIBCO, Grand

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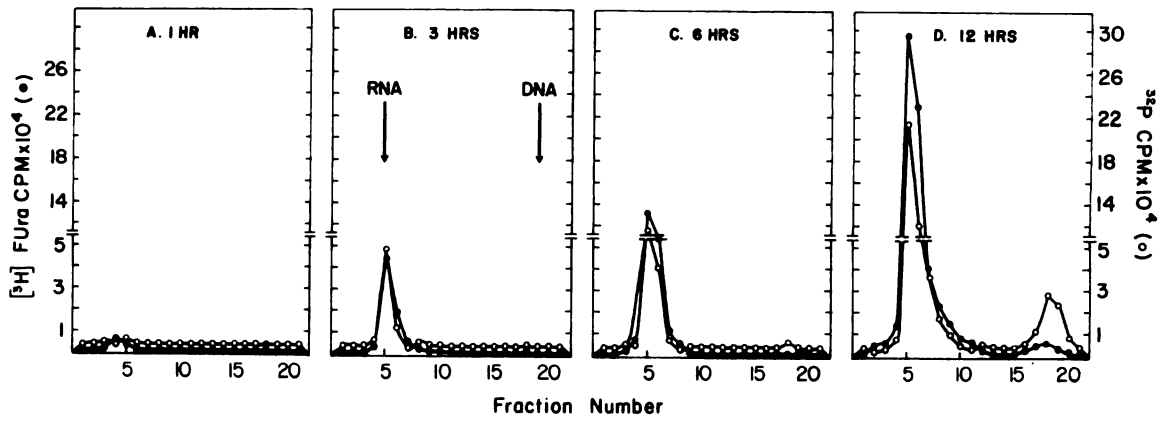

FIG. 1. *Effect of incubation time on incorporation of [^{3}H]FUra and ^{32}P into MEL cell RNA and DNA*

MEL cells (5×10^6) in logarithmic growth phase at a density of 1×10^6 cells/ml were incubated with 10^{-6} M [^{3}H]FUra and ^{32}P (10 μCi/ml) for 1, 3, 6, or 12 hr. Total cellular nucleic acids were purified and analyzed by cesium sulfate density centrifugation.

Island, N. Y.) containing 10% fetal calf serum, streptomycin (100 μg/ml), penicillin (100 units/ml), and 1% L-glutamine.

Clonogenic survival. MEL cells were incubated with FUra at concentrations of 10^{-7} to 10^{-3} M for 3 hr. Following drug exposure, the cells were washed twice in cold PBS and counted in a Model Z Coulter counter. The cells were plated in 0.8% methylcellulose supplemented with 20% fetal calf serum and 6% conditioned media. After incubating for 5 days at 37°, the colonies with 50 or more cells were counted and divided by the number of colonies on an untreated dish and multiplied by 100 to calculate percentage survival. Plating efficiency ranged between 90% and 95%.

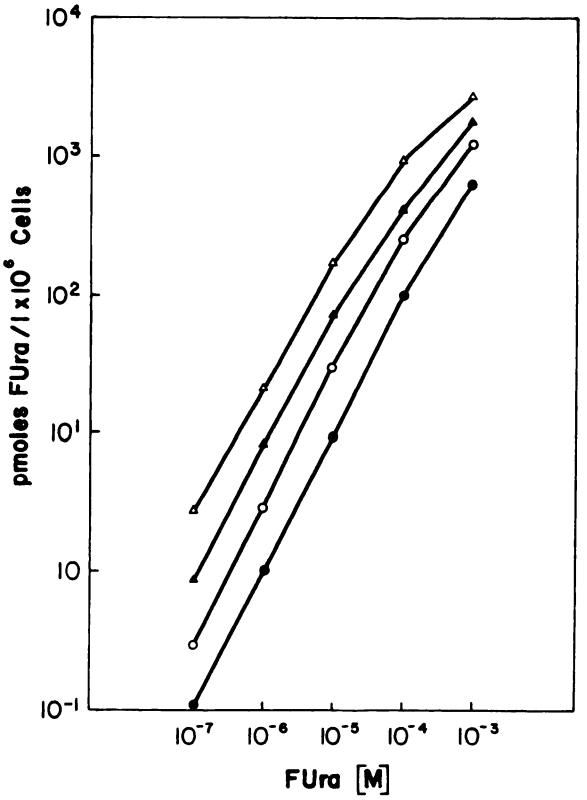

FIG. 2. *Effect of time of exposure and drug concentration on incorporation of FUra into MEL cell RNA*

MEL cells (1×10^6) were labeled with varying concentrations of [^{3}H]FUra for 1 (●), 3 (○), 6 (▲), or 12 (△) hr prior to monitoring for incorporation of [^{3}H]FUra into total cellular RNA.

Incorporation of [^{3}H]FUra into total cellular RNA. MEL cells (5×10^6) at a density of 1×10^6 cells/ml were incubated with 10^{-6} M [^{3}H]FUra (20 Ci/mmole; Moravek Biochemicals, Brea, Calif.) and ^{32}P (10μCi/ml) (carrier-free; New England Nuclear Corporation, Boston, Mass.) for 1, 3, 6, or 12 hr. The cells were washed twice in PBS, and the total cellular nucleic acids were extracted in 2 ml of 0.01 M Tris buffer (pH 7.4) containing 0.01 M EDTA, 0.5% sodium dodecyl sulfate, and 1.5 mg of nuclease-free Pronase (Calbiochem-Boehring Corporation, La Jolla, Calif.). After a 3-hr incubation at 37°, the nucleic acids were precipitated with 0.4 M NaCl and 2 volumes of ethanol at −20° for 48 hr. The resulting pellet was washed in 75% ethanol and resuspended in 1 ml of 0.2 M Tris/0.2 M EDTA (pH 7.4) prior to analysis by Cs_2SO_4 density gradient centrifugation (12).

Isolation of nucleolar and cytoplasmic rRNA (19). PC-4 cells in logarithmic growth phase (2.0 to 4.0×10^7 cells) were incubated with ^{32}P (10 μCi/ml) and 10^{-7} to 10^{-3} M [^{3}H]FUra in minimal essential medium without serum for 3 hr. The following procedures were performed at 4°, and all buffers were treated with diethylpyrocarbonate (100 μl/liter) prior to autoclaving to remove RNase activity. The cells were washed three times with PBS and were resuspended in 2 ml of hypotonic reticulocyte standard buffer (0.01 M NaCl/1.5 mM $MgCl_2$/10 mM Tris-HCl, pH 7.0) and 0.2% Triton X-100. The cells were then incubated for 10 min at 4° followed by mechanical homogenization. The nuclei were pelleted at 1000 × *g* for 2 min and the supernatant containing cytoplasmic RNA was adjusted to 0.02 M EDTA/0.5% SDS prior to incubation for 30 min at 37° with proteinase K (0.25 mg/ml) (Boehringer-Mannheim, Indianapolis, Ind.). The nuclear preparations were washed with 2 ml of reticulocyte standard buffer and resuspended in 2 ml of high-salt buffer (0.5 M NaCl/0.05 M $MgCl_2$/0.01 M Tris-HCl, pH 7.4) prior to vortexing vigorously to disperse the chromatin. The nuclear suspension was then incubated at 37° for 2 min prior to the addition of 20 μl of DNase I (2 mg/ml; Miles Laboratories, Elkhart, Ind.). The mixture was incubated for 3 min at room temperature with gentle vortexing and placed at 4°. The nucleohistone gel was then centrifuged at 17,000 rpm for 15 min on a 15–30% sucrose/high-salt buffer gradient. The pellet of purified nucleoli was resuspended in SDS buffer (0.5% SDS/0.001 M EDTA/0.01 M Tris-HCl, pH 7.4, and 0.01 M NaCl) and treated with proteinase K. The nucleolar and cytoplasmic fractions were extracted with phenol/chloroform/isoamyl alcohol (50:49:1) for 1 min at 60°, followed by chloroform/isoamyl alcohol (50:1) for 1 min at 60°. The RNA was then precipitated with 2 volumes of absolute alcohol and 0.14 M sodium acetate overnight at −20°.

Sucrose density centrifugation. RNA preparations in ethanol were pelleted at 12,000 × *g* for 15 min at 4°. The pellets were resuspended in 550 μl of SDS buffer, heated to 70° for 1 min, and added to 11.4-ml gradients for analysis using an SW 41 rotor. Cytoplasmic RNA preparations were analyzed on 15–30% sucrose-SDS gradients and centri-

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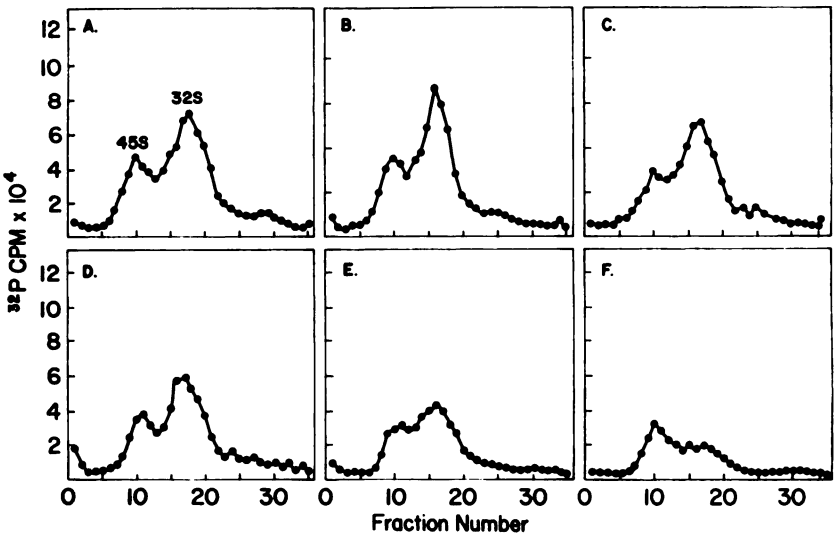

FIG. 3. *Effect of FUra concentration on nucleolar prRNA synthesis*

MEL cells (2×10^7) were incubated with ^{32}P (10 μCi/ml) alone (A) or in combination with 10^{-7} M (B), 10^{-6} M (C), 10^{-5} M (D), 10^{-4} M (E), or 10^{-3} M (F) FUra for 3 hr. The total nRNA was then purified and analyzed using 15–30% sucrose/SDS gradients.

fuged at 24,000 rpm for 16 hr at 24°. Nucleolar RNA was centrifuged at 20,000 rpm for 16 hr at 24°.

Digestion of nucleolar RNA with RNase III (20). RNA preparations were washed with 75% ethanol and then resuspended in 60 μl of RNase III assay buffer [20 mM Tris-HCl (pH 7.9), 10 mM $MgCl_2$, 50 mM KCl, 0.1 mM EDTA, 0.1 mM dithiothreitol, BSA (100 μg/ml)] containing 0.1 unit of RNase III (Bethesda Research Laboratories, Bethesda, Md.) for 1 hr at 37°. Aliquots of the RNA preparations were treated with 0.5 mM ethidium bromide for intercalation of double-stranded regions and inhibition of RNase III cleavage. The reactions were terminated by the addition of SDS buffer, and the cleavage products were analyzed on sucrose density gradients.

RESULTS

The incorporation of FUra into cellular nucleic acids was initially monitored using cesium sulfate gradient centrifugation. Figure 1 illustrates the incorporation of [^{3}H]FUra and ^{32}P into MEL cellular RNA and DNA during incubation periods of 1, 3, 6, and 12 hr. Significant amounts of [^{3}H]FUra were detectable within the RNA region of these gradients, whereas less than 10% of the tritium radioactivity incorporated into the total nucleic acids was detectable in the DNA region. Furthermore, the incorporation of FUra into RNA was dependent upon time of exposure and drug concentration (Fig. 2). The labeling with ^{32}P measures newly synthesized nucleic acid and thus serves as a measure of the effect of FUra on relative RNA and DNA synthetic rates. Under these experimental conditions, DNA synthesis, as compared with RNA synthesis, was more sensitive to the inhibitory effects of FUra.

The effect of 10^{-7}–10^{-3} M FUra on synthesis of nucleolar prRNA was monitored by ^{32}P incorporation during a 3-hr drug exposure. The results obtained (Fig. 3) demonstrate a decrease in the synthesis of both 45 S and 32 S RNA. The percentage of control 45 S and 32 S RNA synthesis is illustrated in Fig. 4. A biphasic decline in 45 S RNA synthesis occurred during exposure to increasing concentrations of FUra. This effect was associated with an increased incorporation of FUra (Fig. 4). Similar

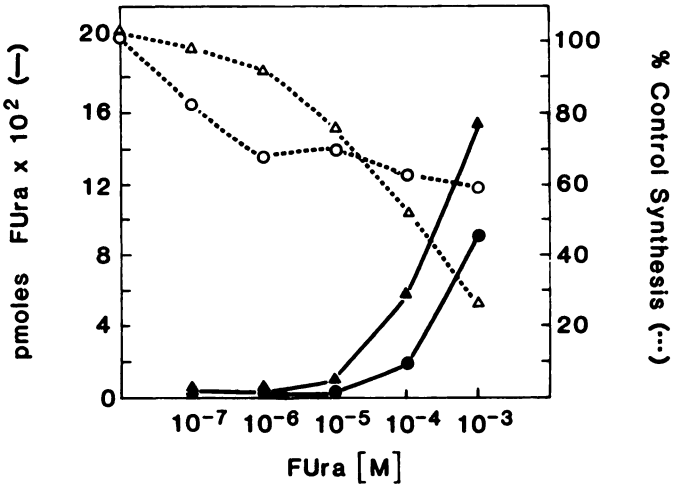

FIG. 4. *Incorporation of FUra into prRNA and percentage of control 45 S and 32 S synthesis*

MEL cells (2×10^7) were incubated with 10^{-7} to 10^{-3} M [^{3}H]FUra and ^{32}P (10 μCi/ml) for 3 hr. The picomoles of FUra incorporated into 45 S RNA (●) and 32 S RNA (▲) were monitored by sucrose/SDS velocity sedimentation. The percentage of control 45 S (○) and 32 S RNA (△) synthesis was determined by monitoring incorporation of ^{32}P into these nucleic acids in the absence of FUra.

results were obtained for 32 S RNA, except that the decline in synthesis was progressive. The extent of FUra incorporation into both 45 S and 32 S RNA as determined by probit analysis (12) was significantly related to drug concentration ($[R] = 0.9754$, $p < 0.0009$).

A similar analysis was performed for the 28 S, 18 S, and 4 S cytoplasmic RNA fractions. As illustrated in Fig. 5, the synthesis of 28 S and 18 S, but not 4 S RNA, follows a pattern similar to that observed for nucleolar RNA. Furthermore, the synthesis of 28 S and 18 S RNA was inhibited even more than that observed for either 45 S or 32 S RNA, and thus the extent of FUra incorporation into 28 S and 18 S RNA was less than that detectable in the nucleolar fractions (Fig. 6). A comparison of relative synthetic rates by ^{32}P incorporation suggests that

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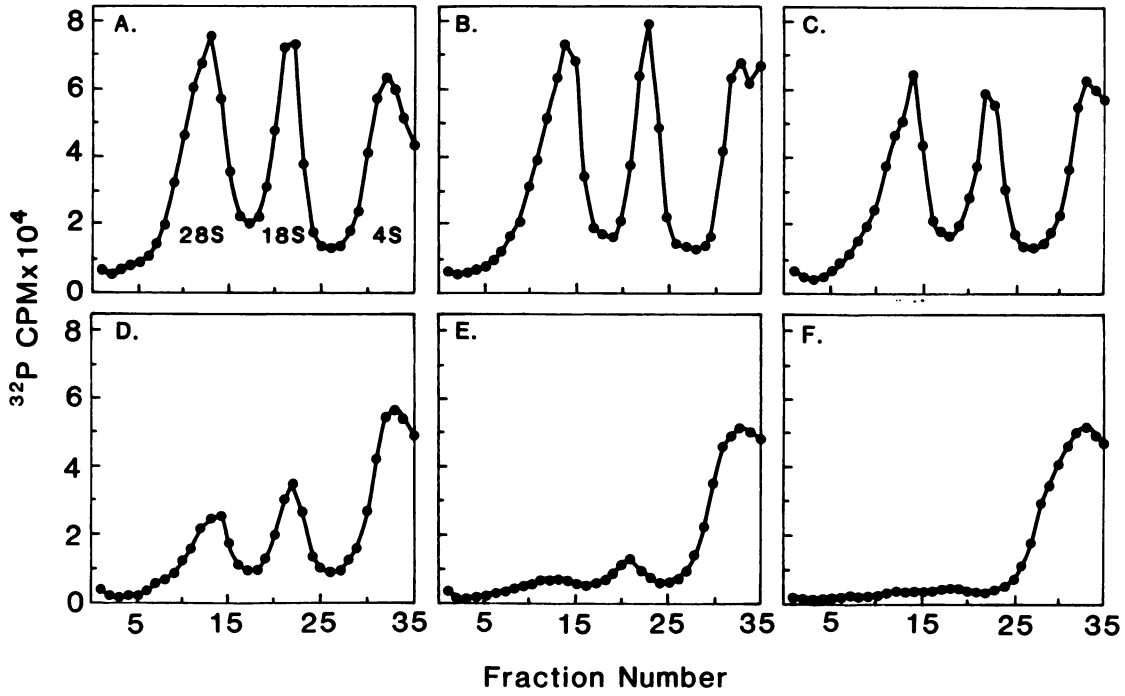

FIG. 5. *Effect of FUra concentration on cytoplasmic rRNA synthesis*

MEL cells (2×10^7) were incubated with ^{32}P (10 μCi/ml) alone (A) or in combination with 10^{-7} M (B), 10^{-6} M (C), 10^{-5} M (D), 10^{-4} M (E), or 10^{-3} M (F) FUra for 3 hr. The cytoplasmic RNA was then purified and analyzed on 15–30% sucrose gradients.

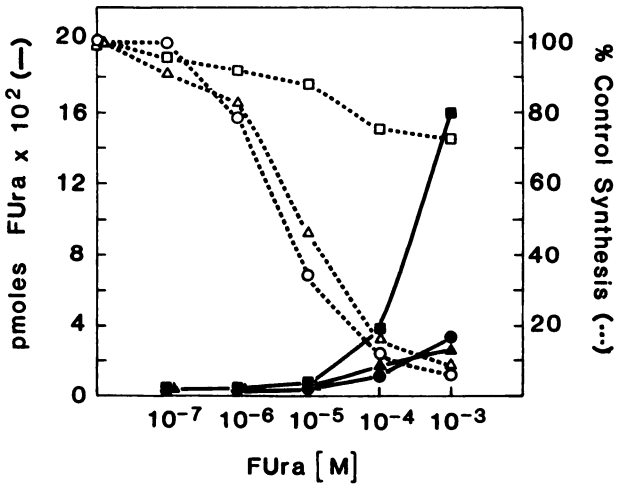

FIG. 6. *Incorporation of FUra into rRNA and percentage of control 28 S, 18 S, and 4 S synthesis*

MEL cells (2×10^7) were incubated with 10^{-7} to 10^{-3} M [^{3}H]FUra and ^{32}P (10 μCi/ml) for 3 hr. The picomoles of FUra incorporated into 28 S RNA (●), 18 S RNA (▲), and 4 S RNA (■) were monitored by sucrose velocity sedimentation. The percentage control 28 S (○), 18 S (△), and 4 S (□) RNA synthesis was determined by monitoring incorporation of ^{32}P into these nucleic acids in the absence of FUra.

DNA synthesis is more sensitive than 28 S and 18 S RNA synthesis to the inhibitory effects of FUra. In contrast, the incorporation of FUra into 4 S RNA increased progressively with drug concentration, and this incorporation was associated with at least 70% of control 4 S RNA synthesis.

The relevance of FUra incorporation into nucleolar and cytoplasmic rRNA to the biological effects of the drug were studied by comparing the amount of FUra incorporation with the clonogenic survival of cells after exposure to drug. Figure 7 illustrates the effect of a 3-hr FUra exposure on the clonogenic survival of MEL cells. The loss of clonogenic survival was a function of drug concentration, as was the formation of (FUra)RNA. Furthermore, the relationship between log percentage survival and picomoles of FUra incorporated into total cellular RNA was highly significant ($[R] = -0.9931$, $p < 0.0001$). The relationship between log percentage survival and picomoles of FUra incorporation into nucleolar prRNA (45 S and 32 S) was also highly significant ($[R] = 0.9951$, $p < 0.0001$). There was a significant correlation

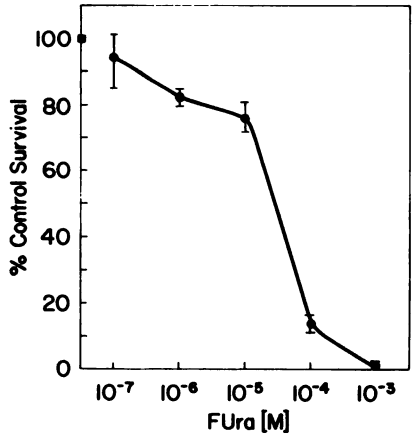

FIG. 7. *Effect of 3-hr FUra exposure on clonogenic survival of MEL cells*

MEL cells in logarithmic growth phase were exposed to 10^{-7} to 10^{-3} M FUra for 3 hr. The cells were then washed and monitored for clonogenic survival. The results are expressed as the mean ± standard deviation of two determinations, each performed in duplicate.

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between the log picomoles of FUra incorporated into prRNA and the percentage synthesis of 28 S and 18 S RNA ($[R] = -0.9482, p < 0.0001$). These findings suggest that the extent of FUra incorporation into prRNA is significantly related to both the synthesis of cytoplasmic RNA (28 S and 18 S) and cell lethality.

The absence of prRNA accumulation despite inhibition of 28 S and 18 S rRNA formation could be due to an enhanced intranuclear degradation of (FUra)prRNA. Since RNase III has been implicated in maturation of rRNA, the effect of this enzyme on (FUra)prRNA was monitored following incorporation of varying amounts of drug. A representative RNase III digest of a 45 S and 32 S nuclear RNA preparation from untreated cells is illustrated in Fig. 8A. In contrast, 45 S and 32 S RNA preparations obtained from cells treated with 10^{-7} to 10^{-4} M FUra were more sensitive to RNase III digestion (Fig. 8B–D). Furthermore, the susceptibility of the prRNA to RNase III increased with the extent of FUra incorporated into these nucleic acids. The specificity of the RNase III reaction for double-stranded RNA regions was also confirmed in each experiment by the use of 0.5 mM ethidium bromide. These findings suggest that FUra incorporation into prRNA enhances sensitivity of double-stranded RNA structures to RNase III and fails to yield identifiable 28 S and 18 S RNA species.

DISCUSSION

Previous attempts to correlate FUra cytotoxicity of human tumor cells with inhibition of DNA or of rRNA synthesis were unsuccessful (21, 22). However, more recent studies have demonstrated a highly significant relationship between the incorporation of FUra into RNA and loss of human breast carcinoma cell clonogenic survival (12). This relationship had not been established previously, although several studies had suggested that the incorporation of FUra into RNA was one mechanism responsible for lethal cellular events (2–4, 23–25). The present study has attempted to extend these findings by examining the relationships between FUra incorporation into nuclear prRNA and lethal cellular events.

The present results demonstrate a significant relationship between the extent of FUra incorporation into 45 S and 32 S RNA and loss of clonogenic survival. Furthermore, FUra incorporation into nucleolar 45 S and 32 S RNA is significantly related to inhibition of the synthesis of 28 S and 18 S RNA. This finding suggests that formation of (FUra)prRNA results in altered recognition sites involved in the processing mechanism. Similar results have been obtained with the MCF-7 human breast cancer cell line.[4] The present findings and those of others (15) are in contrast to recent results which suggest that FUra does not impair processing of rRNA in a colon carcinoma cell line (26). In that study there was no measurement of FUra incorporation into nucleolar prRNA (45 S and 32 S).

Several analogues of purine and pyrimidine bases that incorporate into RNA have similar effects on processing mechanisms. Toyocamycin incorporates into methylated

[4] D. Herrick and D. W. Kufe, unpublished data.

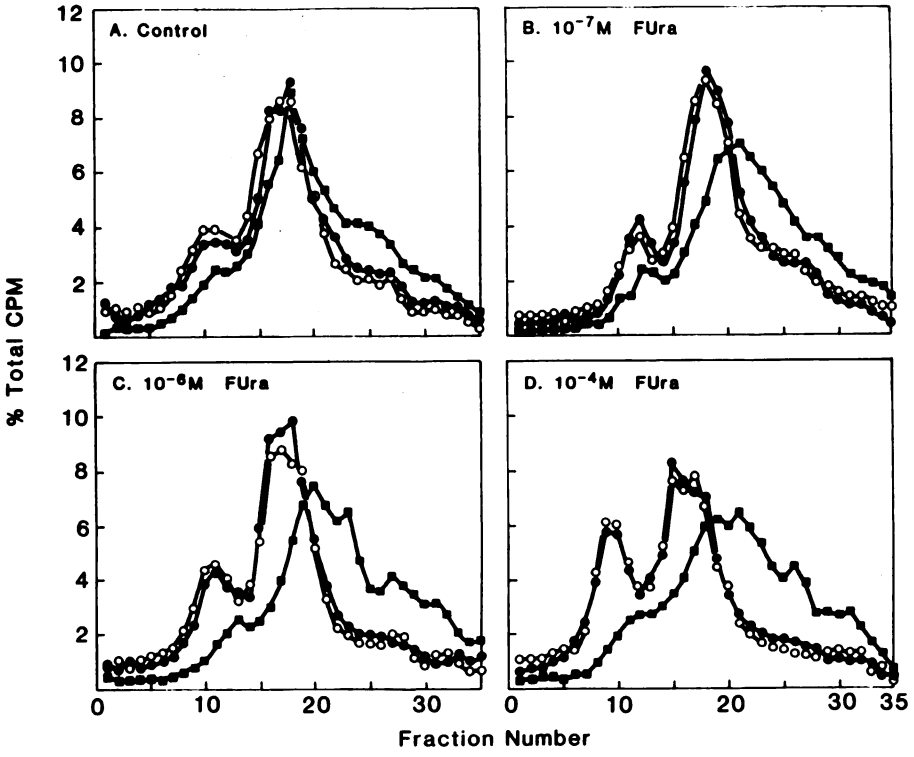

FIG. 8. *Effect of RNase III on (FUra) prRNA*

MEL cells (2×10^7) were incubated with no drug (A), 10^{-7} M (B), 10^{-6} M (C), or 10^{-4} M (D) FUra and ^{32}P (10 μCi/ml) for 3 hr. The 45 S and 32 S RNA were then purified and analyzed by sucrose/SDS velocity sedimentation (●). Aliquots were treated with RNase III (■) or with RNase III and 0.5 mM ethidium bromide (○) prior to gradient analysis.

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45 S prRNA and inhibits the formation of 28 S and 18 S RNA (27, 28). The effects of toyocamycin and FUra on MEL 45 S prRNA have suggested that incorporation of both pyrimidine and purine analogues may alter rRNA processing by similar mechanisms (28). However, exposure of cells to toyocamycin results in accumulation of 45 S RNA (27), whereas FUra treatment is not associated with detectable increases in this RNA. Although the enzymes responsible for rRNA processing have not been clearly defined, RNase III has been implicated in the maturation of *Escherichia coli* prRNA and T7 mRNA (17, 18). This enzyme acts specifically at double-stranded RNA regions. These regions may be important sites in the processing of eukaryotic nucleolar RNA inasmuch as intercalating agents inhibit the maturation of rRNA (29). The RNase III digest yields limited cleavage of HeLa cell 45 S rRNA (30), and this cleavage is inhibited by incorporation of toyocamycin. In contrast, the present study has demonstrated that FUra incorporation into 45 S and 32 S RNA increases susceptibility of these nucleic acids to degradation by RNase III. This unexpected finding could be consistent with the inability to detect 28 S and 18 S RNA despite the absence of 45 S RNA accumulation. Furthermore, the change in susceptibility of the double-stranded regions to RNase III by FUra or the possibility of altered reactivity to other RNases could be due to inhibition of converting uridine to pseudouridine.

These studies correlating the extent of FUra incorporation into prRNA with cell lethality suggest that this is one process responsible for cell death. These findings are supported further by an association between the extent of FUra incorporation into prRNA and inhibition of maturation to 28 S and 18 S RNA. This effect may occur as a result of altered recognition sites within prRNA for enzymes involved in processing of this nucleic acid. It will now be of interest to determine whether incorporation of FUra into mRNA also results in abnormal processing and enhanced degradation of this nucleic acid.

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