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Effect of 5-Azacytidine on Cytoplasmic Ribosomal and Messenger Ribonucleic Acids in BSC-1 Cells

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5-Azacytidine (5-AzaCR) inhibited the accumulation of 28S and 18S ribonucleic acids (RNAs) in the cytoplasm of treated cells. The inhibition of 28S and 18S RNAs in the cytoplasm of BSC-1 cells was dependent upon the concentration of 5-AzaCR employed and the time of exposure. At a concentration of 200 μg/ml for 2 h, 5-AzaCR inhibited the cytoplasmic 28S and 18S RNAs by 80 and 70%, respectively. The 28S and 18S RNAs that appeared in the cytoplasm of treated cultures had no altered secondary structure, as analyzed by polyacrylamide gel electrophoresis. The inhibitory effect on cytoplasmic 28S and 18S RNAs was found to be reversible, and removal of 5-AzaCR from treated cultures allowed the accumulation of cytoplasmic 28S and 18S RNAs to almost normal levels by 20 h. 5-AzaCR appeared to have no effect upon the synthesis and processing of polyadenylic acid-containing messenger RNA in treated cultures. However, the formation of the 80S ribosomal subunit appeared to be inhibited in drug-treated cells. Moreover, 5-AzaCR treatment caused a disaggregation of polyribosomes and an accumulation of 80S ribosomes.

5-Azacytidine (5-AzaCR) is a nucleic acid-base analog that is clinically used in the treatment of some leukemias (1, 3). In both procaryotic and eucaryotic systems, 5-AzaCR is phosphorylated and becomes incorporated into both ribonucleic acid (RNA) and deoxyribonucleic acid (4, 11). 5-AzaCR has been shown to have an inhibitory effect on the processing of precursor ribosomal RNA in Novikoff hepatoma cells (10) as well as to inhibit deoxyribonucleic acid and protein synthesis. Levitan et al. (2) suggested that 5-AzaCR inhibits protein synthesis by the synthesis of 5-AzaCR-substituted messenger RNA (mRNA) that is functionally inactive in protein synthesis. Reichman and Penman (6) reported that the inhibition of protein synthesis by 5-AzaCR occurs at the level of initiating translation.

In the present paper we investigated the effect of 5-AzaCR treatment on ribosomal and mRNA's in BSC-1 cells. Our results show that 5-AzaCR selectively affects the level of cytoplasmic 28S and 18S RNAs. mRNA synthesis and processing appear unaffected; the formation of the 80S ribosomal subunit is inhibited, however. Moreover, 5-AzaCR treatment was shown to cause a disaggregation of polyribosomes.

MATERIALS AND METHODS

RNA preparation. Confluent BSC-1 cell cultures were grown in 150-cm^2 tissue culture flasks at 37°C in minimal essential medium plus 10% fetal calf serum. For both treated and untreated cultures, the medium was removed and replaced with 5 ml of minimal essential medium with or without 5-AzaCR (Sigma Chemical Co., St. Louis, Mo.). Cultures were labeled with 2 ml of medium containing 400 to 500 μCi of [5,6-^{3}H]uridine (Amersham/Searle Corp.; 40 to 50 Ci/mmol) per flask as well as the appropriate concentration of 5-AzaCR. After labeling, the medium was removed, cultures were washed three times with cold tris(hydroxymethyl)aminomethane (Tris)-saline (0.01 M Tris-0.15 M NaCl, pH 7.4), and the cells were scraped off the flasks and collected in the same buffer. Total cells were lysed with 0.5% Nonidet P-40, and nuclei were removed by centrifugation as described (7). The cytoplasmic RNA was extracted with phenol-chloroform-isoamyl alcohol (50:50:1, vol/vol) in the presence of 0.5% sodium dodecyl sulfate, and the cytoplasmic RNA was precipitated with 2 volumes of absolute ethanol and stored overnight at −20°C. The pellet obtained after centrifugation at 10,000 rpm for 10 min was suspended in TKM buffer (0.05 M Tris, pH 6.7, containing 0.025 M KCl and 0.0025 M $MgCl_2$), treated with 25 μg of deoxyribonuclease I per ml for 45 min at 5°C, phenol extracted, and ethanol precipitated as described above. RNA samples were suspended in sodium dodecyl sulfate buffer (0.01 M Tris, pH 7.4, containing 0.5% sodium dodecyl sulfate and 0.1 M NaCl) and layered onto linear 15 to 30% (wt/wt) sucrose gradients prepared in the same buffer. Centrifugation was for 16 h at 29,000 rpm in a Beckman SW40 Ti rotor at 20°C. Fractions were collected from the bottom of the tube into scintillation vials and counted in a toluene-Triton X-100 scintillation cocktail.

Polyacrylamide gel electrophoresis. Polyacrylamide gels containing 2.4% (wt/wt) acrylamide and 0.5% (wt/vol) agarose were prepared as described (9). RNA samples were dissolved in E buffer (0.04 M Tris, pH 7.5, 0.02 M sodium acetate, 0.002 M ethylenediaminetetraacetate [disodium salt], and 0.2% sodium dodecyl sulfate), and electrophoresis was carried out for 5 h at 75 V (6 mA per gel) at room temperature. The gels were sliced into 1-mm fractions, treated with NCS solubilizer (Amersham/Searle Corp.), and counted.

Oligodeoxythymidylic acid-cellulose chromatography. For isolation of polyadenylic acid [poly(A)]-containing RNA, cytoplasmic RNA samples were suspended in 0.01 M Tris (pH 7.4) containing 0.5 M NaCl and passed over a 5-ml column of oligodeoxythymidylic acid-cellulose (8). The column was washed in the above buffer, and poly(A)-containing RNA was eluted in 0.01 M Tris buffer (pH 7.4) and precipitated with ethanol. Samples were suspended in sodium dodecyl sulfate buffer and analyzed on 15 to 30% sucrose gradients as described.

Polysome isolation. Polysomal preparations were obtained according to the procedure recently described by Yakobson et al. (12). Both treated and mock-treated cultures were washed twice with TNM buffer (0.01 M triethanolamine, pH 7.6, 0.025 M NaCl, and 0.005 M $MgCl_2$, containing 0.25 M sucrose). The cells were scraped into TNM buffer containing 1% Nonidet P-40, 0.5% deoxycholate, and 1% diethyl pyrocarbonate and centrifuged for 5 min at 1,500 rpm. The supernatant fluid was recentrifuged for an additional 20 min at 10,000 rpm and collected. Samples were then layered onto 15 to 50% (wt/wt) sucrose gradients in TNM buffer and centrifuged for 2 h at 40,000 rpm in an SW40 Ti rotor at 4°C. Fractions were collected, and the trichloroacetic acid-precipitable radioactivity was determined.

RESULTS

The effect of various concentrations of 5-AzaCR on the cytoplasmic 28S and 18S RNAs in BSC-1 cells was investigated in the current studies. Confluent cell cultures were treated with 5-AzaCR for 2 h and labeled with 200 μCi of [^{3}H]uridine per ml for 3 h. Cells were lysed with Nonidet P-40, and the cytoplasmic RNA was extracted as described in Materials and Methods. In Fig. 1 a sucrose gradient profile of the cytoplasmic RNA extracted from cultures treated with 200 μg of 5-AzaCR per ml and control BSC-1 cells is shown. As shown in the figure, a dramatic reduction in the amount of radioactivity sedimenting in the positions of 28S and 18S was obtained from 5-AzaCR-treated cultures. The majority of radioactivity in cytoplasmic RNA from 5-AzaCR-treated cultures sedimented at 4S and slower sedimenting species and accounted for approximately 80% of the radioactivity observed in untreated cultures. Other studies showed that maximum inhibition occurred with 5-AzaCR at this concentration after treatment for 2 h.

The data in Table 1 show little difference in the incorporation of [^{3}H]uridine into nuclear RNA from controls and cultures treated with 200 μg of 5-AzaCR per ml for 2 h and labeled for 20 min (plus 5-AzaCR). Little effect if any upon the synthesis of total nuclear RNA was observed. Furthermore, the amount of radioactivity incorporated into total cytoplasmic RNA from controls and cultures treated with 5-AzaCR for 2 h was similar after labeling for 2 h. However, upon analysis by sucrose gradient centrifugation, a dramatic difference was observed in the species of RNA labeled (see Fig. 1). After 5-AzaCR treatment for 2 h and labeling for 5 h (plus 5-AzaCR), the incorporation of radioactivity into cytoplasmic RNA in treated cultures decreased significantly in contrast to controls.

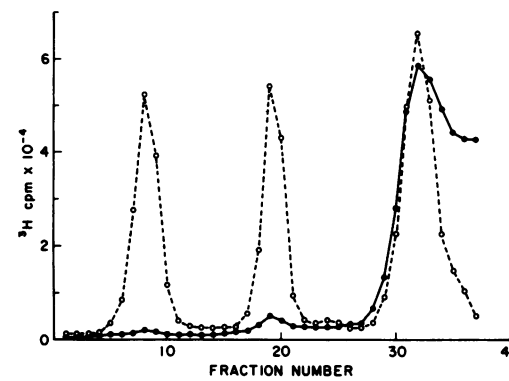

FIG. 1. *Sucrose gradient profile of total cytoplasmic RNA from 5-AzaCR-treated and control cultures. BSC-1 cultures were treated with 200 μg of 5-AzaCR per ml for 2 h and then labeled with [^{3}H]uridine (plus 5-AzaCR) for 2 h. Cytoplasmic RNA was extracted and ethanol precipitated as described in the text. RNA samples were layered onto linear 15 to 30% (wt/wt) sucrose gradients and centrifuged for 16 h at 29,000 rpm in a Beckman SW40 Ti rotor at 20°C. Fractions were collected from the bottom of the tube into scintillation vials and counted.* (O——O) *Control;* (●——●) *5-AzaCR treated.*

TABLE 1. *Effect of 5-AzaCR on the incorporation of [^{3}H]uridine into nuclear and cytoplasmic RNAs*

RNA	Length of labeling	Radioactivity in control cultures (cpm)	Radioactivity in 5-AzaCR-treated cultures (cpm)
Nuclear RNA	20 min	5.5×10^6	6.0×10^6
Cytoplasmic	2 h	1.5×10^7	1.2×10^7
RNA	5 h	5.5×10^7	1.7×10^7

This result confirms that 5-AzaCR enhances the degradation of precursor ribosomal RNAs to fragments that are not precipitable in trichloroacetic acid (5, 10, 11).

The inhibitory effect of 5-AzaCR on cytoplasmic 28S and 18S RNAs was shown to be reversible upon removal of the drug. Treatment of cultures with 200 μg of 5-AzaCR per ml for 2 h drastically reduced the amounts of cytoplasmic 28S and 18S RNAs (Fig. 2A). However, upon the removal of 5-AzaCR from treated cultures, 28S and 18S RNAs began to appear in the cytoplasm by 5 h and reached 60 and 80%, respectively, of normal levels by 20 h (Fig. 2B).

5-AzaCR treatment has been reported to alter the secondary structure of the precursor 45S and 32S ribosomal RNAs. Analysis of the residual 28S and 18S cytoplasmic RNAs present in the cytoplasm of 5-AzaCR-treated cultures was carried out to determine if the cytoplasmic 28S and 18S RNAs also had altered secondary properties. Total cytoplasmic RNA isolated from 5-AzaCR-treated cultures ([^{3}H]uridine labeled) was mixed with total cytoplasmic RNA ([^{14}C]uridine labeled) from untreated cultures and analyzed on 2.4% polyacrylamide gels (Fig. 3). The residual 28S and 18S RNAs present in the cytoplasm of 5-AzaCR-treated cultures co-electrophoresed with control 28S and 18S RNA, indicating little difference if any in the secondary structure as analyzed by this method.

Since other studies on the effect of 5-AzaCR treatment on simian virus 40 transcription (Reuveni and Rosenthal, Intervirology, in press) revealed no inhibition in the synthesis and processing of simian virus 40-specific late 19S and 16S mRNA's, analysis of the cytoplasmic poly(A)-containing RNA from 5-AzaCR-treated cells was performed. BSC-1 cell cultures were treated with 200 μg of 5-AzaCR per ml for 2 h and labeled with [^{3}H]uridine (plus 5-AzaCR) for 2 h and the cytoplasmic RNA was extracted as described in Materials and Methods. Total cytoplasmic RNA was then passed over an oligodeoxythymidylic acid-cellulose column, and poly(A)-containing RNA from 5-AzaCR-treated and control cultures was analyzed on sucrose gradients. The results (Fig. 4) depict similar profiles of poly(A)-containing RNA from 5-AzaCR-treated and control cultures, suggesting little effect if any upon mRNA synthesis and processing. It was therefore apparent from the data in Fig. 1 and 4 that 5-AzaCR treatment had a selective effect on the processing of ribosomal RNA without any major effect on the processing of mRNA.

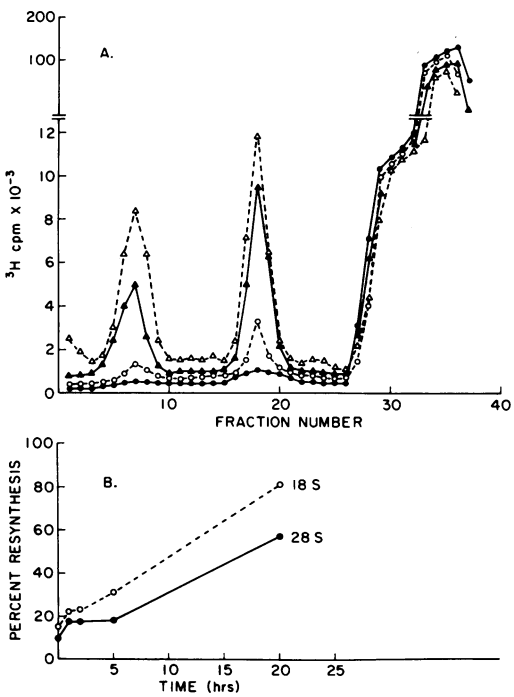

FIG. 2. *(A) Sucrose gradient profiles showing the reversible effect of 5-AzaCR on cytoplasmic 28S and 18S RNAs after treatment with 200 μg of 5-AzaCR per ml for 2 h and removal of the drug for various times. Conditions for sucrose gradients are described in legend for Fig. 1.* (△——△) *Control;* (●——●) *5-AzaCR treatment for 2 h;* (○——○) *removal of 5-AzaCR for 5 h;* (▲——▲) *removal of 5-AzaCR for 20 h. (B) Percent reappearance of cytoplasmic 28S and 18S RNAs after treatment and removal of 5-AzaCR for 1, 2, 5, and 20 h. Results are calculated from gradient profiles in Fig. 2A, except that data from 1 and 2 h were not plotted in Fig. 2A.*

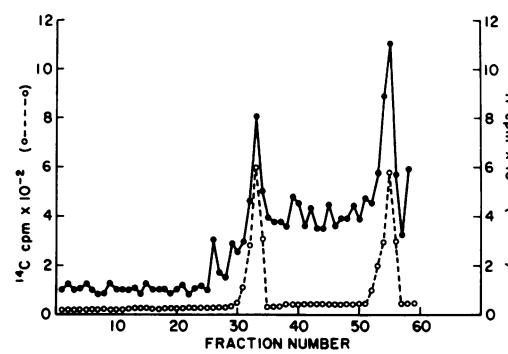

FIG. 3. *Polyacrylamide gel electrophoresis of cytoplasmic 28S and 18S RNAs from 5-AzaCR-treated* (●——●) *cultures labeled with [^{3}H]uridine and control* (○——○) *cultures labeled with [^{14}C]uridine. The cytoplasmic RNA, extracted as described in the text, was subjected to electrophoresis in 2.4% polyacrylamide-0.5% agarose gels for 5 h at 75 V (6 mA per gel) at room temperature.*

To determine whether 5-AzaCR treatment interfered with the attachment of RNA to polyribosomes, thereby inhibiting protein synthesis, BSC-1 cultures were treated with 200 μg of 5-AzaCR per ml for 2 h and labeled with [^{3}H]-uridine for 2 h (plus 5-AzaCR), and polyribosomes were isolated as described in Materials and Methods. In Fig. 5 a representative sucrose gradient profile of polyribosomes from 5-AzaCR-treated and control cultures is presented. As seen in the control, there was a distinct 80S monosome peak in addition to the more rapidly sedimenting polyribosomes. However, in 5-AzaCR-treated cultures, the distinct 80S monosome peak was virtually absent, whereas the polyribosome profile appeared similar to the control. The data suggest that 5-AzaCR affects the formation of the normal 80S ribosome subunit. These results are in agreement with Reichman and Penman (6), who reported that 5-AzaCR treatment inhibited protein synthesis at the stage of initiation.

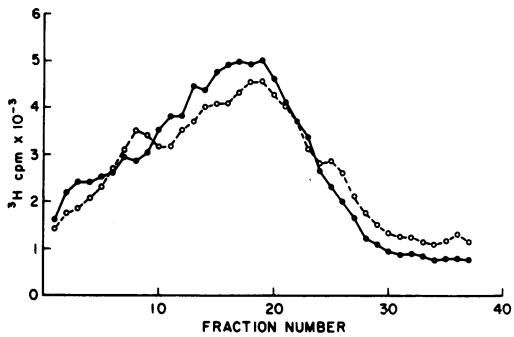

FIG. 4. *Sucrose gradient analysis of cytoplasmic poly(A)-containing mRNA from 5-AzaCR-treated (●——●) and control (○——○) cultures. Samples were layered onto 15 to 30% (wt/wt) sucrose gradients and centrifuged for 16 h at 29,000 rpm in a Beckman SW40 Ti rotor at 20°C.*

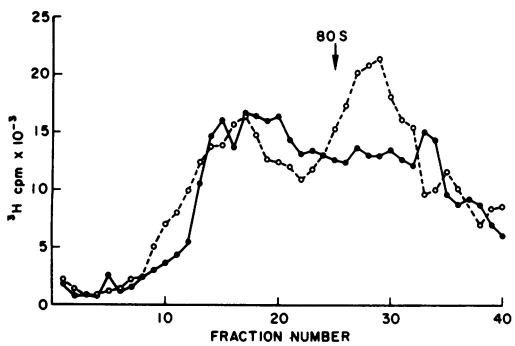

FIG. 5. *Sedimentation profile of polyribosomes isolated from 5-AzaCR-treated (●——●) and control (○——○) cultures. BSC-1 cultures were treated or mock treated with 200 μg of 5-AzaCR per ml for 2 h and then labeled with [^{3}H]uridine for 2 h (with and without 5-AzaCR). Polyribosomes isolated as described in the text were layered onto linear 15 to 50% (wt/wt) sucrose gradients in TNM buffer and centrifuged for 2 h at 40,000 rpm in an SW40 Ti rotor at 4°C. ^{14}C-labeled 80S ribosomes from XC cells were used as an internal sedimentation marker. Fractions were collected, and samples were precipitated with trichloracetic acid and counted.*

To further investigate the effect of 5-AzaCR on polyribosomes, BSC-1 cultures were prelabeled with [^{14}C]uridine for 16 h and treated with 5-AzaCR for 2 h, while controls were prelabeled with [^{3}H]uridine. Polysomes were isolated from both treated and control cultures and analyzed by sucrose gradients. There was an accumulation in the amount of 80S ribosomal subunits in the 5-AzaCR-treated cultures in contrast to untreated cultures (Fig. 6), substantiating that 5-AzaCR treatment causes a disaggregation of polyribosomes (6).

DISCUSSION

It has been established that 5-AzaCR has multiple effects upon both deoxyribonucleic acid and RNA synthesis as well as protein synthesis (1). The importance in understanding these effects is underscored because 5-AzaCR is used in the treatment of some leukemias. The current study has determined the effect of various concentrations of 5-AzaCR on cytoplasmic ribosomal and mRNA's in BSC-1 cultures. Maximal

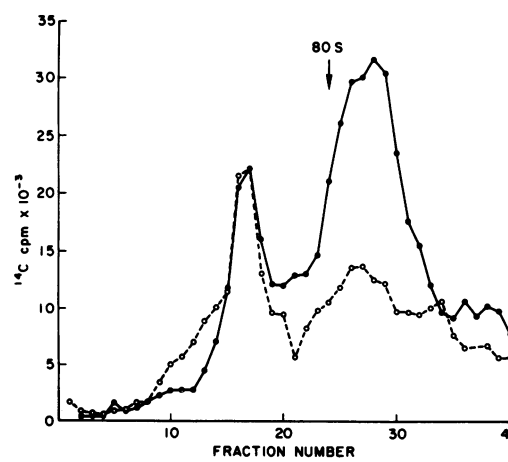

FIG. 6. *Sedimentation profile of polyribosomes from cultures prelabeled with 10 μCi of [^{14}C]uridine per ml for 16 h and then either treated with 5-AzaCR for 2 h (●——●) or mock treated (○——○). ^{3}H-labeled 80S ribosomes were added as a sedimentation marker. Conditions for centrifugation were similar to those described in the legend to Fig. 5.*

inhibition of cytoplasmic 28S and 18S RNAs (Fig. 1) occurred at a concentration of 200 μg/ml for 2 h. In addition, the results presented in Table 1 show that 5-AzaCR affects the processing of precursor ribosomal RNAs and not their synthesis. Incorporation of 5-AzaCR into precursor ribosomal RNAs may produce unstable RNA species which are quickly degraded (5). Upon removal of 5-AzaCR from treated cultures, the 28S and 18S RNAs reappeared in the cytoplasm by 20 h (Fig. 3). The alteration in secondary and tertiary structure of preribosomal 45S and 32S RNAs produced by 5-AzaCR (10) was not observed for the residual cytoplasmic 28S and 18S RNAs present in drug-treated cultures. This may suggest that a small amount of normal processing may occur even in the presence of 5-AzaCR.

The results presented also show that 5-AzaCR selectively inhibits cytoplasmic 28S and 18S RNAs without a concomitant effect upon cytoplasmic poly(A)-containing mRNA. Although the sucrose gradient profiles of poly(A)-selected RNA from 5-AzaCR-treated and untreated cultures appear to be virtually the same (Fig. 4), we cannot rule out some effect that might be observed by more sensitive methods. However, in simian virus 40-infected cultures treated with 5-AzaCR, transcription of simian virus 40-specific 19S and 16S mRNA's also appears to be normal (Reuveni and Rosenthal, Intervirology, in press). The inhibition of methylation in precursor ribosomal RNAs produced by 5-AzaCR has been proposed to explain the effect on normal processing (10). The much lower levels of methylation reported for mRNA in contrast to ribosomal RNA may support the selective inhibition observed.

Finally, the effect by 5-AzaCR on the synthesis of 80S ribosomal subunits (Fig. 5) and the disaggregation of polyribosomes observed after drug treatment (Fig. 6) confirm results from other laboratories on the inhibition of protein synthesis by 5-AzaCR (6). As such, the results of the present study emphasize the selective effect of 5-AzaCR and, hopefully, lead to a greater understanding of its potential application.

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