Alterations in the Maturation and Structure of Ribosomal Precursor RNA in Novikoff Hepatoma Cells Induced by 5-Fluorocytidine†

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ABSTRACT: The effects of 5-fluorocytidine on ribosomal RNA maturation and structure in Novikoff hepatoma cells were investigated. Like other nucleic acid base analogues that are incorporated into RNA, this compound inhibits maturation of the 45S ribosomal RNA precursor. The 45S RNA precursor produced in the presence of 5-fluorocytidine has an abnormal electrophoretic mobility compared with that of the control precursor under nondenaturing conditions, but the two have identical mobilities under denaturing conditions. Under the conditions of these experiments, 5-fluorocytidine inhibited cellular protein synthesis only slightly, whereas equimolar concentrations of 5-azacytidine resulted in nearly 75% inhibition of this process. Despite this difference in the effects of the two analogues as well as the greater chemical lability of the 5-azacytidine, their effects on ribosomal RNA maturation are identical.

Eukaryotic and prokaryotic cytoplasmic species of RNA [mRNA, ribosomal ribonucleic acid (rRNA),[1] and tRNA] are synthesized as large primary transcripts which then undergo various types of processing to become the mature RNA products. In mammalian cells, the primary transcript for the rRNAs is a 45S species with an estimated molecular weight of (4.2–4.6) × 10^6 (Dabeva et al., 1976). This 45S precursor RNA molecule associates with proteins having different rates of synthesis and degradation to form a 45S RNP (Auger-Buendia & Longuet, 1978). Methylation of bases and sugars on the RNA sequences integrated into cytoplasmic ribosomes occurs early during processing, although an immediate, absolute requirement for full methylation may not be present (Weiss & Pitot, 1974c; Caboche & Bachellerie, 1977; Grummt, 1977; Wolf & Schlessinger, 1977). Successive endo- and exonucleolytic cleavages occur on the 45S RNP by at least three possible pathways, resulting in a loss of approximately 50% of the mass of the initial molecule while yielding the mature rRNAs (Bowman et al., 1981).

Various compounds have been found to interfere with this maturation process. Chemicals that inhibit protein synthesis (e.g., cycloheximide) and intercalating agents (proflavin and ethidium bromide) inhibit processing (Craig & Perry, 1970; Stoyanova & Hadjiolov, 1979; Stoyanova & Dabeva, 1980; Snyder et al., 1971; Yannarell et al., 1977). These results have been interpreted as demonstrating a requirement both for continued protein synthesis (presumably of one or more rapidly turning over proteins) and for a specific secondary structure of the precursor, respectively.

Nucleoside and base analogues that are incorporated into RNA have been shown to stop or markedly alter the pattern of maturation of 45S RNA (Weiss & Pitot, 1974a,b; Wilkinson & Pitot, 1973; Hadjiolova et al., 1981). Studies involving nucleoside and base analogues have, with few exceptions, demonstrated no significant difference between normal and abnormal 45S RNAs or 45S RNPs in terms of base composition, density in metrizamide or cesium chloride gradients, length as measured in the electron microscope, or electrophoretic mobility (Weiss & Pitot, 1974a,b, 1975; Auger-Buendia et al., 1978; Auger-Buendia & Tavitian, 1979). In contrast to these results, 5-fluorouracil has been implicated in altering the nearest-neighbor frequency of the 45S RNA (Glazer & Legraverend, 1980), and cordycepin has demonstrated its anticipated RNA chain-terminating effect (Siev et al., 1969).

An effect of 5-AZAC on the secondary structure of isolated 45S RNA has been demonstrated (Weiss & Pitot, 1974, 1975) by comparing the electrophoretic mobility under native and denaturing conditions. This analogue has also been shown to have profound effects on protein synthesis (Lee & Karon, 1976; Reichman & Penman, 1973), DNA synthesis (Jones & Taylor, 1980), DNA and RNA 5-cytidine methylation (Friedman, 1981; Jones & Taylor, 1981; Lu et al., 1976), and functional activities of DNA and RNA molecules (Lee & Karon, 1976; Jones & Taylor, 1980). We report herein the effects of another cytidine analogue, 5-FC, on the synthesis of protein, the inhibition of 45S RNA processing, and the secondary structure of the 45S precursor.

Materials and Methods

5-FC was a generous gift of Dr. W. E. Scott at Hoffmann-La Roche. 5-AZAC, 8-hydroxyquinoline, DNA (calf thymus, highly polymerized), activated charcoal, and diethyl pyrocarbonate were purchased from Sigma Chemical Co. Agarose (grade A), for composite gels, and cycloheximide were obtained from Calbiochem-Behring Corp. Agarose [HGT(P) and LE] for partially and totally denaturing gels was obtained from Marine Colloids. Urea (ammonia free, ultrapure) was obtained from Bethesda Research Labs, and formamide (Spectro grade) was from Eastman Kodak. Sodium dodecyl sulfate (SDS) (specially pure) was purchased from BDH Chemical, LTD. SW77 media and newborn calf sera were purchased from GIBCO. Dextran sulfate was obtained from Pharmacia. Aquassure, Protosol, [5-^{3}H]uridine (26.7 Ci/mmol), [2-^{14}C]uridine (52.4 mCi/mmol), and L-[4,5-^{3}H(N)]leucine (50.4 Ci/mmol) were obtained from New England Nuclear Corp. [8-^{3}H]Guanosine (7.6 Ci/mmol) was obtained from Amersham Corp.

All aqueous solutions involved in RNA isolation and handling were made 0.1% with diethyl pyrocarbonate and then autoclaved. Glassware was siliconized and then treated with

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[1] Abbreviations: rRNA, ribosomal ribonucleic acid; RNP, ribonucleoprotein particle; 5-FC, 5-fluorocytidine; 5-AZAC, 5-azacytidine; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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0.1% diethyl pyrocarbonate in water followed by overnight drying at 80 °C.

Cell Culture Conditions. The Novikoff (N_1S_1) hepatoma cell line was obtained from Dr. V. R. Potter. Cells were grown in continuous culture at 37 °C in a 5% CO_2 atmosphere in SW69 media supplemented with 10% newborn calf serum (Wilkinson & Pitot, 1973). Under these conditions, the doubling time was found to be 12 h. Cells were used for experiments at concentrations of (0.6–1.0) × 10^6 cells/mL.

RNA Extractions. Cells were pelleted by centrifugation and washed twice with ice-cold saline. Total cell RNA was extracted by the hot phenol–SDS method of Wilkinson & Pitot (1973). To the final aqueous phase were added 0.1 volume of 2 M potassium acetate (pH 5.1) and 2 volumes of 100% ethanol. This solution was stored either at −20 °C overnight or at −70 °C for 1 h and then centrifuged to collect the RNA. RNA was washed twice with 75% ethanol. The final pellet was dried under vacuum and then resuspended in double-distilled water at a concentration of 20–30 A_{260} units/mL.

Electrophoresis. Electrophoresis of RNA under nondenaturing conditions was performed by the method of Weiss & Pitot (1975) in 40 mM Tris, 20 mM sodium acetate, and 1 mM EDTA (pH 7.6) buffer on 2.1% acrylamide–0.6% agarose composite gels, with the modification that the gel was prepared the day before and left to polymerize at room temperature overnight. Partially denaturing gels were prepared with minor modifications of the method of Locker (1979), by using the above electrophoresis buffer and gels of 1.2% agarose [HGT(P)], 6 M urea, and 15 mM sodium iodoacetate. The agarose was placed in buffer containing urea and iodoacetate, brought to boiling, and then maintained at 60 °C with constant mixing for 15 min. The solution was then poured into quartz gel tubes and left at room temperature for 1 h. The gels were left at 4 °C overnight. Prior heating of the RNA samples to 65 °C in 10 mM NaCl–0.5% SDS for 15 min prior to electrophoresis had no effect on either the pattern or the magnitudes of the radioactive profiles in these gel systems.

Electrophoresis under totally denaturing conditions was carried out in 0.9% agarose (LE), 2.2 M formaldehyde, 0.018 M Na_2HPO_4, and 0.002 M NaH_2PO_4 as described by Lehrach et al. (1977). The RNA was denatured by being heated to 65 °C for 10 min in buffer composed of 50% formamide, 2.6 M formaldehyde, 0.018 M Na_2HPO_4, and 0.002 M NaH_2PO_4. The formamide had been previously treated as follows: three deionizations of 1 h each with 40 g/L AG 501-X8 (20–50 mesh). The solution was filtered after each deionization. During the second deionization, 10 g of activated charcoal/L was present. The formamide was then extracted twice with an equal volume of anhydrous ether and purged with nitrogen to remove the residual ether.

Gels were sliced into 1.5-mm-thick sections for quantification of radioactivity. Acrylamide–agarose composite gel slices were incubated at 55 °C overnight with 1 mL of 30% H_2O_2 in sealed scintillation vials. Agarose–6 M urea gel slices were dried overnight at 65 °C and then dispersed in 1 mL of H_2O. Agarose–formaldehyde gel slices that had been frozen at −20 °C for slicing were incubated at 60 °C with 1 mL of 30% H_2O_2 for 2 h. Ten milliliters of Aquassure was added to each sample. Radioactivity was quantified in a Nuclear Chicago scintillation counter equipped with single and dual label programs.

Protein Synthesis and DNA Measurement. Protein synthesis was measured by the incorporation of [^{3}H]leucine into trichloroacetic acid insoluble material. After radioactive labeling, cell culture volumes (25–200 μL) were added to 1 mL of 15% trichloroacetic acid, vortexed, and stored at 4 °C for 1 h. The solutions were then filtered through either a Millipore HA 0.45-μm filter or a Whatman GF/C filter. The same type of filter was used for each group of experiments, although comparable results were obtained with either. The filters were then washed with 30 mL of ice-cold 10% trichloroacetic acid. To quantify radioactivity, the Millipore filters were placed in 10 mL of Aquassure overnight at 50 °C; the GF/C filters were incubated at 50 °C for 0.5 h with 1 mL of Protosol followed by the addition of 100 μL of glacial acetic acid and 10 mL of Aquassure.

DNA was measured by a modification of the Giles & Myers (1965) assay. Cells were centrifuged and then resuspended in 1–2 mL of 0.33 M potassium hydroxide and left at room temperature overnight. Equal volumes of 15% trichloroacetic acid–2 N HCl were then added, and the mixture was chilled to 4 °C for 30 min and centrifuged. The pellet was washed with 3 mL of cold 10% perchloric acid. After centrifugation, the pellet was resuspended in 0.6 mL of 5% perchloric acid and hydrolyzed by being heated to 70 °C for 20 min. The solution was cooled and then centrifuged; 0.5 mL of the supernatant was used for the assay. To this sample were added 0.1 mL of 60% perchloric acid, 0.5 mL of 4% diphenylamine in glacial acetic acid, and 30 μL of aqueous acetaldehyde (1.6 mg/mL), and the mixture was placed in the dark overnight. The absorbance was read at 595 nm, and DNA concentrations were determined from a standard curve.

Results

Effect of 5-Fluorocytidine on 45S RNA Processing. The concentration of 5-FC used in the cell culture medium for these experiments was chosen to be the same as that of 5-AZAC used in the experiments of Weiss & Pitot (1974c), since Lu et al. (1979) have shown approximately equimolar efficiency of the two compounds in inhibiting tRNA cytidine methylation. The ability of 5-FC to inhibit processing and to alter the mobility of the 45S RNA was assessed by loading total cell RNA isolated from [^{3}H]guanosine-labeled, 5-FC-treated cells and from [^{14}C]uridine-labeled control cells on the same gel; both agarose–6 M urea and acrylamide–agarose gels were used. Figure 1A,B shows the radioactivity profiles following electrophoresis. Processing occurred in the control cells with the formation of ^{14}C-labeled peaks corresponding to 18S and 28S mature rRNA. In contrast, 5-FC-treated cells displayed no detectable processing of the 45S precursor to the cytoplasmic species with only limited processing to the 32S species. The positions of the 45S species from the 5-FC-treated and control cells are not coincidental under the conditions of either of these electrophoretic systems. The 45S RNA from 5-FC-treated cells migrates more slowly and has a larger apparent molecular weight under these conditions than the control cells.

By use of the control [^{14}C]uridine-labeled internal standards of 18S, 28S, 32S, and 39S RNAs and their respective molecular weights from rat liver (Dabeva et al., 1976), a log molecular weight vs. mobility plot was constructed for the acrylamide–agarose composite gel. Estimates of the molecular weight of 45S RNA from control and 5-FC-treated cells are obtained from a least-mean-squares fit of this plot. The molecular weight of 45S RNA in control cells was estimated to be 4.47 × 10^6, and that of the 5-FC-treated cells was 4.98 × 10^6; this is a difference of 0.51 × 10^6 or 12% of control. Applying the same procedure to the agarose–6 M urea gel (Figure 1A) yields a difference in the molecular weight of the 45S species of 0.31 × 10^6 or 7% of control. In marked contrast to the situation under native and partial denaturing conditions, the 45S RNA from 5-FC-treated and control cells comigrated

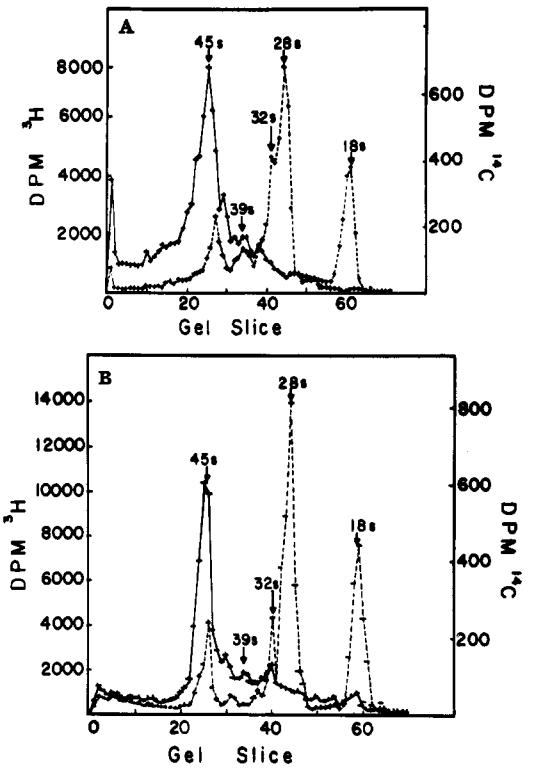

FIGURE 1: Coelectrophoresis of total cell RNA from control and 5-FC-treated Novikoff cells. Two 25-mL cultures of Novikoff cells were prepared. One culture was treated with 5×10^{-4} M 5-FC for 3 h, while the second was treated with 5×10^{-4} M cytidine. For the final 2 h of incubation, the 5-FC culture contained 0.5 μCi/mL (6.6×10^{-8} M) [^{3}H]guanosine, and the control culture contained 0.1 μCi/mL (2×10^{-6} M) [^{14}C]uridine. At the end of 3 h, total cell RNA was prepared from the cultures. Approximately 0.25 A_{260} unit of each was mixed and subjected to electrophoresis. (A) 2.1% acrylamide–0.6% agarose gel, 6 mA/gel for 4.5 h; (B) 1.2% agarose–6 M urea gel, 6 mA/gel for 5 h. Radioactivity (dpm) is plotted vs. distance of migration (gel slice): (—) 5-FC; (- - -) control.

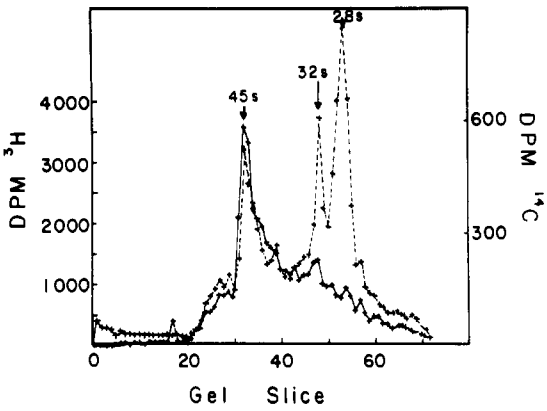

FIGURE 2: Coelectrophoresis of total cell RNA from control and 5-FC-treated Novikoff cells, prepared as described in the legend of Figure 1 except that the control culture was labeled with 0.2 μCi/mL (4×10^{-6} M) [^{14}C]uridine. Samples of approximately 0.25 A_{260} unit were denatured as described under Materials and Methods and electrophoretically separated on a 0.9% agarose–2.2 M formaldehyde gel at 3 mA/gel for 5.5 h. Radioactivity (dpm) is plotted vs. distance of migration (gel slice): (—) 5-FC; (- - -) control.

under totally denaturing conditions (Figure 2).

In addition to the above differences, Figure 1B also demonstrates a marked difference in radioactivity profiles of RNA between 5-FC-treated and control cells around gel slice 31. This difference is not present in either Figure 1A or Figure 2. It is unclear whether this represents an abnormal accumulation of a specific rRNA processing intermediate or an RNA species with an abnormal mobility.

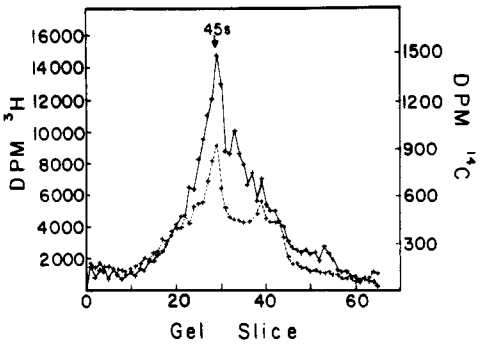

FIGURE 3: Coelectrophoresis of total cell RNA from 5-FC- and 5-AZAC-treated Novikoff cells. 5-FC total cell RNA was prepared as described in the legend of Figure 1. A 25-mL culture of Novikoff cells was incubated with 5×10^{-4} M 5-AZAC for 3 h. During the final 2 h of incubation, 0.2 μCi/mL (4×10^{-6} M) [^{14}C]uridine was present. Total cell RNA was prepared at the end of 3 h. Approximately 0.25 A_{260} unit of each was mixed and subjected to electrophoresis on a 2.1% acrylamide–0.6% agarose gel, 6 mA/gel for 4.5 h. Radioactivity (dpm) is plotted vs. distance of migration (gel slice): (—) 5-FC; (- - -) 5-AZAC.

The inhibition of 45S RNA processing induced by 5-FC resembles that of 5-AZAC, in both the pattern of RNA species labeled and the altered electrophoretic mobility of the precursor (Weiss & Pitot, 1974c, 1975; unpublished results for agarose–6 M urea gels). To investigate further the extent of this similarity, coelectrophoresis under nondenaturing conditions of RNA from 5-FC- and 5-AZAC-treated cells was carried out. Figure 3 demonstrates this result; both analogue-treated 45S RNA precursors migrate together. Figure 3 also demonstrates a number of differences in the specific radioactivity of labeled RNA species from 5-FC- and 5-AZAC-treated cells. The 5-FC-treated cells contain a larger proportion of specific RNA processing intermediates than do the 5-AZAC-treated cells. It is not known whether this is a consequence of the 45S RNA synthesized in the presence of 5-FC being a better substrate for limited enzymatic processing or whether any intermediates formed from this 45S RNA are poorer substrates for further processing and/or degradation as compared with the corresponding RNA molecules of 5-AZAC-treated cells.

Effect of Cycloheximide on 45S Processing. A concentration of cycloheximide that had previously been shown to cause more than 95% inhibition of protein synthesis in HTC cells (Peterkofsky & Tomkins, 1968) (also see below) was used in these experiments. Figure 4 demonstrates these results. Marked inhibition of processing occurred, with limited formation of labeled 28S rRNA. The pattern of radioactive labeling in the gel profile, however, was distinctly different from that induced by either 5-FC or 5-AZAC and is in agreement with previous results for rat liver (Dabeva et al., 1976). A larger amount of processing to the 32S and 36S species occurs. The 45S RNA species from cycloheximide-treated cells, in contrast to the situation with 5-AZAC and 5-FC, comigrates under nondenaturing conditions with those of control cells.

Protein Synthesis and DNA Levels. On the basis of the results demonstrating an interrelation between protein synthesis and 45S RNA processing (Craig & Perry, 1970; Stoyanova & Hadjiolov, 1979; Stoyanova & Dabeva, 1980) and the possible effects of base analogues on protein and DNA synthesis, we felt it necessary to determine the level of these functions in nucleoside analogue-treated and cycloheximide-treated cells. The incorporation of [^{3}H]leucine into protein was measured during the entire time that RNA labeling would normally occur. DNA content was determined at the harvest time of the cells (representing one-fourth of the doubling time

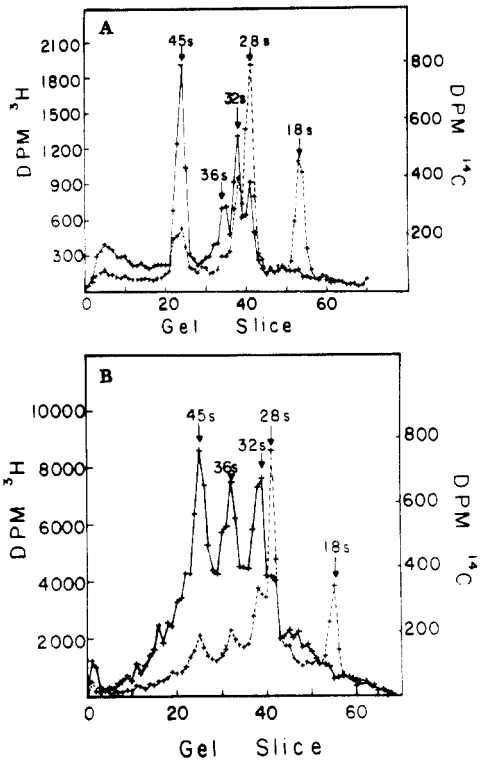

FIGURE 4: Coelectrophoresis of total cell RNA from control and cycloheximide-treated Novikoff cells. Two 25-mL cultures of Novikoff cells were prepared. One culture was treated with 1×10^{-4} M cycloheximide for 3 h. For the final 2 h of incubation, the cycloheximide culture contained 0.5 μCi/mL (2×10^{-8} M) [^{3}H]uridine, and the control culture contained 0.1 μCi/mL (2×10^{-6} M) [^{14}C]-uridine. Total cell RNA was prepared at the end of 3 h. Approximately 0.25 A_{260} unit of each was mixed and electrophoretically separated. (A) 1.2% agarose–6 M urea gel, 6 mA/gel for 4.5 h; (B) 2.1% acrylamide–0.6% agarose gel, 6 mA/gel for 4.5 h. Radioactivity (dpm) is plotted vs. distance of migration (gel slice): (—) cycloheximide; (---) control.

of the cells). Table I summarizes these results.

Cycloheximide at the concentration used was capable of inhibiting protein synthesis by more than 95%. 5-AZAC and 5-FC decreased protein synthesis to 26.4 and 76.4% of the control value, respectively. The difference in the levels of cellular protein synthesis between 5-FC- and 5-AZAC-treated cultures is especially interesting since both analogues inhibited rRNA maturation (and presumably ribosome production) to comparable extents. This implies that, in addition to an effect mediated by decreased rRNA maturation, 5-AZAC must have a second major effect on protein synthesis, which 5-FC does not.

In contrast to the results for protein synthesis, DNA assays performed displayed no significant differences under the several conditions studied. This is a crude estimate of differences in DNA synthesis during the period of treatment and serves to demonstrate only that the number of cells per culture was equal and that there was no gross disturbance of DNA synthesis. Further investigation of this parameter with more sensitive techniques is required before one can accept that cycloheximide, 5-FC, and 5-AZAC have little early effect on DNA synthesis. In this regard, it should be noted that Jones & Taylor (1980) demonstrated a reduction of DNA synthesis in cultured mouse embryo cells (C3H/10T1/2 CL8) after exposure to 5-AZAC for a period of 24 h.

Discussion

The results presented here indicate that the nucleoside analogue 5-FC shared with 5-AZAC the ability to inhibit rRNA maturation and to alter the electrophoretic mobility,

Table I: Inhibition of Protein Synthesis and DNA Content during Treatment with Cycloheximide, 5-FC, and 5-AZAC[a]

	cycloheximide (1×10^{-4} M)	5-AZAC (5×10^{-4} M)	5-FC (5×10^{-4} M)
protein synthesis[b]	2.9 ± 0.9	26.4 ± 8	76.4 ± 2.9
total DNA[b]	99 ± 17	97 ± 22	102 ± 19

[a] The results represent the average values of four experiments. In each experiment, a 100-mL culture of Novikoff cells was divided into four equal 25-mL cultures. One culture was treated with 5-FC (5×10^{-4} M), one with cycloheximide (1×10^{-4} M), and one with 5-AZAC (5×10^{-4} M) for 3 h. At the end of 1 h, 20 mL of medium was removed and kept in culture for 2 h as two 10-mL samples for duplicate DNA determinations. To the remaining 5 mL of culture was added 2 μCi/mL (4×10^{-8} M) [^{3}H]leucine. At the end of the 2 additional hours of incubation, duplicate determinations of [^{3}H]leucine incorporation on at least two different volumes of cells were performed. Values for controls ranged from 4.92 to 6.45 μg of DNA/mL and from 3250 to 5750 dpm/50 μL. The values obtained for protein synthesis are different at the level of $p < 0.001$ (Zar, 1974). [b] Percent of control ± standard deviation.

compared with controls, of the 45S ribosomal RNA precursor under native and partially denaturing conditions of 6 M urea, while not altering the mobility of this (these) RNA molecule(s) under totally denaturing conditions. In addition, 45S RNA precursors synthesized in the presence of 5-AZAC and 5-FC had identical mobilities under nondenaturing conditions. These results, which are in agreement with those of Weiss & Pitot (1974c, 1975), may be interpreted as representing the synthesis of a 45S precursor, which differs only in three-dimensional structure from that of the 45S RNA of untreated Novikoff cells in the presence of these nucleosides. Lin & Glazer (1981) have reported no change in electrophoretic mobility, compared with controls, for poly(A) minus RNA on agarose–6 M urea gels from Ehrlich ascites cells with concentrations of 5-AZAC up to 1×10^{-3} M. This discrepancy may reflect differences in cell type and cell susceptibility to 5-AZAC, or a lack of resolution in their gel system owing to a reduced degree of migration of the RNA species compared with those reported here. Because of the similarity of the effects of 5-FC and 5-AZAC in altering the 45S precursor, we expect that a property common to the two base analogues is the determinant of this change. It should be noted, in addition, that no other analogue, including 5-fluorouracil, has been found to cause a similar change.

In view of reported abnormalities in the 45S ribosomal RNP following the use of cycloheximide (Craig & Perry, 1970), the effect of cycloheximide on the mobility of the precursor was determined. Cycloheximide, at a concentration that inhibited protein synthesis by more than 95%, had no detectable effect on the electrophoretic mobility of the 45S precursor. The inhibition of rRNA processing produced by cycloheximide is very similar to that reported by Stoyanova & Hadjiolova (1979) for rat liver and distinct from that induced by either of the base analogues tested here. We feel, therefore, that neither the inhibition of processing nor the shift of mobility resulting from these analogues is attributable to an indirect effect of decreased protein synthesis.

It has been postulated that hydrolysis of 5-AZAC incorporated into the 45S RNA might account for the observed change in secondary structure (Weiss & Pitot, 1975). Recently 5-AZAC incorporated into DNA has been shown to be more stable than expected (Jones & Taylor, 1981). With our demonstration that the chemically stable analogue 5-FC caused a similar change in the 45S precursor, it is doubtful

that hydrolysis of incorporated 5-AZAC is the origin of the altered mobility.

Speculation on the origin of the change in the secondary and tertiary structure of the 45S RNA induced by treatment with 5-AZAC and 5-FC would include effects, either direct or indirect, dependent on the inhibition of RNA methylation. Methylation of pre-rRNA and rRNA has been shown to be clustered (Salim & Maden, 1981; Brand & Gerbi, 1979; Khan et al., 1978) and to include methylation of the 5-position of cytidine (Klagsbrun, 1973). Both of these analogues have been shown to inhibit methylation of the 5-position of cytidine in tRNA (Lu et al., 1979) and DNA (Friedman, 1981; Jones & Taylor, 1981). Weiss & Pitot (1974c) and Glazer et al. (1980) have demonstrated marked effects of 5-AZAC on the methylation of the 45S precursor RNA. Given the studies demonstrating the direct effects of methylation of the 5-position of cytidine on secondary structures of DNA and RNA (Szer & Shugar, 1966; Ehrlich et al., 1975; Behe & Felsenfeld, 1981), a similar direct effect may be operative for the conformational changes seen in the 45S rRNA precursor. It is unclear, however, whether there are sufficient methylated cytidine residues or whether there is clustering of those that are present to account for a direct stabilization of specific secondary structures. It should, therefore, be noted that a conformational change in fragments of the 16S RNA of a kasugamycin-resistant strain of *Escherichia coli* has been attributed to the lack of methylation of a single adenine residue (Van Charldorp et al., 1981).

Indirect structural effects of a decreased level of methylation might be generated either by specific protein binding or by lack of cleavage of a larger precursor RNA molecule. The former would require a protein(s) that would affect the conformation of the 45S RNA [similar to the results reported by Srinivasan & Jaspars (1982)] while depending on the state of methylation for binding. In this regard, Filipowicz et al. (1976) have described a protein that requires methylation of the cap of mRNA for binding to the RNA; however, the protein(s) postulated here would have to have a much greater affinity for the RNA molecule. The latter hypothesis would incorporate the concepts that methylation is involved in determining sites of cleavage with the 45S RNA and that precursor-specific sequences can alter and determine RNA structures (Stahl et al., 1979). In this case, lack of methylation might cause the accumulation of a larger than normal 45S precursor (not resolved by denaturing gel electrophoresis), which would assume a markedly different conformation.

It should be noted that experiments involving two other inhibitors of methylation, L-ethionine and cycloleucine, have failed to demonstrate any change in electrophoretic mobility of the 45S precursor rRNA (unpublished results). These chemicals, rather than directly inhibiting nucleic acid methylation as is the case with 5-AZAC and 5-FC (Santi et al., 1983), inhibit ATP:L-methionine *S*-adenosyltransferase and deplete cellular levels of *S*-adenosylmethionine (Caboche & Bachellerie, 1977; Swann et al., 1975). It is possible that these compounds may not be so effective at inhibiting specific site(s) of methylation (especially the 5-position of cytidine) in the 45S RNA as the nucleoside analogues; e.g., L-ethionine affects uracil methylation to a greater extent than cytidine in *E. coli* (Tscherne & Wainfan, 1978), and cycloleucine has been reported to reduce methylation of rRNA by 75% (Caboche & Bachellerie, 1977). Also, DNA synthesized in the presence of ethionine and cycloleucine did not have fewer 5-methylcytidine residues as assayed by high-performance liquid chromatography (HPLC) analysis (Jones & Taylor, 1981), whereas 5-AZAC was able to produce a detectable reduction in the numbers of this residue in cellular DNA.

The inhibition of protein synthesis caused by these two analogues differs greatly, whereas the extent of inhibition of 45S RNA processing is very similar. This corroborates the finding of Reichman & Penman (1973) for HeLa cells that protein synthesis inhibition by 5-AZAC is not entirely due to decreased rRNA production. The data presented do not allow evaluation as to whether effects on mRNA, tRNA, or other cellular molecules are responsible for this decrease in protein synthesis. If the results of Lin & Glazer (1981) demonstrating little effect of 5-AZAC on poly(A) RNA levels, on electrophoretic profiles of poly(A) RNA, and on the in vitro translational efficiency of poly(A) RNA from Ehrlich ascites cells (at levels up to 1×10^{-3} M) are applicable to our system of Novikoff hepatoma cells, then the decreased protein synthesis is not due to direct effects of the analogues on mRNA. It is then tempting to speculate that the analogues may interfere with the synthesis of functional tRNA, possibly by affecting the synthesis and function of CCA at the 3′ end of tRNAs.

In addition to the quantitative alterations of protein synthesis after exposure to 5-AZAC and 5-FC, a qualitative change is possible. This would be a postulated result of DNA hypomethylation with subsequent alterations of transcribed DNA sequences and messenger RNA populations (Ehrlich & Wang, 1981; Razin & Friedman, 1981); while this possibility has not been excluded, the short time course of these experiments would argue against this occurring to a significant extent.

Registry No. 5-Fluorocytidine, 2341-22-2; 5-azacytidine, 320-67-2.

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Relative Stability of Guanosine-Cytidine Diribonucleotide Cores: A ^{1}H NMR Assessment†

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ABSTRACT: Proton NMR was used to study the secondary structure and melting behavior of six self-complementary oligoribonucleotide tetramers, each containing two guanosine and two cytidine residues (GGCC, CCGG, GCCG, CGGC, GCGC, and CGCG). GGCC and CCGG formed perfect duplexes containing four G·C base pairs with T_ms of 54 and 47.8 °C, respectively; GCCG and CGGC formed staggered duplexes with two G·C base pairs and four 3′ double-dangling bases, with T_ms of 35.5 and 29.2 °C, respectively; GCGC formed a perfect duplex with a T_m of 49.9 °C, while CGCG formed a staggered duplex with a T_m of 36.9 °C. From these results, an order of stability of the cores containing two G·C base pairs was proposed: GC:GC is more stable than GG:CC which is more stable than CG:CG. The RY model for secondary structure stability prediction was applied to the above tetramers with reasonable success. Suggestions for refinements are discussed.

Duplex formation occurs in several important RNA cellular interactions including the maintenance of the native secondary structures of transfer (Rich & RajBhandary, 1976) and ribosomal (Noller & Woese, 1981) RNA, the control of transcriptional termination (Rosenberg & Court, 1981) and attenuation (Yanofsky, 1981), and tRNA–mRNA recognition in translation. Double-stranded RNA adopts the A helix, which in contrast to the B helix of DNA has 11 base pairs per turn, a shorter pitch (2.3 Å), and a 20° base pair tilt relative to the perpendicular to the helix axis. The ribofuranoside rings in the A helix are 3′-endo in conformation (Dickerson et al., 1982). Short self-complementary oligoribonucleotide sequences have been used as models for double-stranded RNA in the calculation of thermodynamic parameters of formation of the double helix from the single strands, and for the derivation of empirical rules for prediction of secondary structure stability. To date, the most comprehensive set of parameters available is that derived by Borer et al. (1974), applying a nearest-neighbor approximation to the results of optical melting

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