

STUDY ON THE REINITIATION OF TRANSCRIPTION IN 37 RC CELLS AFTER ACTINOMYCIN D INHIBITION

Spectrum of Major RNA Species Resynthesized after Maximal Suppression by the Drug

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ABSTRACT

The reinitiation of the synthesis of major RNA species has been studied in 37 RC cells after maximal inhibition of RNA synthesis by actinomycin D (AMD). During the period of recovery from AMD, resynthesized RNA (rec-RNA) is initially composed of almost exclusively light (4–14S) heterogeneous RNA species. All normal species of RNA can be detected in the rec-RNA spectrum as early as 3 h after AMD removal. The synthesis of low molecular weight methylated RNA species increases slightly during the early period after AMD removal, while the increase of low molecular weight unmethylated species is more significant during the same period. Much of the radioactivity in the polyribosomal fraction is EDTA and puromycin sensitive. Since polysomal, puromycin-sensitive RNA is polyadenylated (as evidenced by the binding to poly-U filters), and is heterogenous in size, it belongs to the m-RNA class. The synthesis of m-RNA increases immediately after AMD removal, whereas the reinitiation of the r-RNA synthesis occurs after a lag period of about 2 h. The kinetics of recovery of the synthesis of major RNA species from AMD inhibition show a size dependency comparable to the size-related sensitivity to AMD inhibition in other cellular systems. This dependency is most clearly seen in HnRNA, the AMD sensitivity of which is measured by the length of the lag period between AMD removal and the appearance of HnRNA fractions in a sucrose density gradient. Low molecular weight HnRNA reappears first, whereas heavier fractions of HnRNA appear in the spectrum after a lag period, the length of which is in direct relation to the position of the HnRNA fraction in the gradient.

Actinomycin D (AMD) suppresses most DNA-dependent RNA synthesis in all cell systems so far studied (15), suppressing transcription irreversibly in the majority of primary cultures and cell lines.

Some cell lines, however, recover RNA synthesis soon after the removal of AMD from the growth medium. This property of recovering transcription seems to characterize exclusively primary cultures

or cell lines that derive from the African Green Monkey kidney (2, 17, 21), and is most clearly observed in the 37 RC cell line (3), where 60% of over-all RNA synthesis in control cultures recovers at 1 h after AMD removal.

Differential AMD sensitivity of major RNA species computed on the basis of dose-effect experiments (14) was shown to depend on the general rule, later elaborated by Bleyman and Woese (4), that the sensitivity to AMD of "in vivo" synthesis of general pulse-labeled RNA is largely a function of RNA size (the larger the RNA size, the more sensitive to AMD its synthesis). Since over-all RNA in 37 RC cells recovers in a relatively short period of time, a study of the kinetics of the reinitiation of transcription of single major RNA species is greatly facilitated, and the compatibility of these kinetics with Bleyman and Woese's rule (4) can be determined.

In order to study the kinetics of recovery of RNA species, and to determine whether or not the liberation of DNA templates from AMD is a sufficient condition for the reinitiation of transcription (18), a detailed analysis of the whole spectrum of RNA recovered in 37 RC cells at various time intervals after AMD removal was undertaken.

During the early period of recovery of RNA synthesis, only small species of heterogeneous RNA may be revealed, but the synthesis of all normal species of RNA may be detected as early as 3 h after AMD removal. The kinetics of the appearance of each single species of RNA in the spectrum of recovered RNA is described. The compatibility of the rule of Bleyman and Woese (4) with our experimental data, and a model explaining the mechanism of rapid recovery of RNA synthesis in 37 RC cells [based also on results of previous studies from this laboratory (2)] will be discussed.

MATERIALS AND METHODS

Cell culture

The cell line used was 37 RC, derived from African Green Monkey kidney and maintained in monolayers in this laboratory for 8 yr with constant controls of metabolic and ultrastructural properties. 37 RC cells were seeded from frozen replicates (-180°C) and routinely passaged in glass containers in Eagle's medium containing 5% fetal calf serum (MEM) as previously described (3). No culture was passed for longer than 24 generations. Only mycoplasma-free cultures were used. When the experiments were carried out with cultures in

suspension, Joklik-modified MEM (Grand Island Biological Co, Grand Island, N.Y.) was used.

Chemicals; Enzymes and

Radioactive Materials

Actinomycin D was obtained from Serva (Heidelberg, West Germany). Electrophoretically purified deoxyribonuclease, ribonuclease-free, was purchased from Worthington Chemical Co. (Freehold, N.J.) and checked for eventual ribonuclease action using purified, labeled RNAs. Crystalline bovine pancreas ribonuclease and puromycin were obtained from Sigma Chemical Co. (St. Louis, Mo.). Crystalline T_1 ribonuclease was purchased from Sankyo Co. Ltd (Japan). Polyadenylate (poly-A), polyuridylyate (poly-U) and [^3H]polyadenylate (81.6 mCi/mmol/phosphorus) were obtained from Miles Laboratories (Kankakee, Ill.) [^3H]uridine (1 Ci/mmol), [^{14}C]uridine (53 mCi/mmol) [^3H]adenosine (5 Ci/mmol), and [*methyl*- ^3H]methionine (187 mCi/mmol) were purchased from The Radiochemical Centre (Amersham, England). Nonidet P-40, Triton X-100, and very pure papain (60,000 U/g) were obtained from Shell Oil Co. (New York). Packard Instrument Co. (Downers Grove, Ill.), and Fluka AG (Buchs, Switzerland), respectively. Sodium deoxycholate (DOC) was obtained from Merck (West Germany). *N*-hydroxyurea and ethidium bromide were purchased from K & K Laboratories (Hollywood, Calif.) and Sigma, respectively.

Effect of AMD on the Synthesis of

Major RNA Species

The growth medium of 37 RC monolayers was replaced with MEM containing 5 $\mu\text{g}/\text{ml}$ of AMD. The cultures were incubated at 37°C for 1 h. After 45 min of incubation, 1 $\mu\text{g}/\text{ml}$ of ethidium bromide was added to all cultures, which were then exposed to [^3H]uridine or, alternatively, to [^3H]adenosine (see legends to Figures and Tables). In some experiments, [^{14}C]uridine incorporation was carried out in the presence of 10^{-8} M cytidine. 1 $\mu\text{g}/\text{ml}$ ethidium bromide was always used in the experiments when AMD was used.

One set of cultures was exposed to radioactive uridine in the presence of AMD; other sets were exposed to the labeled RNA precursor at different time intervals after the growth medium containing AMD had been replaced by the AMD-free medium. At the end of the incorporation period, monolayers were quickly chilled by adding frozen, crushed MEM. The cells were scraped from the bottles with a rubber policeman and collected by centrifugation at 800 *g* for 5 min at $2-4^{\circ}\text{C}$; the cellular pellet was suspended in RSB buffer (10 mM Tris-HCl, 10 mM NaCl, 1.5 mM MgCl_2 , pH 7.4) containing either 0.5% of Nonidet P-40 or 0.5% of Triton X-100 at 4°C . The suspension was vigorously pipetted and then centrifuged at 800 *g* for 2 min at 4°C . The nuclear pellet was suspended in a high ionic strength buffer (0.5 M NaCl, 0.05 M MgCl_2 , 0.01 M Tris-HCl, pH 7.4) and digested with

100 $\mu\text{g}/\text{ml}$ of RNase-free DNase at room temperature. The digestion was stopped by adding sodium dodecylsulfate and ethylenediaminetetraacetate (EDTA) to make 0.5 and 0.01 M concentrations respectively. RNA was extracted from the nuclear fraction by the phenol-chloroform-isoamyl alcohol method (24). The supernate obtained after pelleting nuclei at 800 g was treated with sodium dodecylsulfate, EDTA, and NaCl to make final concentrations of 0.5%, 0.01 M and 0.1 M, respectively. From this solution, representing the cytoplasmic fraction, RNA was extracted at 60°C by the phenol method (19). The nucleoplasmic fraction was obtained from the whole nuclei by pelleting the nucleoli according to Penman (12); sodium dodecylsulfate and EDTA were added to give final concentrations of 0.5% and 0.01 M, respectively. RNA was extracted from this material as indicated for the whole nuclei.

Sucrose Density Gradient Fractionation of RNAs

1 ml of RNA extract, obtained separately from each major cell compartment, was layered on top of a 15–30% linear sucrose gradient prepared in the SW 25.1 rotor of the Spinco ultracentrifuge (Beckman Instruments Inc., Spinco Div., Palo Alto, Calif.); sucrose solutions were prepared in RSB containing 0.1% of sodium dodecylsulfate (SDS). A 1-ml cushion made up of 50% sucrose was put at the bottom of the centrifuge tube; centrifugation was carried out at 5°C and 25,000 rpm for 9 h (nuclear or nucleoplasmic RNA fractions) and 15 h (cytoplasmic RNA fractions). The absorbance of the gradient fractions was monitored at 254 nm with the ISCO Model D fractionator and UV analyzer. The radioactivity of single fractions was measured after precipitation with trichloroacetic acid (TCA).

Polyribosomal Profile in Sucrose Density Gradient

Cytoplasmic extract of AMD-pretreated 37 RC cells in RSB buffer obtained by Dounce homogenization was treated with DOC (0.5% final concentration), layered on top of a linear sucrose gradient 15–30% and centrifuged at 4°C and 25,000 rpm in the SW 25.1 rotor for 90 min. The UV optical absorbance and the radioactivity of the fractions were determined as indicated above.

Measurement of Radioactivity Incorporated into RNA

To determine the labeled RNA precursors incorporated into the total cell material, monolayers were dissolved in Tris-papain solution (0.01 M NaCl, 0.01 M Tris-HCl, 0.0015 M MgCl_2 , 0.01 EDTA, 0.5% SDS, 20 $\mu\text{g}/\text{ml}$ papain, 20 $\mu\text{g}/\text{ml}$ cystein, pH 7.0). The aliquots of the total cell material dissolved with SDS-papain solution were treated with TCA to give a final concentration of 10%. The precipitates were collected on

0.45 μm Millipore filters which were then washed with 5% TCA and 0.5% SDS. The filters were dissolved in Bray's solution (6). The radioactivity was measured in a Beckman LS 133 liquid scintillation counter.

Double-Labeling Procedure

For double labeling using ^3H and ^{14}C , less than 2% of ^3H -counts appeared in the ^{14}C -channel; error due to overlap, then, was negligible. Appropriate corrections were made for 25% of ^{14}C -counts which appeared in the ^3H -channel (1).

Determination of Poly-A Content in Nuclear RNA

The poly-A assay was carried out by the poly-U filter method (23). Samples of nuclear RNA, purified as described above, labeled with [^3H]adenosine and containing cold poly-A in a quantity of 0.25 mg/ml, were digested with DNase (5 $\mu\text{g}/\text{ml}$), pancreatic RNase (1 $\mu\text{g}/\text{ml}$) and T_1 RNase (2.5 $\mu\text{g}/\text{ml}$) at 37°C in a high salt concentration buffer (0.01 M Tris-HCl, 0.22 M NaCl, pH 7.3). The minimal length of the enzymatic treatment was calculated on the basis of the solubilization of ^{14}C -counts in some RNA preparations doubly labeled with [^{14}C]uridine and [^3H]adenosine. The period sufficient for enzymatic treatment was found to be 10 min. Enzymatic treatment was stopped by diluting the samples with 5 vol of ice-cold binding buffer (0.01 M Tris-HCl, 0.12 M NaCl, pH 7.5). After poly-U filter hybridization, the filters were vacuum dried and put into scintillation vials containing 0.2 ml of Triton X-100; after 5 min at 60°C, 10 ml of Bray's solution were added.

Determination of Quantity of Radioactivity Incorporated into Polyribosomal Fraction.

To determine which fraction of the RNA synthesized during the AMD elution phase is bound to polyribosomes, the cells were separated into nuclear and cytoplasmic fractions by Dounce homogenizing and by centrifuging the cell suspension in RSB buffer. The cytoplasmic extract was centrifuged at 105,000 g for 24 h in a discontinuous sucrose gradient (5). The pellet containing ribosomes and polyribosomes was treated with puromycin according to Blobel and Sabatini (5), at a maintained KCl concentration of 0.5 M. After puromycin treatment, the solution was centrifuged at 105,000 g for 2 h and the resulting pellet lysed in SDS buffer (0.01 M Tris-HCl, 0.01 NaCl, 0.05 EDTA, 1% SDS, pH 7.0). TCA was added to the aliquots taken separately from the lysed pellets and the supernate as usual. The radioactivity was measured as described above.

Polyadenylated RNAs Extracted from Polyribosomal Fraction

We applied the poly-U filter method (23) to determine what part is polyadenylated of the RNA extracted from

the ribosomal-polyribosomal fraction obtained by centrifugation of the cytoplasmic extract (4). [³H]adenosine-labeled rec-RNAs were hybridized with poly-U filters without any previous treatment. After poly-U filter hybridization, the radioactivity was measured by the same procedures used for the nuclear rec-RNA samples.

The RNA of 37 RC cells that was initially inhibited by AMD administration and resynthesized during the AMD elution phase will be referred to in the text as rec-RNA (recovered RNA); rec-RNA does not include RNA synthesized in the presence of AMD, which is referred to as AMD-resistant RNA.

RESULTS

Time-Dependent Distribution of rec-RNAs from Nuclear, Nucleoplasmic, and Cytoplasmic Fractions in Sucrose Density Gradients

In previous experiments we found that the capacity to reinitiate and synthesize over-all rec-RNA appears immediately after AMD removal and increases linearly over a period of at least 90 min. The linear accumulation of labeled RNA during the AMD elution phase reflects real RNA synthesis because the nucleotide triphosphate precursor pool measured in parallel during the same period remains unchanged (1). During the early period of the AMD elution phase, the metabolic stability of rec-RNA undergoes significant time-dependent changes: during the first 30 min, rec-RNA is metabolically stable, the turnover rate being similar to that of RNA of control untreated 37 RC cells after only 80 min. The incorporation of [³H]uridine into nuclei at 30 min after AMD removal shows a doubling of values compared with cells labeled immediately after AMD removal. The rate of transport of this RNA to the cytoplasm is extensive (Benedetto and Djaczenko, submitted for publication).

In order to better characterize this early rec-RNA in nuclear and cytoplasmic fractions, we studied its distribution in the sucrose density gradient at different time intervals after AMD removal. In control 37 RC cells, the radioactivity distribution profile of nuclear RNAs shows a broad band with sedimentation coefficient values up to 45 S and more (Fig. 1 *a*). The radioactivity distribution profile of nuclear rec-RNA synthesized in the 30 min immediately after AMD removal shows that a major fraction of the radioactivity has accumulated in a single discrete peak corresponding to low sedimentation coefficient values of 4–14 S (Fig. 1 *b*). The polydispersed

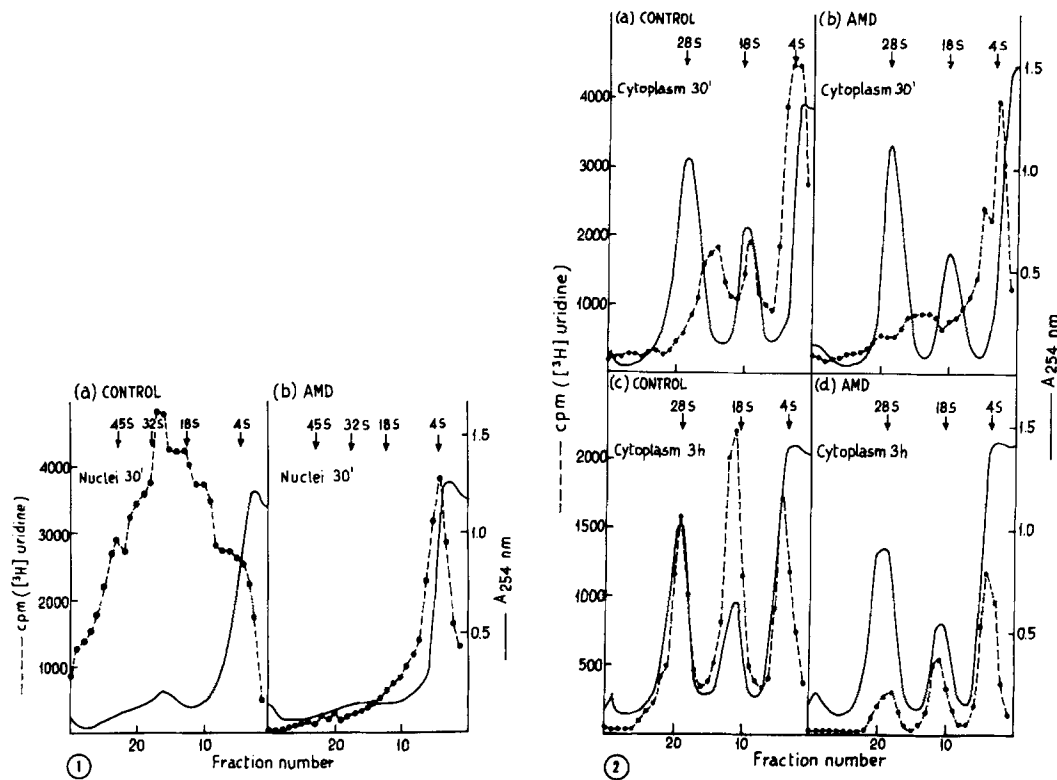
distribution of the radioactive material may be observed in decreasing concentrations over the range of heavier sedimentation fractions. Similar results were obtained with rec-RNAs isolated from the total nuclear extracts or from nucleoplasmic extracts. The radioactivity distribution profile of rec-RNA from various cell fractions depends on the time lapse after AMD removal (Figs. 2 and 3). Fig. 3 shows that, as the over-all rec-RNA synthesis proceeds (insert), the sedimentation coefficients of the nucleoplasmic rec-RNA also increase, although the light components (4–14 S) of the nucleoplasmic rec-RNA prevail.

The time dependence of the cytoplasmic radioactivity distribution pattern of AMD-pretreated 37 RC cells may be inferred from Fig. 2 *b, d*. The radioactivity distribution patterns in control 37 RC cells at 30 min and 180 min after [³H] uridine incorporation are seen in Fig. 2 *a, c*. After 30 min of pulse labeling (Fig. 2 *a*), RNA in control cells is distributed in a large discrete peak banding at the top of the sucrose density gradient, in a smaller peak that coincides with the 18 S band, and in a broad band localized between the 18 S and 28 S regions. In long-labeling experiments, the radioactivity profiles of control cells (Fig. 2 *c*) follow precisely the optical density profiles, with three discrete peaks: 4–14 S (the top component), 18 S, and 28 S.

In AMD-pretreated cells pulse-labeled for 30 min immediately after AMD removal (Fig. 2 *b*), most of the radioactivity accumulates in the top component, with a small amount of the remaining radioactivity being distributed in heavier fractions without discrete peaks. 180-min labeling of AMD-pretreated cells (Fig. 2 *d*) leads to a radioactivity distribution similar in pattern to that of control 37 RC cells, but with a quantitatively lower representation of material from each peak.

Puromycin Sensitivity of rec-RNA Bound to the Ribosomal-Polyribosomal Fraction in AMD-Pretreated Cells

The puromycin sensitivity of the incorporation of rec-RNA into ribosomes and polyribosomes was tested in order to distinguish between r-RNA and non-r-RNA in the rec-RNA class. The data reported in Table I show that in control 37 RC cells, after 60 min of labeling, 25–30% of the total cytoplasmic radioactivity is found in the ribosomal polyribosomal fraction, with half of this amount being dissociable by puromycin. Labeling the cells in the presence of AMD also leads to a 25%



FIGURES 1 and 2 Sucrose density gradient profiles of RNAs extracted from nuclei and cytoplasm of AMD-pretreated cells. 37 RC monolayers grown in Roux bottles (2×10^7 cells per bottle) were divided into four sets of cultures. Two sets were incubated for 1 h with AMD. Immediately after removal of AMD, the cultures were exposed to $4 \mu\text{Ci/ml}$ of $[^3\text{H}]$ uridine, one set for 30 min and the other for 3 h. Two other sets of cultures, exposed to $4 \mu\text{Ci/ml}$ of $[^3\text{H}]$ uridine for 30 min and 3 h, respectively, without any treatment with AMD, were used as controls. The incorporation was stopped by the addition of frozen crushed MEM. The cells were scraped off the bottles with a rubber policeman, and the nuclear and cytoplasmic fractions were separated by the Nonidet P-40 method in all sets of cultures. RNA extracted from each fraction separately was layered on the top of the linear sucrose gradient and centrifuged at 10°C and 25,000 rpm for 9 h (nuclear RNA fraction) and 15 h (cytoplasmic RNA fraction). After monitoring the absorbance at 254 nm, each fraction was collected, TCA precipitated and deposited on Millipore filters for radioactivity measurement. Fig. 1 *a* shows the profiles of RNA extracted from control nuclei and Fig. 2 *a* and *c* show the profiles from control cytoplasm. Fig. 1 *b* shows the profiles of RNA from nuclei of AMD-pretreated cells and Fig. 2 *b* and *d* show profiles from cytoplasm of AMD-pretreated cells.

accumulation of radioactivity in the ribosomal-polyribosomal fraction, but nearly all of this radioactivity disappears after puromycin treatment. The quantity of rec-RNA in the total cytoplasmic fraction and in the ribosomal-polyribosomal fraction increases, but the radioactivity bound to the ribosomal pellet after puromycin treatment shows low values during the early AMD elution phase, increasing only at later periods. The results indicate that a large fraction of the rec-RNA bound to the ribosomal-polyribosomal fraction dissociable by puromycin

may be composed of m-RNA and t-RNA during the early period after AMD removal.

Polyadenylation of the Nuclear rec-RNA

In the preceding paragraph, we noted that only a part of the radioactivity of the ribosomal-polyribosomal fraction remains firmly bound to the ribosomes after puromycin treatment. The radioactivity liberated from the ribosomal-polyribosomal fraction by puromycin may be attributed to the t-RNA species. Since the polyadenylation process is a vital step in the transfor-

mation of m-RNA precursor into mature m-RNA molecules (7, 11, 20), we determined the quantity of [³H]polyadenosine radioactivity associated with nuclear rec-RNA in order to be able to compute the probable fraction of nuclear rec-RNA which

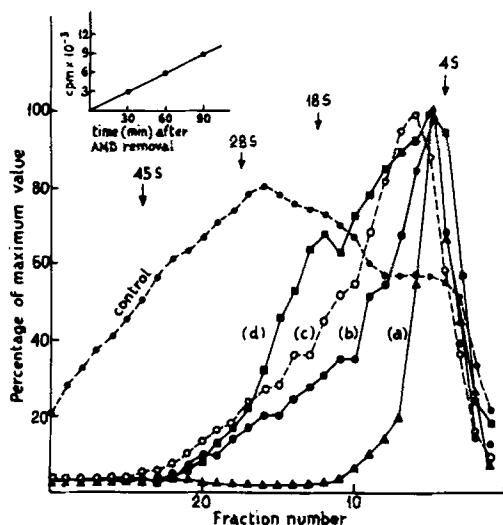


FIGURE 3 Kinetics of nucleoplasmic rec-RNA modifications during the AMD elution phase. 37 RC monolayers grown in Roux bottles were incubated for 1 h with AMD, then washed and refed with AMD-free MEM. Different sets of cultures, 2×10^7 cells each, were pulse-labeled for 15 min with $5 \mu\text{Ci/ml}$ of [³H]uridine at 0 min (▲—▲) (a), 30 min (●—●) (b), 90 min (○—○) (c) and 180 min (■—■) (d) after AMD removal. Untreated 37 RC monolayers were used as controls (●—●). The incorporation was stopped by the addition of frozen crushed MEM. The cells scraped off the bottles with a rubber policeman were lysed in RSB Nonidet P-40 solution. From the nuclear pellet lysed in high ionic strength buffer, the nucleoli and nucleoplasm were obtained by the method of Penman (12). RNA extracted from the nucleoplasmic fraction by the chloroformisoamyl alcohol method (24) was centrifuged on a linear 15–30% sucrose gradient. Each fraction was monitored on an ISCO analyzer and collected for radioactivity measurement. Fig. 3 shows the modification of the profiles of RNAs produced from 0 to 180 min after AMD removal. In the ordinate the radioactivity of each profile is reported as ratios

$$\frac{\text{cpm of single fractions}}{\text{cpm of the most radioactive fraction}} \times 100.$$

Insert to Fig. 3 shows the continuous incorporation of [³H]uridine into the total cell material after 1 h of AMD treatment. The conditions of this experiment were analogous to those of the main experiment. For technical details, see Materials and Methods.

could be defined as a precursor of m-RNA. The results of the polyadenylation experiments are reported in Table II. In 37 RC cells continuously treated with AMD, about 1.4% of the labeled adenosine in rec-RNA is associated with RNase-resistant poly-A segments. The table also shows that, during the recovery phase, the fraction of radioactivity in poly-A remains constant, indicating that the proportion of polyadenylated RNA in nuclear rec-RNA is also constant. This means that the amount of polyadenylated nuclear rec-RNA increases parallel to the increase in the amount of over-all rec-RNA.

Polyadenylation of Polyribosomal rec-RNA

In the preceding section, we have shown that some of the rec-RNA bound to polyribosomes is puromycin sensitive. Now we will extend our observations to the EDTA sensitivity of this rec-RNA bound to polyribosomes, and to its content of poly-A. In experiments illustrated in Fig. 4 a the polyribosomal profiles of AMD-pretreated 37 RC cells, similar in form to that of control untreated 37 RC cells, is followed by the radioactivity profile. This radioactivity profile differs quantitatively from the optical density profile in so far as it exceeds the latter in the region of low sedimentation coefficients. After EDTA treatment of the cytoplasmic extract of AMD-pretreated cells, the whole optical density of the polyribosomal region of the gradient disappears and accumulates at the bottom of the gradient (Fig. 4 b). The high peak corresponding to the position of the larger ribosomal subunit is visible. EDTA treatment of the cytoplasmic extract of AMD-pretreated cells also causes the disappearance of the radioactivity profile from the polyribosomal region. All of the radioactivity accumulates at the top of the gradient. Phenol-extracted rec-RNA from the polyribosomal pellet is distributed in a sucrose density gradient as a large top component, and a small part of this rec-RNA is distributed heterogeneously just below the top component (Fig. 4 c). This rec-RNA probably contains t-RNA and m-RNA. To determine if rec-RNA dissociated from polyribosomes by puromycin and EDTA contains poly-A segments, we applied the poly-U filter method (23). The data obtained from poly-U filter experiments are shown in Table III. In control cells, after a 1-h pulse with [³H]adenosine, about 23% of the polyribosomal RNA is polyadenylated. In AMD-pretreated 37 RC cells, labeled under the

TABLE I
Incorporation of [³H]Uridine into Cytoplasmic Fractions under Various Conditions of AMD Treatment

Conditions of labeling and AMD treatment		Total	Ribosomal-polyribosomal fraction	Released from ribosomal-polyribosomal fraction after puromycin	Ribosomal pellet after puromycin
		<i>cpm</i> × 10 ⁻²	<i>cpm</i> × 10 ⁻²	<i>cpm</i> × 10 ⁻²	<i>cpm</i> × 10 ⁻²
Labeling of untreated cells	A	1,100	310	260	40
	B	3,800	1,050	380	428
Labeling in the presence of AMD	A	200	55	50	0
	B	390	100	90	2.5
Labeling immediately after AMD removal	A	430	120	110	0.5
	B	670	210	200	2.0
Labeling 30 min after AMD removal	A	600	170	160	2.0
	B	1,450	360	250	12
Labeling 60 min after AMD removal	A	1,400	260	200	5
	B	2,350	340	300	25
Labeling 180 min after AMD removal	A	1,650	300	270	10
	B	3,800	500	310	80

A = 30 min of incorporation of [³H]uridine.

B = 60 min of incorporation of [³H]uridine.

37 RC monolayers were divided into several sets of cultures; each set contained 4 × 10⁷ cells. The first set was incubated with 5 μg/ml of AMD for 1 h, washed, refed with AMD-containing MEM and immediately exposed to 5 μCi/ml of [³H]uridine for 30–60 min. Four sets of cultures were incubated for 1 h with AMD, washed, refed with AMD-free growth medium, then exposed to 5 μCi/ml of [³H]uridine for 30–60 min (without further treatment with AMD) immediately, 30 min, 60 min, and 180 min, respectively, after AMD removal. Another set of cultures was exposed to 5 μCi/ml of [³H]uridine for 30–60 min without treatment with AMD. The incorporation was stopped by the addition of frozen crushed MEM. The cells were scraped from the bottles with a rubber policeman and collected by centrifugation at 800 g for 5 min at 2°–4°C. From the cellular pellet resuspended in cold RSB, the nuclei and cytoplasm were isolated with 10 strokes of a fitting ball stainless Dounce homogenizer. The nuclear pellet and cytoplasmic supernate were obtained after centrifugation at 16,000 g. The radioactivity incorporated into the ribosomal-polysomal fraction isolated from the cytoplasm according to Blobel and Sabatini (5) was measured. For technical details, see Materials and Methods.

same conditions as control cells, at 30 min after AMD removal a larger part (48%) of the polyribosomal rec-RNA is bound to poly-U filters than in control, untreated cells. The dissociability of rec-RNA from polyribosomes by EDTA and puromycin action, its polyadenylation, and its heterogeneity as far as size is concerned, all support the assumption that early rec-RNA contains m-RNA.

Methylation Kinetics of rec-RNA

The position in the sucrose density gradient of AMD-resistant RNA (not shown) and early rec-RNA in the cytoplasm of 37 RC cells (Fig. 2 b) suggests that both these RNAs contain t-RNA. To

check this possibility, methyl labels in AMD-resistant RNA and in early rec-RNA of 37 RC cells were analyzed. The methylation pattern of AMD-resistant RNA and rec-RNA is reported in Fig. 3. Quantitative data concerning the methylation are presented in Table IV. In control 37 RC cells, about 60% of the methyl label in the over-all RNA synthesized during a 30-min pulse accumulates in a 4 S peak. The remaining methyl label is distributed predominantly in the 18 S and 28 S regions; the smallest, but nevertheless significant, amounts of ³H-methyl radioactivity are present in fractions heavier than 28 S.

37 RC cells labeled either in the presence of AMD or after removal of AMD contain methyl

radioactivity in a single discrete peak corresponding to a sedimentation coefficient of 4 S. The quantity of methyl radioactivity in the light component is nearly the same for 37 RC cells labeled in the presence of AMD or after removal of the drug. This value is about 10% of that found in control, untreated 37 RC cells. The quantity of [¹⁴C]uridine incorporated into RNAs relative to the quantity of ³H-methyl incorporated was also analyzed. These values for control 37 RC cells, continuously treated with AMD and labeled after AMD removal, together with data on [¹⁴C]uridine incorporation, are reported in Table IV. The data are distributed according to three zones of the

density gradient. All of the radioactive uridine of 37 RC cells continuously treated with AMD is concentrated in the light component (up to 10 S) of the gradient. In 37 RC cells pretreated for 1 h with AMD, approximately double the quantity of [¹⁴C]uridine, as compared with continuously treated cells, is found in the light fraction, and a significant amount of radioactivity is present in the heavier zone of the gradient. In control 37 RC cells, the uridine label is fairly heterogeneously distributed over the whole density gradient, with predominant values in the light zone. Methylation experiments show that the accumulation of radioactivity observed in the light band of the sucrose

TABLE II
Poly-A Content in Nuclear RNA Synthesized under Various Conditions of AMD Treatment

Type of RNA	Input	Radioactivity on the filters		Percent of input retained on the poly-U filter
		Filter with poly-U	Filter without poly-U	
	<i>cpm</i>	<i>cpm</i>	<i>cpm</i>	
[³ H]poly-A standard solution	14,700	12,500	4	89.3
	7,000	6,100	0	87.1
	3,500	3,300	0	94.3
Extracted from control, untreated cells	497,200	8,000	50	1.6
Extracted from AMD-pretreated cells and labeled with [³ H]adenosine in the presence of AMD	56,000	780	2	1.4
Extracted from AMD-pretreated cells and labeled with [³ H]adenosine immediately after AMD removal	100,000	1,200	6	1.2
Extracted from AMD-pretreated cells and labeled with [³ H]adenosine 30 min after AMD removal	160,000	1,480	10	0.9
Extracted from AMD-pretreated cells and labeled with [³ H]adenosine 60 min after AMD removal	210,000	2,400	6	1.1

37 RC monolayers grown in Roux bottles were divided into five sets of cultures, each 2×10^7 cells. One set was incubated for 1 h with 5 μ g/ml of AMD, then washed and refed with AMD containing MEM; three sets were incubated for 1 h with AMD, then washed and refed with AMD-free MEM. All sets of cultures were exposed to 10 μ Ci/ml of [³H]adenosine for 15 min in the presence of 50 μ g/ml of hydroxyurea to block DNA synthesis, in the following way: one set soon after washing and refeeding in the presence of AMD, one set immediately, one set 30 min, and another set 60 min after AMD removal. A control set of cultures was exposed to the same quantity of [³H]adenosine for the same length of time as the experimental sets, but without treatment with AMD. The incorporation was stopped by the addition of frozen crushed MEM. The cells were fractionated into nuclear and cytoplasmic fractions. RNA was extracted from the nuclear fraction and dissolved in high salt concentration buffer (0.01 M Tris-HCl; 0.22 M NaCl; pH 7.3). Aliquots of the resulting solution were treated in the presence of unlabeled poly-A solution with DNase, pancreatic and T₁RNase. Digested samples, after heating to 65°C, were hybridized to poly-U filters which, after treatment with Triton X-100, were dissolved in Bray's solution. For technical details, see Materials and Methods.

A sample of [³H]polyadenosine, originally furnished by Miles, was diluted in a high salt concentration buffer to make standard solutions corresponding to 14,000, 7,000, and 3,500 cpm. The standard solutions were passed through a poly-U filter to check the efficiency of the method.

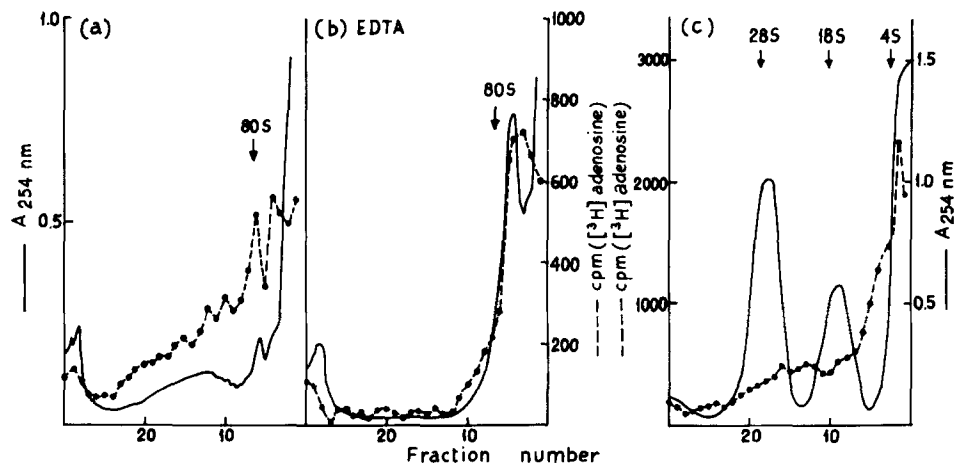


FIGURE 4 EDTA sensitivity and sedimentation coefficients of rec-RNA bound to the polyribosomal fraction. 37 RC monolayers in Roux bottles (4.5×10^7 cells) were treated with prewarmed MEM containing $5 \mu\text{g/ml}$ of AMD for 1 h at 37°C , then washed with cold AMD-free MEM. AMD-treated cells were labeled for 80 min with $5 \mu\text{g/ml}$ of $[^3\text{H}]$ adenosine in the presence of ethidium bromide. The incorporation was stopped by adding frozen crushed MEM to the cultures. The cells were scraped from the bottles with a rubber policeman. Nuclear and cytoplasmic fractions were obtained by Dounce homogenization. 0.1 of the volume of the cytoplasmic extract was treated with DOC up to 0.5% and overlaid directly on a 15–30% linear sucrose gradient in a centrifuge tube (a). Another 0.1 of the volume of the cytoplasmic DOC-treated extract was overlaid on a similar sucrose gradient after EDTA was added to the extract to a final concentration of 0.01 M (b). Both samples were centrifuged at 25,000 rpm for 90 min, and the gradients analysed in the ISCO fractionator. The radioactivity of each 1-ml fraction was determined separately on dry Millipore filters after TCA precipitation. The ribosomal-polyribosomal pellet obtained from the remaining 0.8 of the cytoplasmic extract by centrifugation in discontinuous gradient (4) was lysed in SDS buffer. From this solution, RNA was extracted by the phenol method (19). After alcohol precipitation, one part of this RNA was studied for sedimentation in a 15–30% sucrose density gradient (Fig. 4 c). The remaining part of the polyribosomal rec-RNA was used in poly-U filter hybridization studies; the results are presented in Table III.

TABLE III
Polyadenylation of Polyribosomal RNA

Cell cultures	Input	Radioactivity on the filters		Percentage of poly-A RNA
		Filter with poly-U	Filter without poly-U	
	$dpm \times 10^{-3}$	$dpm \times 10^{-3}$	$dpm \times 10^{-3}$	
Control, untreated cells	54.4	12.5	0.62	23
AMD-treated cells	16.7	8.0	0.58	48

For experimental data, see legends to Fig. 4. Control values refer to similarly labeled untreated cultures.

gradient during the early AMD elution phase is caused predominantly by a synthesis of nonmethylated RNA and only in part by an increase in the amount of t-RNA. The ratio of $[^{14}\text{C}]$ uridine/ ^3H -methyl incorporated into components of the light zone of the density gradient is not significantly modified when $[^{14}\text{C}]$ uridine labeling of AMD-pretreated cells is carried out in the pres-

ence of 10^{-8} M cytidine to prevent ^{14}C -counts from being incorporated into the CCA-3' terminal end of t-RNA (9).

DISCUSSION

Differential AMD sensitivities of major RNA species have always been studied by determining

TABLE IV
Methylation of RNA Synthesized under Different Conditions of AMD Treatment

Conditions of AMD treatment	Zones of the gradient	Methyl groups incorporated into RNA	Uridine incorporated into RNA
		dpm	dpm
Control, untreated cells	a	73,270	139,475
	b	38,293	104,296
	c	9,490	73,368
Cells labeled in the presence of AMD	a	7,916	4,944
	b	149	159
	c	201	110
Cells labeled after AMD removal	a	9,166	11,633
	b	590	4,285
	c	393	2,837

Experiments with incorporation of ^3H -methyl groups into rec-RNAs are described in the legend to Fig. 5. Dpm of [^{14}C]uridine and ^3H -methyl groups incorporated into rec-RNAs are reported here in reference to three main zones (a, b, c) of the density gradient marked with bars in Fig. 5.

the AMD concentration capable of inhibiting the synthesis of single RNA species (13, 14). In this study, for the first time, AMD sensitivities of RNA species are determined by measuring the times and kinetics of the recovery of these species after maximal AMD inhibition.

rec-RNA, initially composed of almost exclusively light (4-14 S) RNA species, tends to normalize as early as 3 h after AMD removal. At this time the sucrose density gradient profile shows the presence of all peaks of RNA typical for control cells, although the quantity of RNA in heavier fractions is less than in control cells. An analysis of sedimentation coefficients of rec-RNA species in 37 RC cells proves that the kinetics of recovery of RNA synthesis from AMD in these cells show a size dependency comparable to the size-related sensitivity to AMD inhibition found by Bleyman and Woese (4).

The synthesis of m-RNA begins immediately after AMD removal. Proof of the presence of m-RNA in early rec-RNA is furnished by the results of the experiments on puromycin- and EDTA-sensitive rec-RNA bound to polyribosomes and by the polyadenylation of this RNA.

Our data indicate that the synthesis of t-RNA proceeds during the phase of AMD inhibition and

only slightly increases during the AMD elution phase. The presence of t-RNA in AMD-resistant RNA is in agreement with reports concerning other cell systems (9).

t-RNA synthesis in 37 RC cells during the

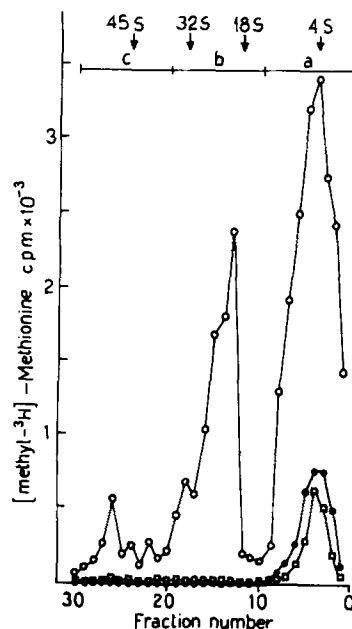


FIGURE 5 Incorporation of ^3H -methyl groups into rec-RNA. 37 RC monolayers were divided into three sets of cultures (24×10^6 cells per set). Two sets were incubated for 1 h with $5 \mu\text{g}/\text{ml}$ of AMD; one set was then washed and refed with AMD-containing MEM (\square — \square); the second set was washed and refed with AMD-free MEM (\bullet — \bullet). These two sets were immediately exposed to $120 \mu\text{Ci}/\text{ml}$ of [^3H]methionine for 10 min at 37°C , then [^{14}C]uridine was added up to $5 \mu\text{Ci}/\text{ml}$. The incorporation was stopped after 20 min of incubation by the addition of frozen crushed MEM. Cytoplasmic and nuclear fractions were separated; the nuclear pellet was dissolved in high ionic strength buffer and digested by RNase-free DNase ($100 \mu\text{g}/\text{ml}$) for 5 min at room temperature, with vigorous pipetting. Digestion was stopped by the addition of SDS-EDTA solution. RNA was extracted from the combined nuclear and cytoplasmic fraction by chloroform-isoamyl alcohol method. After ethanol precipitation, RNA samples were dissolved in SDS buffer and centrifuged on a 15–30% sucrose density gradient for 9 h at 25,000 rpm. The fractions were collected, monitored with the ISCO analyzer and prepared for the radioactivity determination as usual. A third set of 37 RC monolayers (\circ — \circ) exposed to $120 \mu\text{Ci}/\text{ml}$ of [^3H]methionine for 10 min at 37°C and then to $5 \mu\text{Ci}/\text{ml}$ of [^{14}C]uridine for 20 min without any treatment, was processed further as AMD-treated sets of monolayers and used as controls.

AMD elution phase is indicated by experiments with the incorporation of [³H]uridine into the ribosomes, and by the sucrose density-gradient distribution of cytoplasmic rec-RNA. The data show that there is a lag period of at least 2 h between AMD removal and the appearance of clearly defined r-RNA in the rec-RNA.

The kinetics of the recovery of single RNA species after maximal inhibition by AMD have not been reported previously. Therefore, it is necessary to compare the results of the present study with the differential responses to AMD of single RNA species found in other cell systems. Our data show that the AMD sensitivities of the major RNA species in 37 RC cells correspond well to those of other cell systems. t-RNA, known to be highly resistant to AMD inhibition (9), continues to be produced in 37 RC cells even after the administration of high doses of AMD.

In many laboratories it has been shown that m-RNA is relatively AMD resistant (8, 10, 16). Our data are compatible with these results in that, almost immediately after AMD removal, a large part of cytoplasmic rec-RNA belongs to the m-RNA class.

HnRNA is known to consist of several fractions, each of which responds differently to AMD action (13). The fraction of HnRNA which first reappears in the nuclear sap of 37 RC cells is situated in the zone of low sedimentation coefficient, confirming the findings of Perry and Kelley (14) that light N-RNA (with a mol wt of 1.3×10^6) is more AMD resistant than heavy N-RNA (7.0×10^6). The fraction of HnRNA that is the last to appear in the spectrum of rec-RNA of 37 RC cells (Fig. 3) clearly corresponds to the heavy N-RNA fraction of Perry and Kelley (14).

Our experiments did not show which particular biological properties of 37 RC cells are responsible for the rapid recovery of RNA synthesis after AMD inhibition. When we compared 37 RC cells with HeLa cells (a typical cell line that is irreversibly inhibited by AMD), we saw that even weakly inhibited HeLa cells do not recover their RNA synthesis, whereas 37 RC cells recover their RNA synthesis even after strong inhibition by AMD (3). Differences in RNA recovery capacities between these two lines cannot be reduced to differences in the quantitative parameters of rates of diffusion of AMD into the cells. In the 1-h period used in the present study, these rates in 37 RC cells are similar to those of HeLa cells. At 1 h after AMD treatment, however, both the quantity of AMD

bound to nuclear chromatin and the AMD efflux rates differ greatly between 37 RC and HeLa cells. The amount of AMD per length of DNA (expressed as a ratio of nucleotides/AMD) constitutes in 37 RC cells about one sixth of the amount found for HeLa cells. The AMD efflux rates in 37 RC cells are about three times more accelerated than in HeLa cells. These and additional data, such as the lower intracellular concentration of AMD at equilibrium in 37 RC cells as compared with that of HeLa cells, indicate that AMD binds less strongly to 37 RC chromatin than to HeLa chromatin (2). The differences in AMD uptake and binding to chromatin *in vivo* disappear when the AMD binding to DNA extracted from 37 RC and HeLa cells is measured. Therefore, it is reasonable to assume that there are unique properties in 37 RC chromatin that might be responsible for the rapid recovery of RNA synthesis after AMD inhibition. Probably, certain segments of 37 RC chromatin are available each time to AMD molecules, and there could even be an overpopulation of AMD molecules in some segments of chromatin, leading to local conformational changes (22) that would cause the imbalance of early rec-RNA synthesis and turnover (unpublished data from this laboratory).

Williams and Macpherson (25) found that polyoma-transformed BHK cells are more AMD resistant than their normal counterparts. The authors contemplate two hypotheses to account for this phenomenon. In one hypothesis they assume that polyoma-transformed BHK cells have a different mode of association of DNA with chromosomal protein. The second hypothesis implicates the plasma membrane as the structure responsible for the AMD resistance of transformed cells. Williams and Macpherson (26) rule out the first hypothesis on the basis of experiments with isolated nuclei from normal and transformed BHK cells. In isolated nuclei the differences in AMD-binding capacities of the two cell lines disappeared. The authors (26) conclude that the plasma membrane is responsible for the different AMD responsiveness of the two cell lines. Our data strongly suggest that the properties of the chromatin of 37 RC cells are mainly responsible for their particular AMD resistance. Objections based on apparently conflicting results from experiments using isolated nuclei (26) must be waived, as isolated nuclei can lose some of their important functions (such as, for example, the active dissociation of AMD molecules from binding sites). Polyoma-transformed

BHK cells recover the synthesis of all classes of their RNA simultaneously (26), and differences in the AMD resistance of these cells, as compared with other cells, could be a result of their particular properties. Our results do not indicate, either directly or indirectly, that the plasma membrane of 37 RC cells participates in the phenomenon of rapid recovery of RNA synthesis after AMD inhibition. The effect of AMD on RNA synthesis cannot be fully explained by the simple interaction of AMD molecules with guanine and cytosine bases, and particular properties of larger biological units, such as chromatin, must be taken into consideration. Moreover, the results of some experiments carried out in this laboratory (unpublished data) suggest that cellular compartments other than nuclear ones contribute largely to resulting AMD effects in 37 RC cells.

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