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Characterization and Poly(adenylic acid) Content of Ehrlich Ascites Cell Ribonucleic Acids Fractionated on Unmodified Cellulose Columns†

Nelda Sullivan and W. K. Roberts*

ABSTRACT: Conditions are described whereby synthetic poly(A) as well as denatured DNA, heterogeneous nuclear RNA (hnRNA), and heterogeneous cytoplasmic RNA (hcRNA) from Ehrlich ascites cells can be selectively adsorbed to unmodified cellulose columns. In this manner, these species can be isolated essentially free from other types of RNA and double-stranded DNA (ds-DNA). Evidence is presented which indicates that the selective binding of these hRNAs to cellulose is the result of a hydrophobic interaction between poly(A) moieties in the RNAs and the polyaromatic lignins present as minor constituents in all plant celluloses. Hybridization of nuclear and cytoplasmic RNAs to [^{3}H]poly(U) before and after cellulose chromatography revealed that over 98% of the poly(A)-containing RNAs bound to the cellulose. The poly(A) moiety was found to represent approximately 4.2% of the nucleotides in bound hcRNA and 2.0% of the bound hnRNA. The specificity of the cellulose to bind poly(A)-containing RNAs was examined by prehybridizing nuclear and cytoplasmic RNAs to excess poly(U), thereby preventing poly(A) sequences from binding. The amount of bound hcRNA was reduced 90% and the bound hnRNA 80% by this procedure, indicating that most of the RNAs which normally bind to the cellulose do so *via* their poly(A) moieties. In addition, mengovirus RNA was found to contain a poly(A) region approximately 50 nucleotides long, compared with regions 150–200 nucleotides long in hRNA; the viral RNA did not adsorb effectively to the cellulose, suggesting that the length of the poly(A) moiety is important in the binding of RNAs to cellulose. The binding ability of hRNAs synthesized in the presence of 0.04 μg/ml of actinomycin D also was examined. Approximately 50% of the hcRNAs and 40% of the hnRNAs did not bind and, therefore, did not contain significant amounts of poly(A). Finally, the poly(A) content of various sedimentation classes of cellulose-bound hRNAs was determined by hybridization to [^{3}H]poly(U). Poly(A) was detected in all sedimentation classes of hnRNA and hcRNA and there was a relative decrease in the proportion of poly(A) in the hRNA molecules with increasing sedimentation rate.

The functions and interrelationships between the heterogeneous RNAs (hRNAs)[1] in animal cells are not well understood. However, progress toward achieving such an understanding has been aided by the recent development of several procedures for the isolation of hRNA (Edmonds and Caramela, 1969; Kates, 1970; Lee *et al.*, 1971; Sullivan and Roberts, 1971; Sheldon *et al.*, 1972; Kitos *et al.*, 1972). These procedures have facilitated investigations concerning synthesis, processing, transport, and utilization of hRNA, as well as providing the means of isolating hRNA for studies on hybridization and *in vitro* protein synthesis.

One of these methods for isolating hRNA involves chromatography on unmodified cellulose columns (Sullivan and Roberts, 1971; Kitos *et al.*, 1972; Schutz *et al.*, 1972). In this report we show that the basis for the RNA fractionation is a selective hydrophobic bonding of hRNA to polyaromatic lignins, minor constituents of the cellulose. Also, we present evidence that over 98% of the cells' poly(A)-containing RNAs bind to the cellulose, that the single-stranded poly(A) moiety is required for binding, and that hRNA which does not contain poly(A) does not adsorb to the cellulose. Finally, the bound and unbound hcRNA and hnRNAs are characterized with respect to their sedimentation profiles, nucleotide compositions and poly(A) contents.

Materials and Methods

The poly(A), poly(I), poly(C), and [^{3}H]poly(U) (78.1 μCi/mmol of P, >50,000 molecular weight) used in these

† From the Department of Microbiology, University of Colorado Medical Center, Denver, Colorado 80220. *Received February 14, 1973.* This investigation was supported by Public Health Service Research Grant No. CA-10641 from the National Cancer Institute.

[1] Abbreviations used are: hRNA, heterogeneous RNA; hnRNA, heterogeneous nuclear RNA; hcRNA, heterogeneous cytoplasmic RNA; rpRNA, ribosomal precursor RNA.

experiments were all obtained from Miles Laboratories. Lignins (Indulin AT) were purchased from Westvaco. Mixed ester filter membranes (HAWPO2500) with a 0.45 μ pore size were purchased from Millipore. Whatman CF_{12}, CF_{11}, and CF_2 celluloses were obtained from Reeve Angel. SS_{389} and SS_{289} cellulose were purchased from Schleicher and Schuell Inc. Sigma cell 38 and α-celluloses were purchased from Sigma Chemical Corp. The pancreatic ribonuclease (five-times-crystallized A grade) came from Calbiochem. Deoxyribonuclease 1 (RNase free) was purchased from Worthington Biochemical Corp. The [^{3}H]uridine-labeled precursor to 5S RNA was from *Bacillus subtilis* and was kindly supplied by Dr. Norman Pace.

Preparation of Radioactive Nuclear and Cytoplasmic RNA. Ehrlich ascites cells were propagated, harvested, washed, and suspended in modified Earle's saline as previously described (Roberts, 1965). The cell suspensions (10 ml of 2% cell suspensions in petri dishes) were labeled with 0.2 ml of $H^{32}PO_4^{2-}$ solution (carrier free, 0.15 M NaCl and containing approximately 1 mCi of $H^{32}PO_4^{2-}$/ml) for 3 hr at 37° (Roberts and Newman, 1966). After labeling, the cells were washed and fractionated into nuclear and cytoplasmic components, and the RNA was isolated by phenol extraction at pH 9.5 and purified as previously described (Roberts *et al.*, 1966).

Preparation of Radioactive Ribosomal RNA (rRNA). Mice carrying 6 day ascites tumors were injected intraperitoneally with 0.8 mCi of carrier free $H^{32}PO_4^{2-}$ neutralized with 7.5% $NaHCO_3$. Forty-eight hours later, the tumors were harvested and the ribosomes prepared as previously described (Roberts and Coleman, 1971). The ribosomes were further purified by zonal centrifugation (Roberts and Coleman, 1972) and the RNA was extracted from the monosomal region as described before (Roberts, 1965). Seven A_{260} units of this RNA was applied to a cellulose column and chromatographed at 25°. The unbound fraction of RNA from this column was used as an hcRNA-free source of rRNA.

Preparation of Ribosomal Precursor RNA (rpRNA). Nuclear RNA was prepared as described above, and 0.5 A_{260} unit was applied to a cellulose column at 25°. The RNA from the unbound fraction was used as a source of rpRNA in further binding experiments.

Preparation of Native and Denatured DNA. The DNA was prepared from a nuclear fraction of cells labeled and harvested as described for rRNA. The extraction procedure was as previously described for nRNA (Roberts *et al.*, 1966) except that the DNA and RNA were dissolved in TSE (0.3 M NaCl–0.1 M Tris-HCl (pH 7.0)–0.001 M EDTA) and reprecipitated twice by the addition of two volumes of ethanol. The DNA and RNA were then redissolved in TSE, pancreatic ribonuclease added to a concentration of 0.25 mg/ml and the solution incubated at 37° for 45 min. The solution was then placed on ice and brought to 0.15 M in sodium acetate (pH 5.3), and the DNA was precipitated with ethanol as described above. After redissolving the DNA in TSE, 12 A_{260} units was chromatographed on a 15 g cellulose column to remove any hnRNA which may have been protected from the ribonuclease. The fractions containing the unbound DNA were combined, and the DNA was precipitated with ethanol. One-half of this DNA was used as a source of native DNA. The other half was redissolved in 3 ml of 0.1 M NaOH and incubated for 18 hr at 37° to hydrolyze any remaining RNA and denature the DNA. The solution was then neutralized with cold 3 M acetic acid and the DNA was precipitated by adding two volumes of ethanol, redissolved in TSE and used as a source of denatured DNA.

Cellulose Column Chromatography. Unless otherwise indicated all cellulose columns were prepared and used as follows. Four grams of α-cellulose were suspended in 100 ml of distilled water and warmed to 45°. The slurry was then poured into a water-jacketed glass column with a fiberglass plug in the bottom (water jacket temperature was 45°). The cellulose was packed under air pressure to form a column 15 × 1.2 cm. The column was then washed with approximately 100 ml of TSE at 45°, or until the absorbance of the effluent at 260 mμ was that of the TSE. Either 10 A_{260} units of cytoplasmic RNA or 2 A_{260} units of nuclear were layered on top of the column in 5 ml of TSE and allowed to run onto the cellulose by gravity. A pressure head of approximately 50 cm was then applied and a total of eight 5-ml fractions eluted with TSE. The 45° water jacket was then removed and seven 5-ml fractions eluted with distilled water (adjusted to pH 7 with NH_4OH) at room temperature.

Determination of [^{32}P]Nucleotide Composition. The ^{32}P-labeled RNA was precipitated with trichloroacetic acid and hydrolyzed with KOH, and the resulting nucleotides were separated by electrophoresis at pH 3.5 and the radioactivity in each spot was counted in a liquid scintillation counter (Roberts 1965).

Assay for Poly(A). Cellulose-bound and unbound fractions of RNA were dialyzed for 16 hr against 500 ml of 0.1 M NaCl–0.05 M Tris-HCl (pH 7.4)–0.001 M EDTA (STE). Samples of these fractions, as well as total cytoplasmic RNA, total nuclear RNA, or poly(A), in STE, were placed in tubes containing 0.2 ml of [^{3}H]poly(U) (1 μCi/ml) and the mixture was diluted to 2 ml with STE. The tubes were heated to 95° for 1 min, allowed to cool to room temperature over a period of 2 hr, and placed at 4° for 16 hr. To each tube was then added 0.01 ml of pancreatic ribonuclease (10 μg/ml) and the solution incubated at 37° for 10 min. After incubation, the tubes were placed on ice, one drop of a mixture of yeast RNA and 2′(3′)-uridylic acid (both 4 mg/ml in STE) was added to each tube, and the polynucleotides were precipitated by the addition of 1 ml of cold 10% trichloroacetic acid. Following incubation at 0° for 10 min, the precipitates were collected by filtration through filter membranes (EGWP 02500), the precipitates washed twice with cold 5% trichloroacetic acid and once with cold water, and the filter membranes were either glued to planchets and counted in a windowless gas-flow counter or placed in vials and counted using the ^{3}H channel of a liquid scintillation counter (the latter method was used if the RNA assayed had been labeled with ^{32}P).

Results

Fractionation of Ascites Cell RNA on Unmodified Cellulose Columns. Cellulose column chromatography of ^{32}P-labeled nuclear or cytoplasmic RNA in TSE resulted in a portion of these RNAs binding to the cellulose which then could be eluted with water. The bound RNAs had relatively high specific activities and contained [^{32}P]nucleotide compositions very different from the unbound fractions (Table I). The nucleotide compositions of the bound fractions were characteristic of hRNA (Edmonds and Caramela, 1969); the nuclear fraction was high in U and the cytoplasmic fraction high in A, as previously determined for hnRNA and hcRNA from ascites cells (Roberts and Quinlivan, 1969). The unbound fractions contained RNAs rich in G + C. The high G content and low A content of the nuclear unbound fraction is typical of ascites cell rpRNA (Roberts and D'Ari, 1968). The nucleotide composition of the cyto-

TABLE I: [^{32}P]Nucleotide Composition of Bound and Unbound Nuclear and Cytoplasmic RNA Fractions.

Fraction Analyzed	% A_{260}[a]	% Cpm[a]	Mole % C	A	G	U
Bound cytoplasmic RNA	5	30	21.8	30.9	24.0	23.3
Unbound cytoplasmic RNA	95	70	29.0	18.9	30.2	21.9
Bound nuclear RNA	20	45	25.0	24.0	22.3	28.7
Unbound nuclear RNA	80	55	29.7	14.6	34.7	21.1

[a] Calculated as per cent of total cytoplasmic or nuclear RNA that was bound or unbound to cellulose columns. The nuclear RNA in these experiments (ascites cells labeled with $H^{32}PO_4^{2-}$ for 3 hr) represented 10% of the A_{260} units and 48% of the cpm found in the total cellular RNA.

plasmic unbound fraction reflects a mixture of rRNA and tRNA (Roberts and Quinlivan, 1969).

Zonal centrifugation profiles of the bound and unbound fractions of nuclear and cytoplasmic RNAs are shown in Figure 1. In the nuclear unbound fraction there were peaks at 45 and 32 S as expected for rpRNA. The cytoplasmic unbound fraction contained peaks of 28S and 18S rRNA and 4S tRNA. The bound fractions of both nuclear and cytoplasmic RNAs produced heterogeneous sedimentation patterns similar to those previously reported for hnRNA and hcRNA from ascites cells (Roberts and Newman, 1966; Edmonds and Caramela, 1969; Roberts and Quinlivan, 1969; Lee *et al.*, 1971). The mean sedimentation values of the bound fractions are approximately 18 S for the cytoplasmic RNA and 32 S for the nuclear RNA. These sedimentation values are as large as those of hRNAs isolated by other techniques, indicating little if any hydrolysis of the RNA on the cellulose columns.

The zonal centrifugation profiles together with the [^{32}P]-nucleotide compositions of the bound and unbound RNAs indicate that the separation of hRNAs from (G + C)-rich RNAs was fairly complete. Moreover, 98% of the cytoplasmic unbound fraction and 94% of the nuclear unbound fraction did not bind when rechromatographed, indicating that most of the RNA which was capable of binding had been removed by one passage through the cellulose. More than 90% of the bound fractions rebound to the cellulose if they were dialyzed rapidly and rechromatographed immediately, but these RNAs appeared to be very labile. Breakdown may have been due to shear (Perry *et al.*, 1972) or to a cellular nuclease which has been shown to remain with the poly(A)-containing RNA through extraction and purification procedures (Rosenfeld *et al.*, 1972).

Specificity of Cellulose with Respect to the Binding of Various Polynucleotides. Attempts were made to elucidate the factors which determine the specificity of the binding reaction. The capacity of the cellulose to bind nuclear and cytoplasmic RNAs, various homopolymers, rRNA, rpRNA, bacterial 5S RNA, native and denatured DNA, poly(A)·poly(U), and 2′(3′)-adenylic acid is presented in Table II. Cellulose had 30 times greater affinity for poly(A) than poly(U) and 8 times greater affinity for poly(A) than for poly(I) or poly(C), at 25°. No binding of adenylic acid or bacterial 5S RNA could be detected. Poly(A)·poly(U), rRNA, rp-

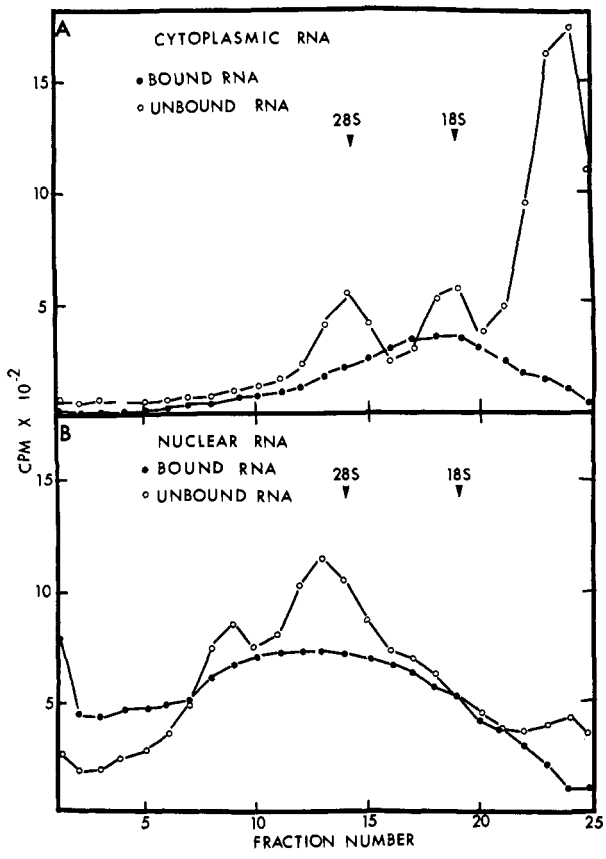

FIGURE 1: Zonal centrifugation of bound and unbound fractions of ^{32}P-labeled RNA. The (a) cytoplasmic and (b) nuclear RNA were chromatographed on unmodified cellulose columns as in Materials and Methods. Fractions containing bound RNA were combined, 1.0 mg of unlabeled cytoplasmic RNA was added as carrier, the solution made 0.15 M in sodium acetate buffer (pH 5.3), and the RNA precipitated by the addition of two volumes of ethanol. The RNA was then dissolved in 1.0 ml of gradient buffer (0.1 M NaCl–0.01 M sodium acetate (pH 5.3)–0.001 M $MgCl_2$) and layered onto 26 ml of a 5–20% linear sucrose gradient in gradient buffer. Centrifugation was in an SW25.1 Spinco rotor for 16 hr at 4°. Fractions of 1.0 ml were collected and 0.5 ml of each was plated and counted: (●) bound RNA; (○) unbound RNA.

RNA, and native DNA bound very little. We conclude, therefore, that adenylic acid, present as a single-stranded polymer, is important for the binding of polynucleotides to cellulose. However, since single-stranded DNA has a strong affinity for cellulose (Table II; Kitos *et al.*, 1972), DNA must contain poly(dA) sequences, or poly(A) and single-stranded DNA must share a common property necessary for binding, such as a properly extended configuration.

Effects of Temperature and Salt Concentration on the Binding of Polynucleotides to Cellulose. The binding capacities summarized in Table II revealed that small but significant amounts of rRNA and rpRNA bound to the cellulose columns at 25°. Therefore, the column temperature and the NaCl concentration in the chromatography buffer were varied in an attempt to avoid the binding of these RNAs while retaining the capacity of the cellulose to adsorb hRNA. Lowering the column temperature to 0° caused a significant increase in the amount of rRNA and rpRNA bound to the cellulose. However, increasing the column temperature from 25 to 45° resulted in a greater than 80% decrease in bound rRNA and rpRNA without affecting significantly the binding of poly(A) or hRNA to the cellulose. Salt concentration also was found to play an important role in the capacity of

TABLE II: Binding Capacity of Cellulose for Various Polynucleotides.[a]

Polynucleotide Examined	A_{260} Units of Polynucleotide Bound/g of Cellulose
Poly(A)	0.850
Poly(I)	0.100
Poly(C)	0.100
Poly(U)	0.025
Cytoplasmic RNA	0.325
Nuclear RNA	0.325
Denatured DNA	0.260[b]
Native DNA	0.017
Poly(A)·poly(U)	0.032
rRNA	0.030
rpRNA	0.032
5S rRNA from *B. subtilis*	<0.002
2′(3′)-Adenylic acid	<0.002

[a] Capacities were determined by passing a solution of excess polynucleotide in TSE through the cellulose column at 25° as described in Materials and Methods. [b] This figure does not represent a maximum capacity, as the column adsorbed all the denatured DNA applied; there was no excess.

TABLE III: Lignin Content and Binding Capacities of Various Celluloses.[a]

Type of Cellulose	A_{285}	A_{260} Units of Poly(A) Bound/g of Cellulose
Whatman CF_{12}	0.116	0.072
Whatman CF_{11}	0.147	0.125
Sigma 38	0.160	0.188
SS 286	0.779	0.627
Sigma α	0.807	0.855
SS-acid washed 389	0.488	1.010
Sigma α-acid washed	0.460	1.007

[a] Binding capacities were determined as in Table II. Lignin content was approximated by dissolving 0.1 g of cellulose in 10 ml of Cross and Bevan's Reagent ($ZnCl_2$–concentrated HCl, 1:2, w/w), and determining the absorbance at 285 mμ of the resulting solutions.

cellulose to bind polynucleotides; raising the NaCl concentration in the buffer to 0.5 M caused significant amounts of rRNA and rpRNA to adsorb to cellulose, whereas decreasing the concentration to 0 M eliminated the binding of most of the hRNA. Therefore, 0.3 M NaCl and 45° were selected as optimum chromatography conditions for the selective binding of hRNA.

Requirement of Lignins for the Binding of Polynucleotides to Cellulose. The observations that the capacity of cellulose to adsorb polynucleotides is increased by raising the salt concentration or lowering the temperature suggest that the binding of polynucleotides to cellulose takes place *via* hydrophobic rather than ionic bonds. A strong hydrophobic interaction between polymers of planar bases and polymers of sugars which have rings in the chair formation seems unlikely. There are, however, minor constituents in all refined plant celluloses, called lignins, which have molecular structures that could be expected to form hydrophobic bonds with polynucleotides in solution. Lignins are polymers of benzaldehyde derivatives which have an ultraviolet adsorption maximum at 285 mμ (Siegel, 1968). Since different celluloses have different lignin contents, their capacities to bind polynucleotides should vary directly with their absorption at 285 mμ, if lignins are the active components. This was found to be the case for the five commercial cellulose powders examined; the capacities of the celluloses to bind poly(A) were roughly proportional to their absorption at 285 mμ when dissolved in Cross and Bevans reagent (Table III). Although acid washing removes part of the A_{285} material, the capacity of the cellulose for binding poly(A) increases, suggesting that this procedure leaves the remaining lignins more available for polynucleotide binding.

Additional evidence that lignins are responsible for polynucleotide binding would be the ability to decrease or increase the capacity of celluloses to bind polynucleotides by removing or adding lignins, respectively. Table IV demonstrates that the extraction of lignins by two different procedures was found to decrease the capacities of celluloses to bind polynucleotides. NaOH is used commerically to remove lignins (Siegel, 1968; Noller, 1965), whereas dioxane is one of the best known lignin solvents (Siegel, 1968). In another type of experiment, commercially purified lignins were attached to a low-capacity cellulose (CF_{12}) by treatment of the cellulose and the lignins in buffer with ultraviolet light. This procedure was found to increase the capacity of the cellulose to bind poly(A) by more than sixfold. Recently, DeLarco and Guroff (1973) also have presented evidence suggesting that the binding of polynucleotides to cellulose is due to a lignin-like contaminant in the cellulose.

The binding capacities of the filter membranes (Table IV) were determined by stirring 0.1 g of membranes for 15 min at 25° in 10 ml of TSE containing 10 A_{260} units of poly(A). Under these conditions, the membranes had a similar capacity for adsorbing poly(A) as cellulose powder treated in a parallel manner, and the binding showed a similar requirement for a high salt concentration in the buffer. This, together with the loss of poly(A) binding capacity following dioxane treatment of the membranes, suggests that the binding of hRNA to nitrocellulose filter membranes (Lee *et al.*, 1971; Brawerman *et al.*, 1972) also may result from a hydrophobic interaction between the RNA and lignins present in the membranes.

Poly(A) Assay: Hybridization to [^{3}H]Poly(U). Hybridization with [^{3}H]poly(U) has been used to estimate the amount of poly(A) sequences in nucleic acids (Kates, 1970; Gillespie *et al.*, 1972). Therefore, samples of increasing amounts of unlabeled poly(A) were hybridized to [^{3}H]poly(U) as a control for the determination of the poly(A) content of ascites cell RNA (Figure 2). The [^{3}H]poly(U) hybridized was proportional to the amount of poly(A) added over the concentration range tested, with approximately 4×10^6 cpm hybridized per A_{260} unit of poly(A). This number was used to calculate the poly(A) content of RNA.

Poly(A) Content of Nuclear and Cytoplasmic RNA Fractionated on Unmodified Cellulose Columns. Poly(A) assays were performed on nuclear and cytoplasmic RNA before and after fractionation on cellulose columns (Figures 3 and 4). The linearity of the curves and the fact that essentially 100% of the poly(A) assayed in total cytoplasmic RNA was recovered in the bound cytoplasmic RNA fraction

TABLE IV: Binding Capacities of Different Celluloses after Addition or Extraction of Lignins.

Type of Cellulose	Treatment[a]	Polynucleotide Investigated	A_{260} Units or % Cpm Bound[b]
Whatman CF_2	None	Nuclear RNA	64%
Whatman CF_2	Dioxane, 24 hr	Nuclear RNA	39%
Whatman CF_2	1% NaOH, 100°, 1 hr	Nuclear RNA	18%
Whatman CF_{12}	None	Poly(A)	0.29 A_{260} unit
Whatman CF_{12}	Lignins + uv, 24 hr	Poly(A)	1.85 A_{260} units
HAWP Millipore filter membranes	None	Poly(A)	7.1 A_{260} units
HAWP Millipore filter membranes	Dioxane, 8 hr	Poly(A)	2.3 A_{260} units

[a] For the dioxane treatment, either 4.0 g of cellulose or 0.1 g of Millipore filter membranes was stirred in 100 ml of dioxane or 10 ml of dioxane, respectively, at 25° for the time indicated. The cellulose was removed by filtration and washed as described in Materials and Methods. The filters were removed with tweezers, washed with 10 ml of water, dried, and weighed. For NaOH treatment, 4.0 g of cellulose was boiled in 100 ml of 1% NaOH for 1 hr. The cellulose was removed by filtration and washed as usual. Addition of lignins was done by suspending 1.0 g of Indulin AT in 10 ml of PSE (0.2 M NaCl–0.1 M KPO_4 (pH 6.7)–0.001 M EDTA) for 1 hr and filtering off the insoluble lignins. Ten milliliters of the supernatant was then added to 4.0 g of cellulose suspended in 100 ml of PSE. The suspension was stirred overnight 20 cm from a General Electric 30-W ultraviolet germicidal lamp. The cellulose was removed by filtration and washed as usual. [b] Cellulose binding capacities for nuclear RNA were determined as in Table I. Cellulose binding capacities for poly(A) were determined as in Table II.

indicate that this assay can quantitatively detect poly(A) moieties in the presence of excess RNA which does not contain poly(A). Over 98% of the poly(A)-containing RNAs from the cytoplasm and nucleus were found in the bound fractions after one passage through the cellulose. From Figures 3 and 4 it can be calculated that the poly(A) moiety represents 4.2% of the nucleotides in the bound cytoplasmic RNA and 2.0% of the nucleotides in the bound nuclear RNA (Table V), assuming that [^{3}H]poly(U) hybridizes with the poly(A) moieties in hRNA as effectively as with synthetic poly(A) (Figure 2). It should be noted that this method measures the total poly(A) in hRNA, in contrast to the more usual assays which measure radioactive poly(A) present in newly synthesized hRNA.

Dependence on Poly(A) for Cellulose Binding. In an attempt to determine if the single-stranded poly(A) moiety is essential for the binding of hRNAs to cellulose and if there are animal cell hRNAs without poly(A) that are still able to bind, we prehybridized ^{32}P-labeled nuclear and cytoplasmic RNA with excess unlabeled poly(U) before cellulose chromatography. This procedure prevented the binding of approximately 90% of the hcRNA and 80% of the hnRNA, indicating that single-stranded poly(A) regions are required for the adsorption of most hRNA molecules to cellulose.

Poly(A) Distribution among hRNAs. RNAs from ascites cells labeled with $H^{32}PO_4^{2-}$ for 3 hr in the presence of 0.04 μg/ml of actinomycin D were chromatographed on cellulose columns to determine the poly(A) distribution among these RNAs. This concentration of actinomycin D is known to

TABLE V: Poly(A) Content of RNA Fractions.

Fraction Analyzed[a]	% Poly(A)[b]
Total cytoplasmic RNA	0.21
Bound cytoplasmic RNA	4.20
Unbound cytoplasmic RNA	<0.003
Total nuclear RNA	0.42
Bound nuclear RNA	1.97
Unbound nuclear RNA	<0.003

[a] Total nuclear and cytoplasmic RNA were isolated and purified as described previously (Roberts and Newman, 1966) and redissolved in STE for determination of poly(A) content by hybridization to [^{3}H]poly(U). Bound and unbound RNAs were obtained by fractionation of total nuclear or cytoplasmic RNA by chromatography on unmodified cellulose columns and then dialyzed against STE for determination of poly(A) content as above. [b] Per cent poly(A) was calculated by comparison of [^{3}H]poly(U) hybridized per A_{260} unit of RNA to that hybridized per A_{260} unit of synthetic poly(A) (Figure 2), using molar extinction coefficients of 7.9×10^3 for RNA and 9.5×10^3 for poly(A).

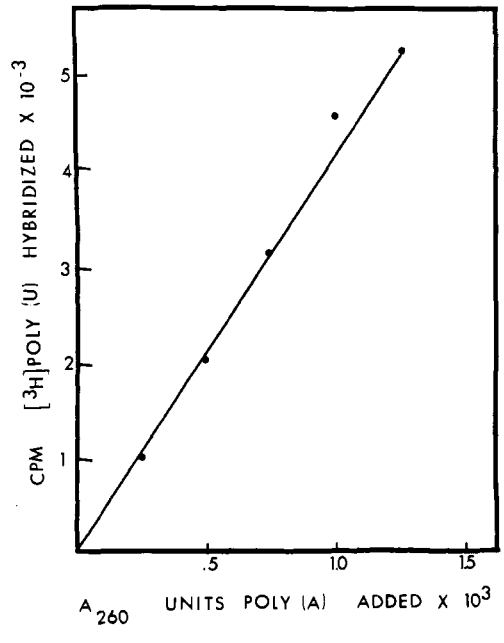

FIGURE 2: Hybridization of [^{3}H]poly(U) to poly(A). Increasing amounts of poly(A) were hydridized to [^{3}H]poly(U) and analyzed as described in Materials and Methods.

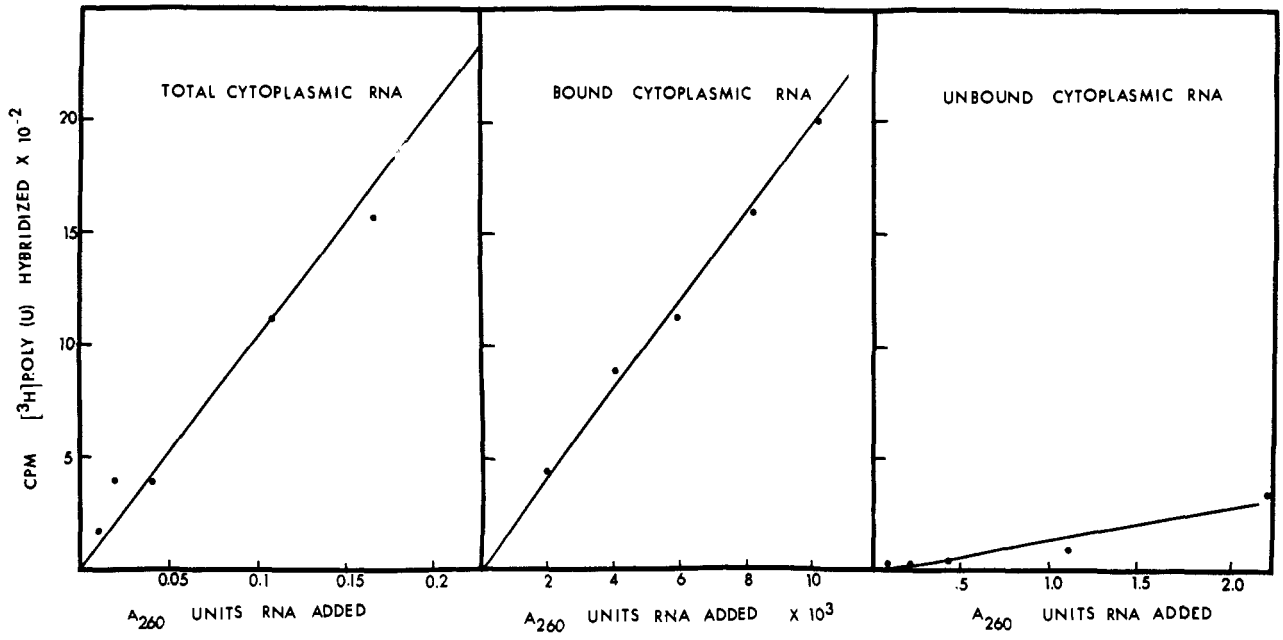

FIGURE 3: Hybridization of [^{3}H]poly(U) to cytoplasmic RNA fractions. Total cytoplasmic RNA was fractionated into bound and unbound RNA by chromatography on cellulose columns. Increasing amounts of either total cytoplasmic RNA or bound or unbound fractions of cytoplasmic RNA were hybridized to [^{3}H]poly(U) and analyzed as described in Materials and Methods.

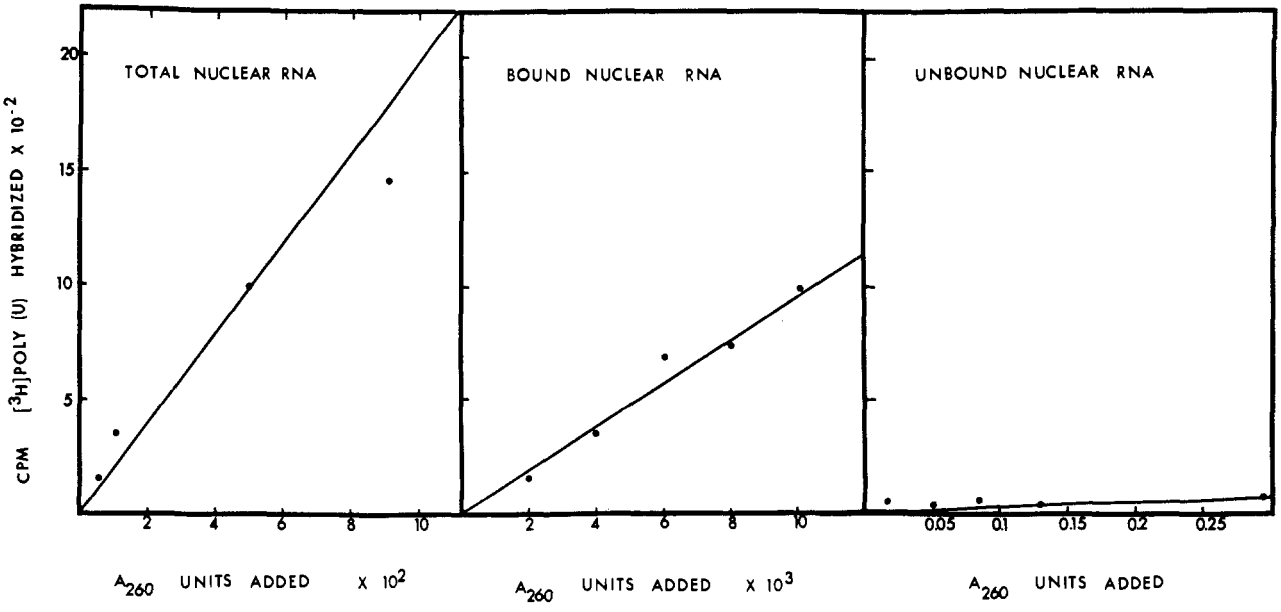

FIGURE 4: Hybridization of [^{3}H]poly(U) to nuclear RNA fractions. Total nuclear RNA was fractionated into bound and unbound RNA by chromatography on cellulose columns. Increasing amounts of total nuclear RNA or bound or unbound fractions of nuclear RNA were hydridized to [^{3}H]poly(U) and analyzed as described in Materials and Methods.

inhibit the synthesis of rRNAs while permitting the synthesis of hRNA and limited amounts of tRNA (Perry, 1963; Roberts and Newman, 1966; Penman *et al.*, 1968; Perry and Kelley, 1970). Of the hRNAs which were synthesized in the presence of actinomycin D and which had sedimentation coefficients greater than 8 S (thereby excluding newly synthesized tRNA), approximately 50% of the hcRNAs and 40% of the hnRNAs did not bind to the cellulose and, therefore, did not contain poly(A). The bound and unbound hRNAs were compared with respect to their sedimentation profiles and nucleotide compositions (Figure 5). The bound and unbound RNAs from the same cellular component showed similar sedimentation profiles, but had significantly different nucleotide compositions, particularly with respect to the mole per cent adenylic acid.

Distribution of Poly(A) in Various Sedimentation Classes of Cellulose-Bound hnRNA and hcRNA. Poly(A) assays were carried out on ^{32}P-labeled cellulose-bound hcRNAs and hnRNAs which had been fractionated into different sedimentation classes by zonal centrifugation. Figure 6 shows the sedimentation profiles of these RNAs along with the Cpm of [^{3}H]poly(U) hybridized by each fraction. All fractions contained poly(A), as judged by their hybridization with significant amounts of [^{3}H]poly(U). However, the per cent poly(A) in the hRNA molecules, which is proportional to the ratio of ^{3}H/^{32}P, varied with their sedimentation rates;

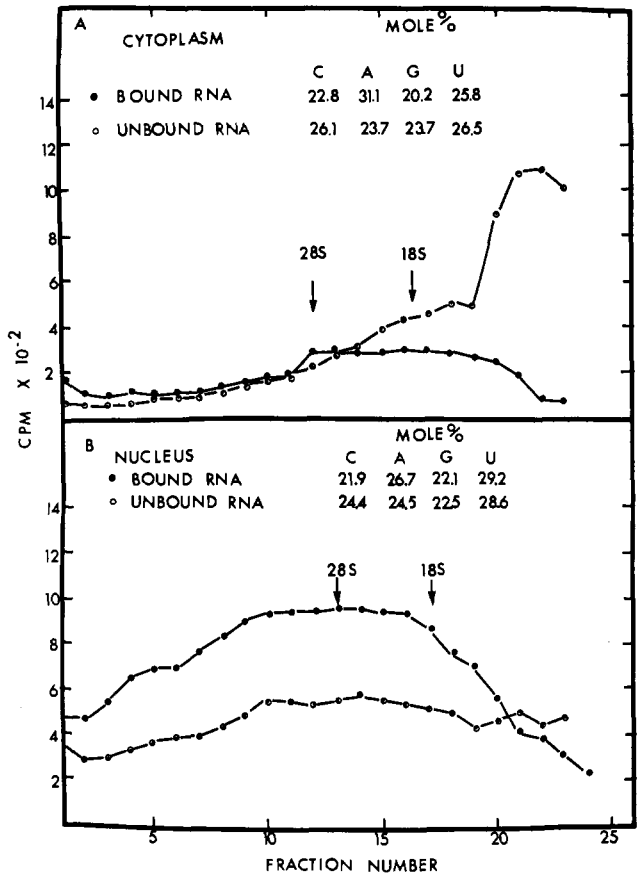

FIGURE 5: Zonal centrifugation patterns of bound and unbound RNAs synthesized in the presence of actinomycin D. Either 10 A_{260} units of cytoplasmic RNA or 2 A_{260} units of nuclear RNA (labeled with $H^{32}PO_4^{2-}$ for 3 hr in the presence of 0.04 μg/ml of actinomycin D) was chromatographed on unmodified cellulose columns. The RNAs from the bound or unbound fractions were combined, ethanol precipitated, and analyzed by zonal centrifugation as in Figure 1. Nucleotide compositions were performed as reported earlier (Roberts, 1965).

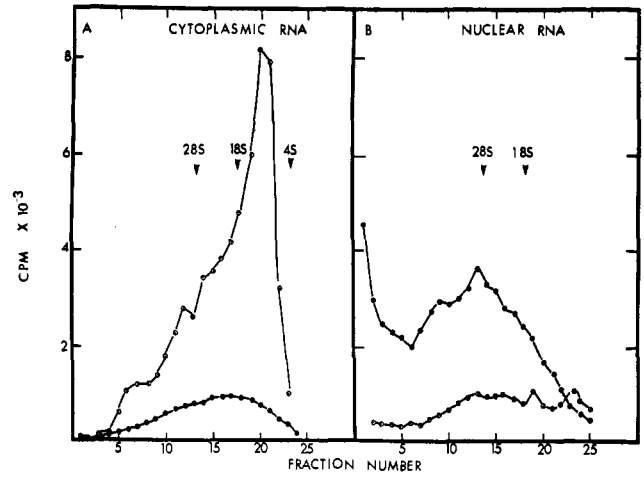

FIGURE 6: Poly(A) content of different sedimentation classes of bound hRNAs. Either 10 A_{260} units of cytoplasmic RNA or 2 A_{260} units of nuclear RNA (labeled with $H^{32}PO_4^{2-}$ for 3 hr) was chromatographed on cellulose columns. The RNAs from the bound fractions were combined, ethanol precipitated, and analyzed by zonal centrifugation as in Figure 1. [^{3}H]poly(U) was hydridized to one-half of the RNA in each fraction and analyzed as described in Materials and Methods: (●) ^{32}P-labeled cellulose-bound RNA; (○) [^{3}H]poly(U) hybridized to cellulose-bound RNA

both hcRNA and hnRNA showed a maximum poly(A) content in molecules with sedimentation coefficients of approximately 8–12 S, with the per cent poly(A) decreasing progressively with increasing sedimentation rate.

Cellulose Column Chromatography of Mengovirus RNA. We wanted to investigate the importance of the length of the poly(A) region on the binding of RNA to cellulose. The hRNAs have been shown to contain poly(A) moieties 150–200 nucleotides long (see Discussion), whereas picornaviruses contain RNAs with poly(A) regions which are only about 20–80 nucleotides in length (Armstrong *et al.*, 1972; Johnston and Bose, 1972; Miller and Plagemann, 1972; Yogo and Wimmer, 1972). Therefore, mengovirus was prepared from Ehrlich ascites cells according to the procedure of Burness (1969); the RNA then was extracted and intact viral RNA isolated from the 35 S region of a zonal centrifugation run (Roberts *et al.*, 1966). The poly(A) content of this RNA was determined as in Figures 3 and 4; an average value of approximately 50 nucleotides per poly(A) moiety was found, assuming a molecular weight of 2.8×10^6 daltons for mengovirus RNA and that every RNA molecule contained one poly(A) region (Miller and Plagemann, 1972). The ability of mengovirus RNA to bind to cellulose was investigated by passing 2.0 A_{260} units of mengovirus RNA through a cellulose column at 45°. The column bound approximately 0.04 A_{260} unit of viral RNA per gram of cellulose, less than 15% the binding capacity of the column for hRNA. This indicates that the length of the poly(A) region must be fairly long for an RNA species to bind effectively to cellulose, compared with the shorter poly(A) lengths which are sufficient for binding to poly(dT)- or poly(U)-cellulose (Kates, 1970).

Discussion

The technique described in this report promises to be a useful procedure for the isolation of hnRNAs from animal cells. It is simple, rapid, efficient, and results in no apparent degradation of the RNAs. Furthermore, conditions of isolation can be adjusted to prevent significant contamination of the hRNA with rRNAs, rpRNAs, or tRNAs. Major advantages of this technique are that the bound RNAs may be recovered in two to three minutes at a low temperature with very few manipulations, and free from contaminating poly(U) which sometimes accompanies hRNA eluted from filter membranes containing immobilized poly(U). In addition, since this technique utilizes a different principle of fractionation than those which isolate hRNAs by hydrogen bonding to immobilized poly(U) or poly(dT), it may affect a somewhat different fractionation of RNAs, which could prove useful. For example, the experiment with mengovirus RNA indicates that the length of the poly(A) moiety required for binding is different for the two procedures, and suggests that cellulose columns may be useful in helping fractionate RNA molecules which contain poly(A) moieties of varying lengths. The capacity of cellulose to adsorb hRNA, using the cellulose and conditions described in this communication, is rather low (approximately 0.32 A_{260} unit/g of cellulose). This capacity can be raised by increasing the lignin content of the cellulose. Also, the capacity of cellulose to bind polynucleotides can be increased markedly by lengthening the contact time between cellulose and polynucleotide or by decreasing the particle size of the cellulose (N. Sullivan and W. K. Roberts, unpublished observations). However, the utilization of high capacity conditions for isolating hRNA is

not always useful, since it can lead to significant contamination of the hRNA with rRNA and rpRNA.

Our results and those of DeLarco and Guroff (1973) indicate that polynucleotides adsorb to cellulose because of a hydrophobic interaction between the polynucleotide and lignins in the cellulose. Under the proper conditions, the adsorption of RNAs can be limited essentially to those which contain poly(A) moieties, as judged by adsorption of over 98% of the poly(A)-containing RNAs (Figures 3 and 4), lack of contamination with rRNA, rpRNA, and tRNA (Table II), and the inhibition of hRNA binding by prehybridization with poly(U). The small amounts of hcRNA and hnRNA which did bind to cellulose in the presence of poly(U) were found to have sedimentation coefficients significantly larger than the average values for bulk hcRNA and hnRNA. This could mean that because of their large size these RNAs can present sufficient non-poly(A) binding sites to the cellulose to permit adsorption in the presence of poly(U). This could be the explanation, too, for the adsorption of single-stranded DNA to cellulose (Table II; Kitos *et al.*, 1972), which also is not prevented by prehybridization with poly(U) (P. A. Kitos, personal communication).

Approximately 50% of the total hcRNA and 40% of the total hnRNA did not bind to the cellulose and, therefore, did not contain poly(A). The unbound hcRNA might be expected to include a small amount of histone mRNA and perhaps other mRNA that does not contain poly(A) (see Adesnik and Darnell, 1972; Adesnik *et al.*, 1972), part of the non-polysomal hRNA (Penman *et al.*, 1968, 1970), and bound hcRNA from which the poly(A) has been cleaved by shearing (Perry *et al.*, 1972), by poly(A)-specific nuclease (Rosenfeld *et al.*, 1972), by or by random endonucleolytic cleavage (Adesnik *et al.*, 1972). Our value of 50% for the amount of hcRNA isolated from total cytoplasm which binds to cellulose compares favorably with the values of 50–80% for the amount of hcRNA isolated from total cytoplasm which binds to filter membranes or immobilized poly(U) (Lee *et al.*, 1971; Sheldon *et al.*, 1972; Adesnik *et al.*, 1972). The unbound hcRNA (>8 S) had a sedimentation profile similar to bound hcRNA, but had a higher mole per cent of all nucleotides except adenylic acid (Figure 5A). This is consistent with the view that the bulk of the unbound hcRNA may represent bound hcRNAs which have lost their poly(A) sequences by *in vivo* or *in vitro* cleavage. The unbound hnRNAs also might contain hnRNAs from which the poly(A) moieties have been cleaved, as well as hnRNAs which do not contain poly(A) because their functions do not require it or because they have not been selected for transport into the cytoplasm. In addition, the unbound hnRNAs may contain some hnRNAs to which post-transcriptional addition of poly(A) (Kates, 1970; Darnell *et al.*, 1971; Philipson *et al.*, 1971; Mendecki *et al.*, 1972) had not yet occurred. We found a higher percentage of poly(A)-containing hnRNA (60%) than had been reported previously (Sheldon *et al.*, 1972) for nucleoplasmic RNA from HeLa cells (12–20%) or from rabbit embryos (Schultz *et al.*, 1973). It is not clear whether this discrepancy reflects differences in tissues, RNA isolation procedures, or poly(A) binding techniques. A comparison of bound and unbound hnRNA sedimentation profiles and nucleotide compositions illustrates the similarities between these two fractions (Figure 5B), which again suggests that the bulk of the unbound hnRNA may be identical to bound hnRNA except for the loss of poly(A) moieties.

The poly(A) content per A_{260} unit in bound hcRNA is approximately 20 times higher than the poly(A) content per A_{260} unit of total cytoplasmic RNA and approximately five times greater in bound hnRNA than in total nuclear RNA (Table V). These figures are about what would be expected if the poly(A)-containing RNAs account for 5% of the total cytoplasmic RNAs and 20% of the total nuclear RNAs (Table I). Assuming that [^{3}H]poly(U) hybridizes as effectively to hRNA as to synthetic poly(A), one can calculate that poly(A) constitutes approximately 4.2% of the total nucleotides in bound hcRNA (Figures 2 and 3) and approximately 2.0% of the total nucleotides in bound hnRNA (Figures 2 and 4). These values are similar to those reported by other investigators (Kates, 1970; Edmonds *et al.*, 1971; Darnell *et al.*, 1971; Mendecki *et al.*, 1972; Sheldon *et al.*, 1972). The poly(A) moieties have been shown to consist of polynucleotide regions 150–200 nucleotides long (Edmonds *et al.*, 1971; Kates, 1970; Mendecki *et al.*, 1972; Philipson *et al.*, 1972; Darnell *et al.*, 1971) located on the 3′ terminus of the hRNA molecules (Kates, 1970; Mendecki *et al.*, 1972). Using a model in which each bound hRNA molecule has a single poly(A) sequence 200 nucleotides long and the calculated percentages of poly(A) given above, the bound hcRNAs would have an average molecular weight of about 1.1×10^6 daltons and the bound hnRNAs an average molecular weight of about 2.4×10^6 daltons; these are the approximate mean sizes found for these RNAs by zonal centrifugation analysis (Figures 1, 5, and 6). Such a model also predicts that the larger an hRNA molecule is, the smaller the per cent poly(A) it will contain. Calculating the ratio of [^{3}H]poly(U) hybridized per bound [^{32}P]hRNA from Figure 6 demonstrates that the poly(A) content does indeed decrease with increasing sedimentation rate of bound hRNA. However, the decrease is not as rapid for the estimated molecular weights of the bound hRNAs as the model predicts. The reason for this unexpectedly slow decrease is not known, but it suggests the possibility that some of the larger hRNA molecules may contain multiple tracts of poly(A).

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Two Physically and Functionally Distinct Forms of Eukaryotic 40S Ribosomal Subunits†

Richard E. H. Wettenhall,‡ Ira G. Wool,* and Corinne C. Sherton

ABSTRACT: Preparations of the small subunit of rat liver ribosomes made in buffer containing puromycin and high concentrations of potassium (0.8 M) contain two forms of the particle; one of the two species sediments as a dimer in buffers having low concentrations of potassium (80 mM) and the other as a monomer. The two forms of the 40S ribosomal subunit are not in equilibrium; rather they remain physically distinct when sedimented through several cycles of buffers containing high and low concentrations of potassium. The monomer ($40S_m$) always has a sedimentation coefficient of 40; the dimer ($40S_d$) sediments at 40 S in high concentrations of potassium but at 55 S in low concentrations. The predominant RNA of both particles is 18S ribosomal RNA, and they have the same set of ribosomal proteins; electron microscopy confirmed that one is a dimer of the other. The buoyant density of the monomer is 1.522 g/cm^3, whereas that of the dimer is 1.514 g/cm^3. The difference in buoyant density suggested the former might contain more RNA than the latter, and indeed each $40S_m$ particle was found to have a molecule of deacylated tRNA associated with it, whereas only a small percentage of the $40S_d$ species had deacylated tRNA bound to them. The $40S_d$ species was more active than the $40S_m$ particle in the binding of aminoacyl-tRNA in the reaction catalyzed by eukaryotic initiation factor 1 (EIF-1). The dimers were also more active when assayed with an excess of 60S subunits: in the synthesis of polyphenylalanine at 12 mM magnesium (when only eukaryotic elongation factors 1 and 2 are required) as well as at 3.5 mM (when EIF-1 and EIF-2 are needed also), and in the translation of encephalomyocarditis RNA.

The small subparticles of prokaryotic and eukaryotic ribosomes can occur *in vitro* as either monomers or dimers (Pestka and Nirenberg, 1966; Tashiro and Morimoto, 1966; Igarashi and Kaji, 1969; Terao and Ogata, 1970; Petermann and Pavlovec, 1971; Martin *et al.*, 1971; Nonomura *et al.*, 1971). Whether dimers are formed *in vivo* as well is not known. The physical basis for the dimerization of ribosomal subparticles is not certain either, although electrostatic forces and divalent salt bridges are likely to play a role since dimers occur more readily in dilute than in concentrated potassium chloride buffers (Igarashi and Kaji, 1969; Petermann and Pavlovec, 1971) and dimerization is dependent on the magnesium concentration (Pestka and Nirenberg, 1966; Tashiro and Morimoto, 1966; Petermann and Pavlovec, 1971; Zamir *et al.*, 1971). It is possible that particular ionic conditions favor a conformation which allows dimerization (Zamir *et al.*, 1971). It is not known if monomers and dimers of small ribosomal subunits are in equilibrium, or

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‡ Present address: Queen Elizabeth Medical Center, Birmingham, England.