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Isolation of Poly(adenylic acid)-Rich Ribonucleic Acid from Mouse Myeloma and Synthesis of Complementary Deoxyribonucleic Acid†

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ABSTRACT: An RNA fraction containing poly(adenylic acid) sequences has been isolated from membrane-bound polysomes of mouse myeloma tumor. Annealing to a poly(thymidylic acid)-cellulose column, significant RNase-resistance (20%), and the size of the RNase-resistant fragment (7 S) all suggest that this RNA fraction contains large tracts of poly(adenylic acid)-rich sequences. This is confirmed by the finding of 80% AMP in the RNase-resistant fraction. Conditions of temperature and ionic strength for chromatography on poly(thymidylic acid)-cellulose are established for optimal recovery of RNA containing poly(adenylic acid) with minimal contamination by ribosomal RNA. The isolated poly(adenylic acid)-rich fraction functions as an efficient RNA template for the synthesis of complementary DNA with the RNA-dependent DNA polymerase of avian myeloblastosis virus. This reaction is completely dependent upon added RNA as template, and oligothymidylic acid as primer. Priming activity is inversely related to the size of the oligothymidylic acid. Density equilibrium centrifugation of the native product shows that some of the radioactivity is associated with RNA. However, after denaturation, all the radioactivity bands as DNA. The average size of the product synthesized, as determined by alkaline sucrose sedimentation, depends on the concentration of the deoxyribonucleoside triphosphates used in the reaction. The specificity of the product synthesized is demonstrated by hybridization.

One of the intriguing questions concerning the immune response is the generation of antibody diversity. As yet, there are no experimental data to decide between the two main theories proposed: the germ line theory and the theories involving somatic mutation and recombination (for review see Smith *et al.*, 1971). A measure of the number of genes for immunoglobulin chains in cells of different tissues, including the genes for the variable part, could answer that question. Such a measure could be achieved by DNA–DNA hybridization under conditions of large DNA excess, using a "Cot" analysis (Britten and Kohne, 1968; Gelb *et al.*, 1971). One advantage of such an approach is that *in vitro* synthesized DNA could be obtained at high enough specific activities to give meaningful data. In addition, DNA–DNA hybridizations are less susceptible to degradation at high temperature when compared to those performed with RNA.

After the discovery of the RNA-dependent DNA polymerase in tumor viruses (Baltimore, 1970; Temin and Mizutani, 1970), and the subsequent demonstration that it could use 9S hemoglobin (Hb)[1] mRNA as template for complemen-

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[1] Abbreviations used are: Hb, hemoglobin; cDNA, complementary deoxyribonucleic acid; medium A-100, 50 mM Tris-HCl (pH 7.6)–100 mM KCl–5 mM $MgCl_2$; AMV, avian myeloblastosis virus; SSC, standard saline citrate (0.15 M NaCl–0.015 M sodium citrate); DEP, diethylpyrocarbonate; poly(A), poly(adenylic acid).

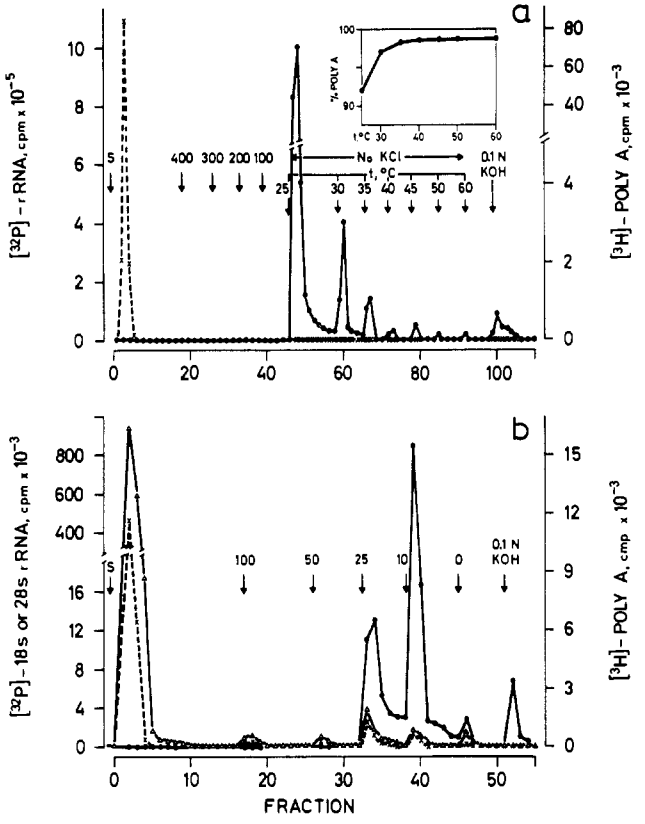

FIGURE 1: (a) A 1.5-ml column of poly(dT), prepared as described in Methods and Materials, was equilibrated with 500 mM KCl and 10 mM Tris-HCl, pH 7.6. A 300-μl sample, S, of [^{32}P]rRNA (3 A_{260} units and 1.65×10^6 cpm) prepared from pooled myeloma subunits, and [^{3}H]poly(A) (no measurable A_{260} and 1.5×10^5 cpm) in 500 mM KCl and 10 mM Tris-HCl, pH 7.6, was applied and washed through the column with four column volumes of the same buffer at 25°. Two column volumes of the following were then successively washed through the column at 25° (arrows): 400 mM, 300 mM, 200 mM, and 100 mM KCl, all containing 10 mM Tris-HCl, pH 7.6. This was followed by 4 column volumes of 10 mM Tris-HCl, pH 7.6, with no KCl at 25°. The flow rate was 0.7–1.0 ml/(cm^2 min). The flow was stopped, the temperature was raised stepwise as indicated (arrows), allowing 15–20 min for equilibration, and two column volumes of the same buffer were passed through the column. Finally, the column temperature was returned to 25° and washed with two column volumes of 0.1 N KOH. Fractions of 0.4 ml were collected directly into counting vials and counted in 10-ml scintillator (Bray, 1960), using a Beckman LS-250 counter. Radioactivity is indicated for rRNA (×) and poly(A) (●). Corrections were made for spillover of ^{32}P into the ^{3}H channel. (b) The poly(dT) column was prepared as described above. A 300-μl sample, S, of ^{32}P 18S rRNA (1.0 A_{260} unit and 5.0×10^5 cpm) and [^{3}H]poly(A) (no measurable A_{260} and 6.0×10^4 cpm) was applied to the column in 500 mM KCl and 10 mM Tris-HCl, pH 7.6, and washed with four column volumes of the same buffer at 30°. This was followed with a KCl step gradient at 30°: 100 mM, 50 mM, 25 mM, 10 mM, and no KCl (arrows), all containing 10 mM Tris-HCl, pH 7.6. Fractions were collected and counted as above. The column was then regenerated with 0.1 N KOH. A separate identical experiment was run with 28S rRNA from myeloma (2 A_{260} units and 1.0×10^6 cpm). Radioactivity is indicated for 18S rRNA (×), 28S rRNA (△), and poly(A) (●).

tary DNA (cDNA) synthesis (Ross *et al.*, 1972; Verma *et al.*, 1972; Kacian *et al.*, 1972), it became apparent that the same approach might be applied in the case of the light chain of immunoglobulin. This could be possible only if the mRNA for the light chain can serve as a template for cDNA synthesis. In the case of Hb mRNA, the presence of poly(A) sequences apparently allows it to be used for cDNA synthesis (Ross *et al.*, 1972; Verma *et al.*, 1972; Kacian *et al.*, 1972).

The existence of poly(A) in certain eukaryotic tissues has been known for some time (Hadjivassiliou and Brawerman, 1966; Edmonds and Caramela, 1969). It is associated with rapidly labeled polysomal RNA, heterogeneous nuclear RNA (Darnell *et al.*, 1971; Lee *et al.*, 1971; Edmonds *et al.*, 1971; Sheldon *et al.*, 1972; Burr and Lingrel, 1971), and the RNA of certain viruses (Kates, 1970; Armstrong *et al.*, 1972; Lai and Duesberg, 1972; Weinberg *et al.*, 1972). The histone mRNA may be an exception (Adesnik and Darnell, 1972). The occurrence of poly(A) in mRNA can be used to separate mRNA from other cytoplasmic RNA (Lee *et al.*, 1971; Edmonds *et al.*, 1971; Sheldon *et al.*, 1972; Aviv and Leder, 1972; Rosenfeld *et al.*, 1972).

Methods and Materials

Preparation of Myeloma Polysomes. All operations were performed under sterile conditions. Polysomes were prepared from solid MOPC-41 myeloma tumors (generous gift of Dr. M. Potter). ^{32}P was injected intraperitoneally 8 hr before harvesting of the tumors. A postmitochondrial supernatant was prepared in medium A-100[1] and 0.8 M sucrose (Mach *et al.*, 1968). Microsomes were prepared as for rat lymph nodes (Vassalli, 1967), except that the centrifugation was for 20 min at 30,000 rpm in a Spinco 50 Ti rotor over a 2-ml cushion of 0.8 M sucrose in medium A-100. Microsomes were resuspended by gentle agitation in medium A-100 and rat liver postribosomal supernatant. Nonidet P-40 (1% final concentration) was added. The polysomes were recovered by centrifugation through a cushion of 2 M sucrose in medium A-100 for 2.5 hr at 50,000 rpm in a Spinco 50 Ti rotor. The pellet was resuspended in medium A-100 and either used fresh or stored at −70°. Polysomes freshly prepared in this way were capable of initiating the synthesis of new polypeptide chains *in vitro* (Jones and Mach[2]). Ribosomal subunits were prepared from the above polysomes, using 0.5 M KCl and puromycin (Faust and Matthaei, 1972).

Extraction of RNA. Polysomes (100 A_{260} units/ml) in medium A-100 were diluted with 4 vol of water, and RNA was extracted by any of the following three procedures: (1) 0.1 vol of 1 M Tris-HCl, pH 9, and 0.1 vol of 10% sodium dodecyl sulfate were added, and phenol extraction and RNA precipitation were performed as described by Lee *et al.* (1971); (2) proteinase K (Wiegers and Hilz, 1971) (200 μg/ml) and 0.1% sodium dodecyl sulfate (final concentrations) were added, and the sample was incubated at 0° for 10 min before proceeding as described in procedure 1 above; and (3) same as procedure 2, except that the incubation was at 23° for 3 min only. RNA was stored at −20°, either lyophilized or dissolved in 1 mM EDTA in small aliquots.

Fractionation of Polysomal RNA by Poly(dT)-Cellulose Chromatography. The preparation of the poly(dT) and its coupling to cellulose has been described earlier (Gilham, 1964). The only modification was a 0.5 N KOH washing at 30° (at least four column volumes) before its initial use. The column was washed with 10 mM Tris-HCl, pH 7.6, to neutrality and stored in 0.5% sodium dodecyl sulfate. After removal of the sodium dodecyl sulfate, the column was equilibrated with 500 mM KCl and 10 mM Tris-HCl, pH 7.6, before use. Each polysomal sample in the above buffer was applied

[2] Jones, G., and Mach, B., manuscript in preparation.

to the column (1.5 ml), followed by two column volumes of the same buffer. The bulk of the RNA not adsorbed represents peak I. The column was washed with four column volumes of 50 mM KCl and 10 mM Tris-HCl, pH 7.6. An RNA fraction (5–6% of the total ^{32}P) was eluted with two column volumes of 10 mM Tris-HCl, pH 7.6 (peak II). Unlabeled polysomal RNA (1000 A_{260} units) was prepared (procedure 2) on an identical 15-ml column. Peak II contained about 2.5% (A_{260}) of the total RNA and was used for studies on DNA synthesis.

Purification of RNA-Dependent DNA Polymerase from AMV. Plasma from ten leukemic chickens (about 50 ml) was collected and virus particles pelleted and taken up in 2.5 ml of 0.05 M Tris-HCl, pH 7.5, 0.1 mM EDTA, 0.5 mM 2-mercaptoethanol, and 5% glycerol. Virus particles were disrupted with Nonidet P-40 (2% final concentration) and incubation at 37° for 5 min. The lysate was diluted fivefold in the above buffer and adsorbed onto DEAE-cellulose column (15 × 0.9 cm). The enzyme activity was eluted from the column at 0.1–0.15 M NaCl by a 100-ml linear NaCl gradient. The enzyme was then dialyzed against 0.05 M sodium phosphate buffer, pH 7.6, containing EDTA, 2-mercaptoethanol, and glycerol as above, and subsequently adsorbed onto a phosphocellulose column (10 × 0.9 cm). The enzyme (270 μg/ml) was eluted at 0.15–0.2 M NaCl by a 60-ml linear NaCl gradient, and the fractions containing activity were immediately stored at −70°. At this temperature the enzyme did not lose activity for at least 6 months, while at 0° the half-life of the enzyme was about 1 hr. The purity of the enzyme was estimated as 90% (Kacian *et al.*, 1971).

Preparation of cDNA. Poly(dT) (60 μg/ml) was digested with pancreatic DNase A at different concentrations to obtain an oligo(dT) fraction which maximally stimulated DNA synthesis. RNA and oligo(dT) were added to a reaction mixture containing the following components: 2–4 μl of AMV polymerase; 1 mM unlabeled deoxytriphosphates and labeled deoxytriphosphates as indicated; 10 mM KCl; 50 mM Tris-HCl, pH 7.5; 1.5 mM 2-mercaptoethanol; and actinomycin D (25–50 μg/ml), in a final volume of 20 μl. The reaction was stopped by the addition of sodium dodecyl sulfate to 1% and EDTA to 10 mM. The product was brought to 0.2 N NaOH and incubated for 20 min at 80° and subsequently layered onto a linear alkaline sucrose gradient. The DNA was collected, neutralized, alcohol precipitated with T4 carrier DNA, and used in the annealing experiments.

Chemicals used and their sources are: [^3H]poly(A), gift from Dr. D. Kolakofsky; [^{32}P]orthophosphate (10 mCi/ml), [^3H]dTTP (11.3 Ci/mmol), [^3H]dCTP (12 Ci/mmol), [^3H]-dGTP (9.8 Ci/mmol), Amersham; poly(A), Miles Laboratories; poly(dT) (chain length = 700), gift from Dr. S. Modak; proteinase K (chromatographically pure), Merck; RNase T1, Calbiochem; pancreatic RNase A and DNase A (electrophoretically pure), Worthington; actinomycin D, Sigma; Whatman DEAE-52, PC-11, and CF-11, Reeve Angel; *N,N'*-dicyclohexylcarboimide, Merck; diammonium thymidine 5'-monophosphate, Schwarz-Mann; Nonidet P-40, Shell; DEP ("Baycovin"), Bayer; dimethyl sulfoxide, Merck.

Results

Behavior of rRNA and Synthetic Poly(A) on Poly(dT)-Cellulose Columns. The bulk of the rRNA (98.6%) is not adsorbed (Figure 1a). At lower ionic strength, only traces of rRNA are eluted (Figure 1b). The 18S and 28S rRNA were examined separately to determine their relative contributions to the slight contamination of the poly(A) fraction. Most of the 18S and 28S rRNA was eluted between 500 and 50 mM KCl. Altogether, 1.6% of 18S and 0.8% of 28S rRNA are eluted below 50 mM KCl.

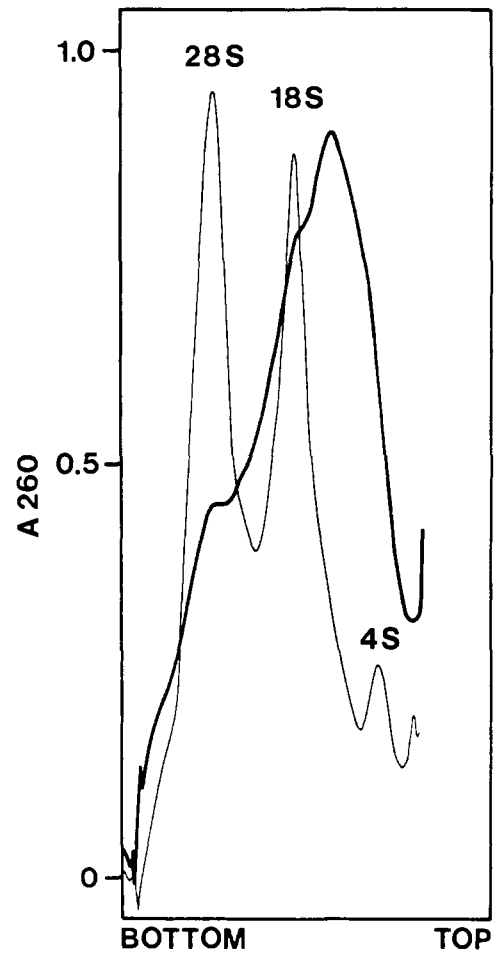

FIGURE 2: Sedimentation profile of RNA from peaks I and II. A 200-μl sample of RNA (1 mg/ml) from either peak I (thin tracing) or peak II (heavy tracing) of the poly(dT) column (obtained as described in Methods and Materials) was layered onto separate 5–20% linear sucrose gradients containing 0.02 M sodium acetate, pH 5, in a Spinco SW 41 rotor. Centrifugation was for 7 hr at 40,000 rpm and 2°. The profiles were obtained by reading A_{260} with a flow-through cuvet. The S values were estimated from a parallel run in a separate gradient with rRNA and tRNA standards. The main peak of peak II RNA is 14 S.

Two separate experiments, one with a tenfold excess of [^3H]- over [^{32}P]- and one with only [^3H]poly(A), demonstrated that no poly(A) is eluted at 500 mM KCl. Figure 1a shows the effect of temperature on the elution of poly(A) at low ionic strength. At 25°, 92% of all poly(A) is eluted with 10 mM Tris-HCl. More poly(A) can be released by increasing the temperature, as is illustrated in the inset of Figure 1a. At 30°, 97% is recovered, and this temperature was chosen for all subsequent experiments. The poly(A) not eluted (1.5%), even at 60°, can be released with 0.1 N KOH. The elution of poly(A) at 30° is presented in Figure 1b. No poly(A) elutes at 50 mM KCl, while 38 and 53% are recovered at 25 and 10 mM, respectively. An additional 6% is eluted with 10 mM Tris-HCl alone.

Preparation of RNA from Myeloma Polysomes. The use of proteinase K at 0 at 23° results in a clear aqueous phenol interface and in a slight increase of polysomal RNA yield (maximally 20%). However, the enzyme is probably more

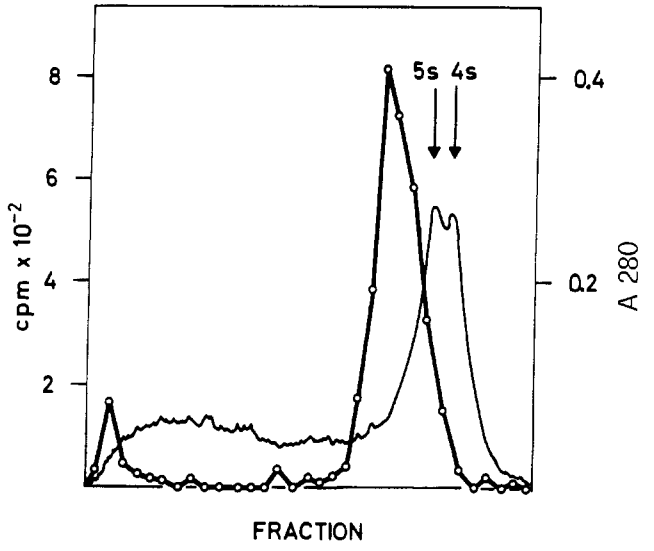

FIGURE 3: Acrylamide gel electrophoresis of the RNase-resistant fraction from peak II. The RNase-resistant fraction was prepared as described in Table II, and made 85% (v/v) in dimethyl sulfoxide and incubated at 37° for 20 min before loading onto the gel (modified procedure of Katz and Penman, 1966). The gels were prepared according to Loening (1967), as modified by Mirault and Scherrer (1971), and preelectrophoresed at 10 V/cm for 1–2 hr. The electrophoresis buffer was 0.04 M triethanolamine 0.02 M sodium acetate, 0.002 M EDTA, 2.5% glycerol, and 0.2% sodium dodecyl sulfate. A sample aliquot was electrophoresed through a 5% acrylamide cushion (1 cm) over a 3% gel (11 cm) for 190 min at 10 V/cm. Internal absorbance markers of 4S tRNA and 5S rRNA are indicated. Radioactivity, as ^{32}P, was determined in toluene–Omnifluor on 4-mm gel slices, after hydrolysis in concentrated NH_4OH overnight and evaporation to dryness.

important in protecting against traces of RNase (Weigers and Hilz, 1971).

Characterization of Peak I and Peak II RNA. The RNA was fractionated into two peaks on poly(dT)-cellulose (see Methods and Materials) and was shown to be 98–100% sensitive to alkali hydrolysis and 100% resistant to digestion with pancreatic DNase. Peak I consists of RNA that does not adsorb to the column, while peak II RNA sticks to the column at high salt. The sedimentation profiles of peaks I and II are given in Figure 2. [^{32}P]RNA from both peaks was examined for RNase sensitivity (Table I).

Digestion with RNase A and T1 shows that the RNA of peak II is resistant (15–20%), while peak I RNA is not. There is thus a correlation between adsorption to poly(dT)-cellulose and RNase resistance. The RNase-resistant RNA was analyzed by gel electrophoresis (Figure 3), and its nucleotide composition was measured (Table II). It migrated as a sharp and nearly symmetrical band in gels (Figure 3) (about 7 S),

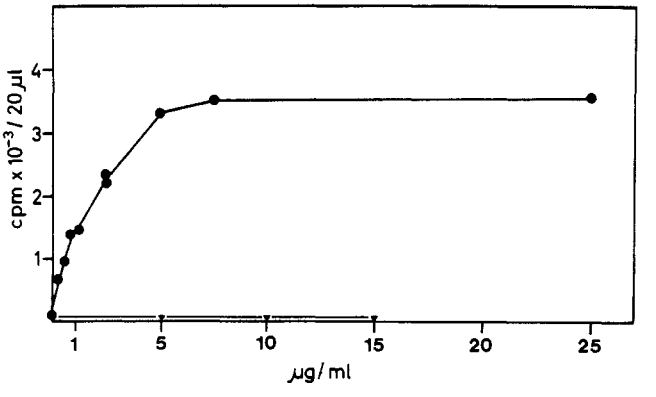

FIGURE 4: Dependence of incorporation on the concentration of peak II RNA (poly(A) containing RNA) and of peak I RNA as control. A constant amount (0.06 µg/ml) of the oligo(dT) digested with DNase A at a concentration of 20 µg/ml (see Table III) was used, and the incubation was done for 30 min at 37° in the presence of [^{3}H]dTTP at a concentration of 4 µM and of a specific activity of 5000 cpm/pmol. The total sample was precipitated with trichloroacetic acid and counted.

TABLE I: RNase Resistance (%) of RNA Fractions Obtained from Poly(dT) Chromatography.[a]

RNA Fraction	Procedure of RNA Extraction		
	1	2	3
Peak I	0.4 (430)	0.7 (1160)	0.9 (2080)
Peak II	15 (12,490)	16 (13,930)	21 (8720)

[a] Preparation of peaks I and II from [^{32}P]polysomal RNA by chromatography on poly(dT)-cellulose was as described in Methods and Materials, and the RNA was precipitated with ethanol. Assays for RNase resistance were performed essentially according to Darnell *et al.* (1971). The RNA, redissolved in water, was incubated in 2 × SSC with RNase A (10 µg/ml) and RNase T1 (1 µg/ml) at 37° for 30 min. RNA was precipitated at 0° in the presence of carrier poly(A) (20 µg/ml), and washed twice at 0° with perchloric acid (0.5 N). The radioactivity in the supernatant was determined and the acid-precipitable radioactivity was calculated. This is expressed as a per cent of the total radioactivity of each fraction. Radioactivity (cpm), as determined on an aliquot, is indicated in parentheses. Procedures 1, 2, and 3 for the preparation of polysomal RNA are described in Methods and Materials.

TABLE II: Nucleotide Composition (%) of RNase-Resistant RNA from Peak II.[a]

Nucleotide	Procedure of RNA Extraction		
	1	2	3
CMP	1.1	1.0	0.0
AMP	78.5	75.0	82.5
GMP	7.4	10.7	8.1
UMP	13.0	13.3	9.5

[a] The experiment was performed on the acid-precipitable [^{32}P]RNA following RNase digestion (see Table I). [^{32}P]RNA was hydrolyzed in 0.4 N KOH for 18 hr at 37° and the nucleotides (700–1200 cpm) were separated on Whatman 52 paper by high voltage electrophoresis (4000 V for 75 min) using a pyridine–acetate buffer, pH 3.5, according to Salzman and Sebring (1964). The nucleotide spots were identified, cut out, and counted directly in toluene–Omnifluor. Procedures 1, 2, and 3 for the preparation of polysomal RNA are as described in Methods and Materials.

TABLE III: Determination of Optimal Size of Oligo(dT) Primer.[a]

Primer dT Concn in Assay (μg/ml)	DNase A Concn (μg/ml)	Incorp per 20 μl	% of Max Incorp Obsd
None	None	282	1.9
3	None	2,601	17.6
3	0.2	2,878	19.5
3	2.0	3,729	25.3
3	20.0	6,116	41.5
3	200.0	14,713	100

[a] The DNase A treatment was done as indicated in Methods and Materials. The resulting oligo(dT) was treated with 0.1% DEP at room temperature for 5 min and boiled for 10 min prior to use in the enzyme reaction. A constant amount of RNA from peak II (12.5 μg/ml) was used and the [^{3}H]dGTP had a specific activity of 5000 cpm/pmol. The incorporation was done for 30 min at 37°, followed by precipitation with trichloroacetic acid.

suggesting a relatively constant length of RNase-resistant segments. The percentage of AMP (80%) and the size of the RNase-resistant RNA are consistant with the existence of some poly(A)-rich sequences in peak II. In addition, significant amounts of GMP and UMP also occur in the RNase-resistant fraction of peak II.

Properties of the Poly(A) RNA-Dependent DNA Synthesis. The synthesis of cDNA is completely dependent upon poly(A) RNA (Figure 4) and oligo(dT) primer (Tables III and IV) (Ross *et al.*, 1972; Verma *et al.*, 1972; Kacian *et al.*, 1972). If RNA from peak I (mainly rRNA) is used as template, negligible incorporation occurs (Figure 4). The dependence of the reaction on the size of the oligo(dT) is given in Table III. The smallest oligo(dT) stimulates the incorporation to a much larger extent than the longer polymers. If a constant amount of RNA (12.5 μg/ml) is used with increasing amounts of the smallest oligo(dT) (Table IV), the incorporation reaches its maximum around 0.3 μg/ml. An excess of oligo(dT) appears to inhibit the reaction of the enzyme.

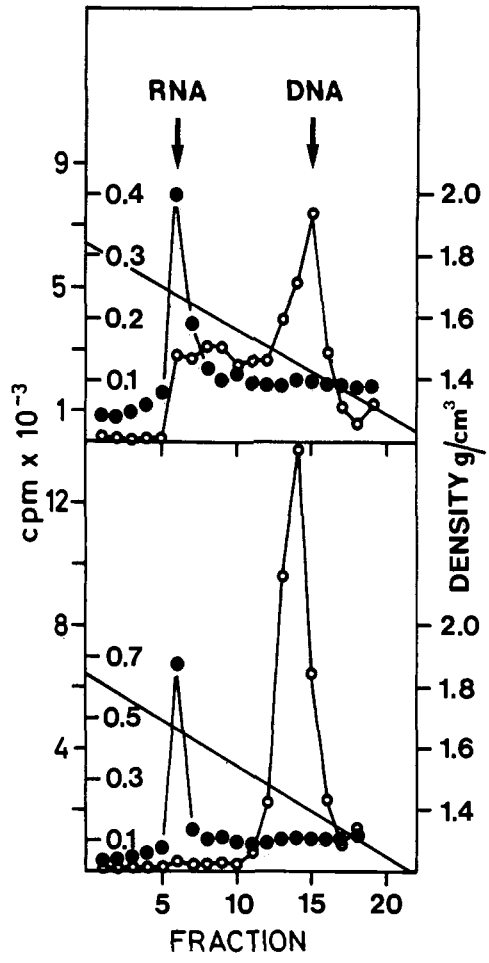

FIGURE 5: Cesium sulfate density gradient centrifugation of native (above) and heat-denatured (below) cDNA made from poly(A) RNA. The products labeled with 41.6 μM [^{3}H]dCTP were put on 4 ml of Cs_2SO_4 with an initial density of 1.56 g/cm^3 and centrifuged in an SW 50.1 rotor at 35,000 rpm and 15° for 66 hr in a Spinco ultracentrifuge. *E. coli* rRNA was added as an absorbance marker. Fractions were collected, the density and the A_{260} were determined, and the radioactivity was measured after precipitation with trichloroacetic acid: A_{260} (●) and cpm (○).

TABLE IV: Determination of Optimal Concentration of Oligo(dT) with a Constant Amount of Poly(A) RNA (Peak II).[a]

Oligo(dT) Concn (μg/ml)	Radioactivity (cpm) Incorp	% Max Incorp
None	408	2.05
0.03	9,351	47.2
0.09	9,849	49.7
0.15	18,449	93.1
0.30	19,808	100
0.90	17,579	88.7
1.50	15,427	77.8
3.0	14,713	74.2
9.0	11,664	58.8

[a] The smallest oligo(dT) fraction obtained by digestion with 200 μg/ml of DNase A (see Table III) was used, and the reaction conditions were identical with those in Table III and as indicated in Methods and Materials.

Characterization of the cDNA Product. Analyses on Cs_2SO_4 equilibrium density gradients conclusively demonstrate that the radioactivity is in DNA (Figure 5). The native product is very heterogeneous in density, and above 50% of the radioactivity bands are at a density higher than that of free DNA. After heat denaturation, all the radioactivity is shifted into the DNA-density region. This is the behavior expected for DNA pieces synthesized from an RNA template.

The size of the product synthesized (Figure 6) depends strongly upon the concentration of deoxynucleoside triphosphates used. At the highest dGTP concentration, the average size is 7.6 S and the largest molecules synthesized are about 10 S. The small amount of synthesis with rRNA (Figure 4) yields a small product (Figure 6).

The faithfulness of the DNA product was studied by hybridization (Table V). The results show that actinomycin D is effective in preventing double-stranded DNA from being synthesized (Verma *et al.*, 1972). Only 4.8% of the input counts are resistant to the single-strand specific nuclease. No an-

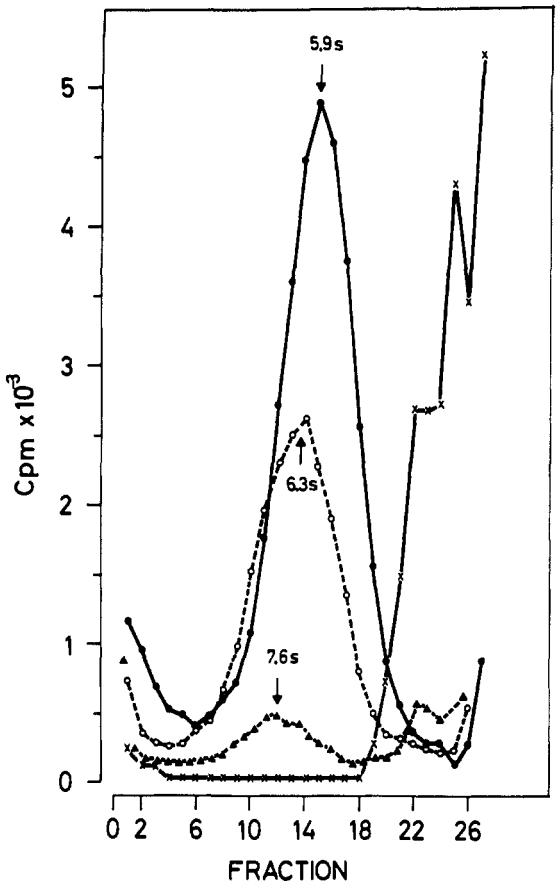

FIGURE 6: Alkaline sucrose gradient centrifugation of poly(A) RNA (peak II) products made in the presence of different concentrations of [^{3}H]dGTP and 1 mM unlabeled triphosphates. A 5–20% linear sucrose gradient, containing 0.2 N NaOH–10 mM EDTA–100 mM NaCl, was centrifuged for 15 hr at 45,000 rpm and 4° in a Spinco SW 50.1 rotor. Fractions were collected and precipitated with trichloroacetic acid and counted. Bovine serum albumin (4.6 S) was centrifuged as a sedimentation marker in a parallel neutral sucrose gradient. The following symbols indicate the products made in the presence of 4.16 μM [^{3}H]dGTP (●—●); 41.6 μM [^{3}H]dGTP (○- -○); 40.0 μM [^{3}H]dGTP + 360 μM unlabeled dGTP (▲- -▲); and product from peak I (rRNA) in 400 μM [^{3}H]dGTP (X- -X).

TABLE V: Hybridization of cDNA Made from Poly(A) RNA (Peak II).[a]

Sample	Input (cpm)	Hybridized (cpm)	% Hybridized
cDNA	439	21	4.8
cDNA + 5 μg/ml of poly(A) RNA (peak II)	439	179	40.7
cDNA + 25 μg/ml of poly(A) RNA (peak II)	439	268	61.0
cDNA + 50 μg/ml of poly(A) RNA (peak II)	439	306	69.7
cDNA + 10 μg/ml of rRNA (peak I)	439	48	10.9
cDNA + 50 μg/ml of rRNA (peak I)	439	75	17.1
cDNA + 5 μg/ml of 10S rabbit Hb mRNA	439	13	3.0
cDNA + 25 μg/ml of 10S rabbit Hb mRNA	439	30	6.8
cDNA + 50 μg/ml of 10S rabbit Hb mRNA	439	34	7.7
cDNA + 100 μg/ml of *E. coli* rRNA	439	16	3.6

[a] cDNA (average size 5.9 S in alkali) labeled with 41.6 μM [^{3}H]dCTP and 51.0 μM [^{3}H]dGTP was hybridized to different RNA in a 10 μl total volume, containing 0.3 M NaCl–40 mM Tris-HCl (pH 7.5)–0.1% sodium dodecyl sulfate, and was sealed in a 50-μl "Microcap" for 2 hr at 65°. The samples were then blown into 200 μl of 0.03 M sodium acetate (pH 4.6)–1 mM $ZnSO_4$–5% glycerol–0.05 M NaCl containing 4 units of single-strand specific nucleases from *Aspergillus oryzae* (Vogt, V. (1972), personal communication) (1 unit = 10 μg of DNA digested in 10 min at 45°) and 5 μg of denatured, sonicated salmon sperm DNA and incubated at 45° for 30 min. Trichloroacetic acid precipitable radioactivity was counted. A blank sample background of 20 cpm has been subtracted.

nealing occurs with *Escherichia coli* rRNA or 10S rabbit Hb mRNA. A small amount of hybridization occurs with RNA from peak I, and the highest amount with template RNA. A 70% saturation level was obtained with template RNA.

Discussion

When conditions minimizing rRNA contamination are established, poly(dT) chromatography is a powerful tool for the purification of RNA containing poly(A) sequences. The contamination of peak II by rRNA (1% of total polysomal RNA) suggests that the poly(A) RNA of peak II (2.5% of total polysomal RNA) is at most 60% mRNA. This implies that a given mRNA would have to be present as a large percentage of all other mRNAs in order to be easily identified.

The fact that poly(A) is eluted in several peaks (Figure 1b) does not necessarily imply that these peaks represent different classes of poly(A) molecules with different binding properties. Instead, it probably reflects the binding to different chain lengths of poly(dT) on the column, as suggested from the data of Gilham (1964). The same may apply to the elution of natural mRNA.

RNA from myeloma polysomes does contain poly(A) sequences. Contrary to the situation in other tissues (Darnell *et al.*, 1971; Edmonds *et al.*, 1971; Lee *et al.*, 1971), the RNase-resistant RNA fraction is not exclusively poly(A), but contains significant amounts of GMP and UMP (Table II). The uniformity of the 7S RNase-resistant peak implies that either there are many different moieties of mRNA in that fraction, all with poly(A) sequences of the same length, or that peak II contains one predominant species of mRNA.

The presence of poly(A) sequences in the RNA fraction of peak II suggests that it could serve as a template for cDNA synthesis. Our results show that a DNA product of appreciable size can be synthesized with high efficiency from such a crude fraction of poly(A) RNA.

The product specificity, when examined by hybridization with RNA from peak I, indicated a small but significant complementarity (Table V). The reason for this is unknown, but one explanation could be the following. Peak I RNA, consisting mainly of rRNA (Figure 2), contains no detectable poly(A) by the following criteria: no synthetic [^{3}H]poly(A) eluted in peak I (Figure 1), and no RNase resistance was

observed. Furthermore, peak I RNA has no significant template activity for cDNA synthesis (Figure 4). Therefore, it seems unlikely that the positive annealing observed is due to a contamination of peak I with poly(A)-rich RNA of peak II. A more likely explanation might be that sequences similar to those occurring in the poly(A)-rich RNA of peak II also occur in peak I, but without poly(A).

The fact that the annealing with template RNA is not complete at concentrations which give complete hybridization with pure Hb cDNA (Imaizumi *et al.*[3]) could be explained as follows. The template RNA used is a heterogeneous fraction, and we have no indication that the RNA-dependent DNA polymerase uses all RNA templates equally well. Therefore, the spectrum of cDNA synthesized may not necessarily reflect the spectrum of different RNA molecules in peak II.

The experiments performed so far show that the approach successfully used with Hb mRNA (Ross *et al.*, 1972; Verma *et al.*, 1972; Kacian *et al.*, 1972) may work equally well with myeloma mRNA, and may have general applicability to other systems. The isolation of a 14S RNA from the poly(A)-rich RNA described in this report, its purification to a single band on acrylamide gel, and its activity in the synthesis of protein and of cDNA will be reported elsewhere (Mach *et al.*, 1973; Diggelmann *et al.*, 1973). Since the writing of this manuscript, a recent independent report (Swan *et al.*, 1972) has shown that RNA prepared from the same tumor by poly(dT) chromatography can direct the synthesis of light-chain immunoglobulin. That RNA was heterogeneous in size, but it is of interest that the highest template activity coincided with the 14S peak mentioned above. It is also of interest to note that the degree of rRNA contamination in our crude poly(A)-rich RNA from peak II (Figure 2) is less than in the same fraction of Swan *et al.* (1972). This difference may be explained as a result of the slightly modified poly(dT)-cellulose preparation and the different elution conditions used with the poly(dT)-cellulose column.

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