# Estimation of the Number of Nucleotide Sequences in Mouse DNA Complementary to Messenger RNAs Specifying a Complete Mouse Immunoglobulin†

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ABSTRACT: A fraction of ribonucleic acid (RNA) enriched in messenger RNA (mRNA) coding for immunoglobulin G (IgG) was isolated from cells of mouse myeloma RPC5 using specifically purified antibodies to immunoprecipitate polyribosomes engaged in IgG $\gamma$ heavy and $\kappa$ light chain synthesis. More than 85% of the RNA present consisted of IgG mRNA as determined by an analysis of the products translated in its presence in the wheat germ system. IgG mRNA labeled with $^{125}I$ was hybridized with mouse liver DNA. Approximately 95% of the RNA hyridized with mouse a $C_0t_{1/2}$ of $4.0 \times 10^3$, indicating that the complementary DNA sequences were present less than five times per haploid genome. In contrast, approximately 75% of poly(adenylic acid) containing RNA prepared from unfractionated polyribosomes of RPC5 cells hybridized with a $C_0t_{1/2}$ of $3.3 \times 10^3$; 25% of such RNA formed hybrids at lower $C_0t$ values.

The development of methods for isolating messenger RNAs[1] for individual and identifiable species of protein molecules has allowed investigations by molecular hybridizations into the proportion of the genome devoted to their synthesis. Using this experimental approach, attempts have been made to determine the total number of genes specifying immunoglobulins (Cohen, 1967; Raska and Cohen, 1968; Faust et al., 1974; Honjo et al., 1974; Stavnezer et al., 1974; Milstein et al., 1975). The constant regions of mouse $\kappa$ light chains appear to originate from few DNA sequences (less than five per haploid genome). The number of sequences specifying the variable region is less certain and little information is reported on the sequences involved in specifying an entire lg molecule. Transcription of immunoglobulin mRNA using reverse transcriptase proceeds for approximately 300–700 nucleotides from the 3′ terminus of the RNA used as template and yields a transcript mainly of the light chain constant region (Honjo et al., 1974). To determine the reiteration frequency of sequences specifying the variable portions of immunoglobulin chains, hybridization using labeled immunoglobulin mRNA as a "probe" is necessary. Using partially purified mRNAs coding for $\kappa$-, $\lambda$-, or $\alpha$-immunoglobulin chains, several investigators have estimated the number of sequences specifying the variable regions of different species of immunoglobulins. The results of different laboratory groups have been at times at variance on this subject in part because of the difficulties in obtaining RNA "probes" of sufficient purity (Delovitch and Baglioni, 1973; Leder et al., 1974, 1975; Milstein et al., 1975; Premkumar et al., 1974; Tonegawa et al., 1974a, b; Tonegawa, 1976).

In the study reported here, we used preparations of immunoglobulin mRNA obtained from mouse plasmacytomas engaged in the synthesis of both immunoglobulin $\kappa$ and $\gamma$ chains. The mRNAs were isolated by methods similar to those developed by Shapiro et al. (1974), for the isolation of ovalbumin mRNA, using in our experiments specifically purified antibody to immunoprecipitate immunoglobulin-producing polysomes. The mRNA obtained was estimated to contain more than 85% IgG mRNA. The results of this study using such mRNA in hybridization reactions revealed essentially a monophasic transition with a $C_0t_{1/2}$ of $4 \times 10^3$. The presence of a significantly large rapidly hybridizing fraction of mRNA was not detected.

This is the first report of molecular hybridizations using RNA for a complete immunoglobulin molecule isolated by the highly specific polyribosome precipitation method.

## Materials and Methods

*Tumor Source and Animals.* The following mouse plasmacytoma cell lines were obtained from Michael Potter, National Institutes of Health, Bethesda, Md.: (1) RPC5 (AdjPC5), producing $\gamma$ G2a immunoglobulin containing $\kappa$ L chains;[1] (2) MOPC41, producing $\kappa$ L chains.

The cells were maintained by serial transfer in histocompatible recipients; $10^7$ cells suspended in HBSS were injected subcutaneously into female Balb/c mice 8–12 weeks of age.

IgG was isolated from the sera of mice bearing advanced RPC5 tumors or from the ascitic fluid of mice injected ip with RPC5 cells.

*Immunoprecipitation of Polyribosomes from RPC5 Cells. (a) Preparation of Antisera.* Young female rabbits were immunized by injecting intramuscularly in complete Freund's adjuvant (1:1 v/v mixture of protein solution and adjuvant) 1 mg of IgG isolated from serum of mice bearing RPC5 tumors. Ten days later, 2 mg of the same protein in incomplete Freund's adjuvant was injected sq. Serum was obtained on the 20th day. Immunized rabbits were injected periodically with 1 mg of protein in saline before bleeding 10 days later.

The globulin fraction from rabbit antisera was prepared by ammonium sulfate precipitation; the antibodies present were

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[1] Abbreviations used are: EDTA, ethylenediaminetetraacetic acid; SSC, 0.15 M sodium chloride–0.015 M sodium citrate; RNase, ribonuclease; PBS, equimolar sodium phosphate buffer (pH 6.8); HTP, hydroxylapatite; $A_{260}$, absorbance at 260 $\mu$m; DNase, deoxyribonuclease; poly(A), poly(adenylic acid); $T_m$ temperature of 50% thermal denaturation; Ig, immunoglobulin; mRNA, messenger ribonucleic acid; L chain, light chain; H chain, heavy chain; HBSS, Hanks balanced salt solution; ip, intraperitoneal; sq, subcutaneous.

fractionated further by adsorption to and recovery from RPC5 IgG bound to Sepharose.

"RPC5-Sepharose" was prepared by mixing 20 mg of IgG from RPC5 cells in 0.1 M sodium carbonate (pH 9.0) with 1 g of cyanogen bromide activated Sepharose (Pharmacia), washed previously with $10^{-3}$ M HCl and equilibrated before use with sodium carbonate. The mixture was stirred for 1 h at room temperature and incubated further at 4 °C for approximately 18 h. It was sedimented by centrifugation and incubated with 1 M ethanolamine for 2 h at room temperature. The Sepharose was washed twice with 0.01 M sodium phosphate (pH 7.2), containing 0.015 M NaCl, and then poured into a 1 × 10 cm column. To remove adsorbed but noncovalently bound IgG, the column was washed successively with 30 ml of 0.1 M sodium acetate (pH 4.8)–1 M NaCl, and then with 30 ml of 0.1 M sodium carbonate (pH 7.6)–1 M NaCl. After three washing cycles the column was equilibrated with 50 ml of PBS (pH 7.2).

The globulin fraction of anti-RPC5 IgG serum (about 400 $A_{280}$ units of protein) was dissolved in 5 ml of PBS and passed slowly through the "RPC5-Sepharose" column over a period of about 1 h. The column was washed successively with 30 ml of phosphate buffer, followed by three alternate washes with each of the acetate buffer (pH 4.8) and the carbonate buffer (pH 7.6). Antibody bound to the column was eluted with 1 N acetic acid and immediately dialyzed against cold PBS for 12 h and then against cold water for an additional 24 h. It was lyophilized and stored in sealed vials at −20 °C.

(*b*) *Quantitative Precipitation of Column Fractionated Anti-RPC5 IgG with Goat Anti-Rabbit IgG.* Column fractionated anti-RPC5 IgG (100 μmg; 2 mg/ml in PBS) was mixed in a constant volume with varying quantities of the IgG fraction of goat anti-rabbit IgG serum (42 $A_{280}$ units/ml in PBS; Cappel Laboratories, Downington, Pa.) and then incubated at 0 °C for 1 h. The quantity of antigen–antibody precipitate which formed was estimated by optical density (600 nm). One-half of a milliliter of the IgG fraction of goat anti-rabbit IgG serum (21 $A_{280}$ units) led to maximal precipitation of 50 μl (100 μg) of rabbit anti-RPC5 IgG and this ratio was used in all relevant experiments. RNase activity was not detected in the antibodies used for polysome precipitation.

(*c*) *Immunoprecipitation of Polysomes.* Tracer amounts of radioactivity were introduced into the RNA of RPC5 cells by injection of 100 μCi of isotonic [$^3$H]uridine directly into the tumors 1 h before the animals were killed. A suspension of tumor cells was washed twice with 0.14 M NaCl–0.01 M Tris-Cl (pH 7.4) buffer and 1.5 mM $MgCl_2$. The cells were resuspended in a small volume of polysome buffer (PB500) (0.025 M Tris-Cl (pH 6.6)–0.025 M NaCl–5 mM $MgCl_2$–500 μg/ml heparin); sufficient 10% Triton X-100–10% sodium deoxycholate in the same buffer was added to yield a final concentration of 0.5–1.0% each, and the mixture was incubated at 0 °C for 15 min. Cell nuclei were sedimented at 1000*g* and discarded. After centrifugation for 10 min at 27 000*g*, polysomes were isolated from the supernatant which was layered over a discontinuous sucrose density gradient consisting of 1 ml of 2.5 M sucrose in PB500 and 2 ml of 1.0 M sucrose in PB500 in a five-ml centrifuge tube and centrifuged for 2 h at 180 000*g* at 2 °C in a Beckman-Spinco SW50L rotor. The polysomes forming a visible band were removed through a needle inserted through the tube wall and dialyzed for 6 h against a buffer solution containing 0.025 M Tris-Cl (pH 7.1)–0.15 M NaCl–5 mM $MgCl_2$–500 μg/ml heparin. Heparin (500 μg/ml) was added to all buffer solutions as an inhibitor of ribonuclease (Rhoads et al., 1973).

Polysomes were removed from the dialysis bath and centrifuged at 27 000*g* for 10 min immediately before they were immunoprecipitated with specific antiserum. Column fractionated anti-RPC5 IgG (150 μg/ml final concentration) was added to a polysome suspension of approximately 10 $A_{260}$ units/ml and the mixture was incubated 30 min at 0 °C before an immunologic equivalent of goat anti-rabbit IgG was added and the incubation continued for an additional 30 min. It was layered over a discontinuous sucrose gradient consisting of 3 ml of 0.5 M sucrose and 6 ml of 1.0 M sucrose in the same buffer along with 1% Triton X-100 and 0.5% sodium deoxycholate. The tubes were centrifuged for 20 min at 27 000*g* at 4 °C and the precipitate was resuspended in 1 ml of the buffer and centrifuged as previously. After the second centrifugation, the precipitate was resuspended in buffer–3% sodium dodecyl sulfate; an aliquot of 50 μl was removed, and the radioactivity present was determined after the addition of 0.3 ml of Nuclear Chicago Solubilizer (NCS) and 10 ml of Liquifluor. The remaining suspension was diluted to about 10 ml with a solution of 0.1 M NaCl–0.01 M Tris-Cl (pH 7.2)–0.001 M EDTA–0.5% sodium dodecyl sulfate and extracted at least three times with 1:1 phenol and chloroform. The RNA was precipitated from the final aqueous layer by adding 0.1 volume of 1.5 M NaCl and 2.5 volumes of cold ethanol. It was stored at −20 °C in ethanol until further use (Shapiro et al., 1974).

The proportion of polysomes precipitated by specific antiserum was determined by comparing the radioactivity in immunoprecipitated polysomes with the radioactivity present in an equivalent amount of polysomes precipitated by $Cl_3CCOOH$.

*Preparation of Poly(A) Containing RNA from Polysomes Immunoprecipitated with Specific Antibody.* Poly(A) containing RNA was separated from other polysomal RNAs by chromatography on cellulose (Schutz et al., 1972; Sullivan and Roberts, 1973). The column containing cellulose (Sigmacell 38 (Sigma)), 1 g of dry weight per 100 $A_{260}$ units of RNA, was washed with water and then equilibrated with 0.01 M Tris-Cl (pH 7.6)–0.05 M KCl–1.5 mM $MgCl_2$. RNA obtained previously by ethanol precipitation was centrifuged at 20 000*g* for 10 min at 4 °C, and the pellet dissolved in 1.0 ml of water, and then diluted to 10 ml with 0.5 M KCl buffer and applied to the column. The column was washed extensively with the application buffer until the $A_{260}$ of the fractions collected was less than 0.01. The RNA adsorbed to the cellulose was eluted with water adjusted to pH 7.0 and was adsorbed to and eluted from fresh cellulose a second time under similar conditions and then precipitated by adding 0.1 volume of 1.5 M NaCl and 2.5 volumes of ethanol. It was stored at −20 °C until further use.

Radioactive RNA was prepared by the method of Commerford (1971), with the exception that as catalyst Chloramine-T was substituted for thallium trichloride.

*Translation of RNA in a Cell Free Protein Synthesizing System Derived from Wheat Germ.* Protein synthesis directed by poly(A) RNAs obtained from RPC5 cells was investigated in a wheat germ cell free system in collaboration with Dr. D. S. Shih and Dr. P. Kaesberg (Biophysics Laboratory and Biochemistry Department, University of Wisconsin, Madison, Wis.) and with S. Chan, P. Keim, and Dr. D. Steiner (Department of Biochemistry, University of Chicago, Chicago, Ill.) (Shih and Kaesberg, 1973; Chan et al., 1976). To determine the total radioactivity incorporated into polypeptides, 5 ml of 5% $Cl_3CCOOH$ was added to a 10-μl aliquot of the mixture and the precipitate was heated at 90 °C for 15 min. The precipitates were collected on 0.45-μm pore size Millipore

filters, washed with $Cl_3CCOOH$, and dried. The radioactivity present was determined after the addition of Liquifluor in a liquid scintillation spectrometer (Davies and Kaesberg, 1973; Shih and Kaesberg, 1973).

The polypeptides synthesized in the cell free system were immunoprecipitated using rabbit anti-mouse IgG serum (RAM IgG) or rabbit anti-mouse $\gamma$ heavy chain sera (RAM$\gamma$) (Bionetics Laboratory Products, Kensington, Md.), possessing no detectable specificity for $\kappa$ chains from mouse myeloma MOPC41 cells.

*DNA Isolation and Preparation for Experimental Use.* DNA was extracted from washed nuclei of the cells of the livers of Balb/c mice by the Marmur method, with modifications (Bishop, 1972; Marmur, 1961). The method of Bishop (1972) was followed to prepare the DNA for use in hybridization experiments. DNA in 0.1 × SSC was concentrated under vacuum to a final concentration of approximately 500 $\mu$g/ml. It was sheared during two passages at 20 000 psi through the needle valve of precooled French pressure cell, and then dialyzed against SP-50 buffer (0.3 M NaCl-0.01 M sodium acetate (pH 5.5)). It was applied to a 2 × 35 cm column of Sephadex SP-50. The material eluting with SP-50 buffer in the exclusion volume was collected, precipitated with 2 volumes ethanol and stored at −20 °C.

The molecular weight of the sheared DNA was determined from its sedimentation coefficient in 0.9 M NaCl-0.1 M NaOH according to the method described by Studier (1965). Its mean alkaline $s_{20,w}$ was 5.3, corresponding to a single-strand molecular weight of $10^5$ and an average length of about 300 nucleotides. No reactive contaminants were detected to the limits of the Lowry method (less than 3 $\mu$g/ml) in a solution of DNA at a concentration of 300 $\mu$g/ml (Lowry et al., 1971). No RNase activity was detected in the DNA preparations used.

*RNA-DNA Hybridization: Experimental Method.* Hybridization reactions were carried out according to methods described by Davidson et al. with minor modifications (Hough and Davidson, 1972; Smith et al., 1974). RNA was labeled with $^{125}I$ as described previously. Sheared DNA at a concentration of approximately 5 mg/ml in 0.01 M PBS was denatured during incubation in a boiling water bath for 10 min and then transferred to a second water bath maintained at the incubation temperature used for hybridization. Sodium phosphate buffer containing sodium dodecyl sulfate was added to a final concentration of 0.12 M PBS and 0.1% sodium dodecyl sulfate, along with $^{125}I$-labeled RNA. The time at which the sodium phosphate concentration was adjusted was taken as the beginning of the reaction.

In some instances, the sodium ion concentration in the reaction mixtures was raised as high as 0.72 M using 0.48 M PBS. $C_0t$ values were corrected for the relative increase in hybridization rates resulting from increases in the monovalent cation concentration.

*Separation of RNase Resistant Hybrids by Sephadex G-200 Chromatography.* Hybrid samples were treated with 10 $\mu$g/ml RNase for 20 min at 37 °C as described above and then passed through a 1 × 35 cm Sephadex G-200 column in 0.12 M PBS. The proportion of the radioactivity in hybrid form was taken as the total radioactivity in the exclusion volume after RNase digestion divided by the total $Cl_3CCOOH$-precipitable radioactivity recovered from the column. From this value was subtracted the proportion of radioactivity present in the exclusion volume for the samples taken at zero time. If less than 85% of the radioactivity applied to the column was recovered, the preparation was discarded.

*Thermal Denaturation of Hybrids.* After RNase digestion and passage through a column containing Sephadex G-200 in 0.12 M PBS, the material recovered in the exclusion volume was applied to a 1 $cm^3$ Bio-Rad DNA grade hydroxylapattite (HTP) column equilibrated previously at 60 °C in the same buffer with 0.1% sodium dodecyl sulfate. The nucleic acids retained by the column under these conditions were eluted by raising the temperature in increments of 5 or 10 °C up to 100 °C. At each temperature 5 volumes of buffer were passed through the column, and the radioactivity in the material recovered was determined.

*Recovery of RNA from RNA-DNA Hybrids.* RNA-DNA hybrids isolated after RNase digestion by passage through a Sephadex G-200 column were passed through a second column containing Sephadex G-200 in 0.3 M sodium acetate (pH 6.8). The material in the exclusion volume of the second column was precipitated with ethanol and incubated at −20 °C overnight and then centrifuged at 27 000$g$ for 10 min at 4 °C and redissolved in about 0.2 ml of water. It was brought to 6% (v/v) with neutralized formaldehyde and 0.01 M EDTA (pH 6.8) and then incubated at 37 °C for 1 h. Sufficient 1.5 M NaCl was added to give a final concentration of 0.1 M, and the mixture was centrifuged at 10 °C in a 7.5-22% sucrose density gradient in 0.1 M NaCl-0.01 M EDTA-6% formaldehyde (pH 6.8) for 8 h at 28 000 rpm in a SW50L rotor. Fractions were collected, and the radioactivity in each fraction was determined.

*Reassociation of DNA.* Samples taken from hybridization reaction mixtures after incubation to various $C_0t$ values were applied to hydroxylapatite columns in 0.2 M sodium phosphate (pH 6.8)-8 M urea-1% sodium dodecyl sulfate at 40 °C; double-stranded nucleic acids were eluted by raising the column temperature to 80 °C and washing with the same buffer. To measure the reassociation of DNA, the $A_{260}$ of each fraction from hydroxylapatite columns was determined for samples taken at each $C_0t$ value and the proportion of DNA reassociated was calculated (Smith et al., 1974).

## Results

*Polyribosome Precipitation.* The optimum concentration of antibody used to precipitate polysomes from RPC5 cells was determined by incubating freshly isolated polysomes with varying quantities of specifically purified antibody followed by an immunologic equivalent of goat anti-rabbit IgG (IgG fraction (21 $A_{280}$ units/100 $\mu$g of anti-RPC5 IgG)). The results (Figure 1) indicate that the proportion of [$^3$H]uridine-labeled polysomes precipitated increased with the quantity of antibody added until at saturation the proportion of polysomes precipitated was approximately 9%. In other experiments, the proportion of polysomes precipitated in this manner varied from 7 to 13%. Nonspecific precipitation of the polysomes of RPC5 cells amounted to less than 1% at concentrations of anti-IgG up to 150 $\mu$g/ml (Figure 1) using nonimmune rabbit IgG rather than specific antibody. As the nonspecific precipitation of polysomes was lowest at an antibody concentration of 150 $\mu$g/ml, this concentration of anti-RPC5 IgG was used in all subsequent experiments.

Using purified antibody, the proportion of polysomes precipitated equaled approximately 9% (for nine determinations) (Table I); nonspecific precipitation of aliquots of the same preparations using normal rabbit IgG was less than 1.0%. The use of normal rabbit IgG, absorbed before use by incubation with an excess of RPC5 IgG (to block antigen combining sites in normal rabbit IgG which might be specific for RPC5 IgG), reduced the nonspecific precipitation of polysomes to an average of 0.6%. This figure was approximately the same as that obtained when RPC5 polysomes were incubated without

TABLE I: Immunoprecipitation of Polysomes from RPC5 and Liver Cells.

| Polysomes | Antibody | % of Total cpm Precipitated |
|---|---|---|
| RPC5 | | 0.7 |
| | Normal rabbit IgG | 1.0[a] |
| | Normal rabbit IgG, preincubated with antigen | 0.6[b] |
| | Rabbit anti-RPC5 IgG | 9.3[a] |
| | Rabbit anti-RPC5 IgG, preincubated with IgG | 0.7[b] |
| | Rabbit anti-RPC5 IgG, preincubated with $\kappa$ chain | 5.5[c] |
| Liver | | 0 |
| | Normal rabbit IgG | 0 |
| | Rabbit anti-RPC5 IgG | 0 |

[a] Average of nine determinations. [b] Antibodies were mixed with 2 mg of RPC5 IgG and incubated for 30 min at 0 °C before being used to precipitate polysomes. Average of two determinations. [c] Antibody was mixed with 1 mg of $\kappa$ light chain and incubated for 30 min at °C before being used to precipitate polysomes. Average of two determinations. Polysomes from RPC5 or liver cells (8 $A_{260}$ units in 1 ml) were immunoprecipitated as described in the text. The concentration of rabbit anti-RPC5 IgG and normal rabbit IgG was 150 $\mu$g/ml. An equivalent amount of goat anti-rabbit IgG (IgG fraction) was added for indirect precipitation. The percent of radioactivity precipitated was determined by comparing radioactivity precipitated by antibody with the total $Cl_3CCOOH$-precipitable radioactivity in the polysome preparation.

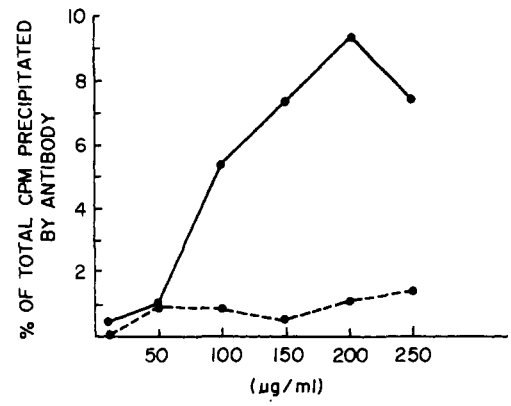


FIGURE 1: Immunoprecipitation of polyribosomes from RPC5 cells with rabbit anti-RPC5 IgG. Reaction mixtures contained 5 $A_{260}$ units of polysomes (2194 cpm/$A_{260}$ unit) in 0.5 ml of precipitation buffer (0.15 M NaCl–0.025 M Tris-Cl (pH 7.1)–5 mM $MgCl_2$) with 500 $\mu$g/ml heparin. The indicated amounts of rabbit anti-RPC5 IgG or normal rabbit IgG were added. An immunologic equivalent of goat anti-rabbit IgG (IgG fraction) was used to precipitate the rabbit antibodies. The precipitates were washed, aliquots were dissolved in 0.5 ml of NCS, and radioactivity was determined in 10 ml of Liquifluor. (●—●) Rabbit anti-RPC5 IgG; (●- - -●) normal rabbit IgG.

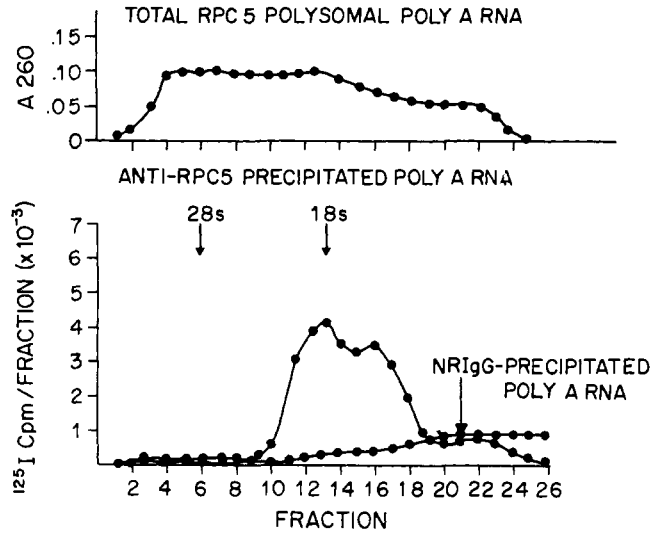


FIGURE 2: Sucrose gradient patterns of poly(A) RNA recovered from immunoprecipitates. Approximately 1 $\mu$g of the poly(A) RNA recovered from polyribosomes precipitated by rabbit anti-RPC5 IgG or normal rabbit IgG was labeled with $^{125}$I as described in the text. The specific activity of the RNA precipitated by anti-RPC5 IgG was 9 × $10^4$ cpm/$\mu$g; the specific activity of the RNA precipitated by normal rabbit IgG was 4 × $10^4$ cpm/$\mu$g. The RNAs were centrifuged in 10–30% sodium dodecyl sulfate gradients in 0.01 M NaCl–1.5 mM $MgCl_2$–0.01 M Tris-Cl (pH 7.4) for 3.5 h at 180 000$g$ at 25 °C. The radioactivity per fraction was determined. The sucrose gradient pattern is compared with that of poly(A) RNA obtained after two cellulose fractionations of total polysomal RNA.

antibody at all. As an additional control, the specific precipitation of polysomes by anti-RPC5 IgG could be blocked almost completely by preincubation of the antibody with excess RPC5 IgG (Table I). Preincubation of anti-RPC5 IgG with an excess amount of purified $\kappa$ light chains alone led to a decrease of approximately 40% in the specific preciptation of polysomes from RPC5 cells forming both light and heavy chains.

*Separation of Poly(A) Containing RNA from Polysomes of RPC5 Cells Immunoprecipitated with Specific Antibody.* Poly(A) containing RNA was obtained from immunoprecipitates of polysomes of RPC5 cells by fractionation on columns containing Sigmacell 38. The average specific activity of RNA binding to the column was 1.1 × $10^4$ cpm/$A_{260}$ unit. The $A_{260}/A_{280}$ ratio of the RNA bound to cellulose was consistently greater than 2.0.

In a typical experiment, approximately 9% of the polysomes isolated from RPC5 cells were precipitated by specific rabbit anti-RPC5 IgG. After two fractionations of the RNA by cellulose chromatography, a total of 14 $\mu$g of poly(A) RNA was recovered from approximately 12 $A_{260}$ units of the RNA extracted from the immunoprecipitated polysomes (2.3%). Small amounts of poly(A) RNA were recovered from immunoprecipitates formed with normal rabbit IgG. About 1 $\mu$g of poly(A) RNA was obtained after two cellulose fractionations of RNA extracted from about 0.1 $A_{260}$ unit of polysomes nonspecifically precipitated with normal rabbit IgG.

*Characterization of the RNA Obtained from RPC5 Polysomes.* Poly(A) containing RNA recovered from polysomes of RPC5 cells precipitated by rabbit anti-RPC5 IgG or normal rabbit IgG and labeled with $^{125}$I was centrifuged in sucrose–sodium dodecyl sulfate density gradients. The results (Figure 2) indicate that poly(A) containing RNA from specifically immunoprecipitated polysomes possessed two identifiable size components sedimenting at about 18 and 14 S corresponding to RNAs of molecular weights of approximately 7 × $10^5$ and 4 × $10^5$, respectively. These are the approximate size classes of RNA expected for $\gamma$ and $\kappa$ chain mRNAs.

RNAs isolated from immunoprecipitated polysomes of RPC5 cells were translated in a cell-free system derived from wheat germ. The relative activities of the total and purified RNAs, the molecular weight of the proteins specified by the RNAs, and their precipitation by antibodies to IgG were measured. Altogether, 4.0 $\mu$g of RPC5 total poly(A) RNA led to the incorporation of 106 pmol of [$^{14}$C]leucine, 7 $\mu$g of MOPC41 total poly(A) RNA stimulated the incorporation of 252 pmol of [$^{14}$C]leucine, and 10 $\mu$g of IgG mRNA caused

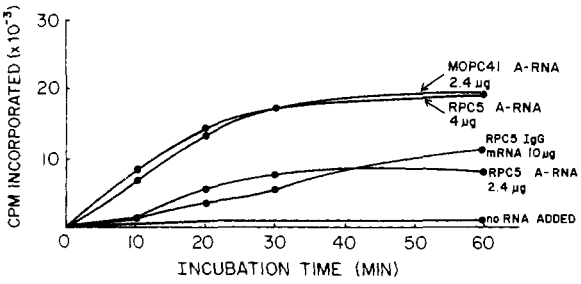


FIGURE 3: Time course of protein synthesis directed by RNAs added to wheat germ extracts. Reaction mixtures of 200 µl were assembled as described in the text; they were incubated at 30 °C. Ten microliters was removed from each mixture at the times indicated and $Cl_3CCOOH$ precipitated. The radioactivity precipitated by $Cl_3CCOOH$ was determined by counting in 10 ml of Liquifluor.

the incorporation of 63 pmol of [$^{14}$C]leucine. BMV RNA (0.5 µg) and background incorporation were 473 and 7 pmol of [$^{14}$C]leucine, respectively. No attempt was made to determine the optimal conditions for translation of the myeloma RNAs. The incorporation stimulated by myeloma RNAs ranged from 8 to 43 times background values (Figure 3), allowing analysis by gel electrophoresis of the products.

Proteins synthesized in the wheat germ system in the presence of myeloma RNAs were subjected to electrophoresis in 10% urea–sodium dodecyl sulfate–polyacrylamide gels. Figure 4 is the electrophoretic pattern of cell-free products directed by RPC5 IgG mRNA, MOPC41 total poly(A) RNA, and RPC5 total poly(A) RNA. Total poly(A) RNA from myeloma MOPC41, a κ chain producing cell line, specified one major protein which migrated with a molecular weight equivalent to 25 000 and numerous other peptides migrating at lower molecular weights than κ chains. RPC5 total poly(A) RNA specified one main protein species of approximately 25 000 molecular weight, and numerous other polypeptides, 25% of which were larger in size. Like total poly(A) RNA from RPC5 cells, the major protein synthesized in the presence of IgG mRNA migrated with a molecular weight of 25 000; three peaks were identified corresponding to molecular weights of approximately 50 000, 37 000, and 10 000 along with numerous other species.

To determine whether the proteins synthesized in the presence of RPC5 IgG mRNA shared antigenic determinants with κ and γ chains from RPC5 cells and to determine the proportion of the total proteins synthesized sharing such determinants, the products of the cell-free reaction mixture were incubated with rabbit antibody specific for IgG from RPC5 cells. The results (Table II) indicate that in the presence of total poly(A) RNA from RPC5 cells, about 10% of the total material synthesized was immunoprecipitated by anti-RPC5 IgG. This value agrees with the proportion of RPC5 polysomes that were precipitable by anti-RPC5 IgG (9%) and is somewhat less than the proportion of total protein synthesized by RPC5 cells immunoprecipitable from cytoplasmic extracts of the cells (13%). Considerably greater proportions of radioactivity were immunoprecipitated from extracts incubated with IgG mRNA from RPC5 cells. Between 79 and 90% of the [$^{14}$C]leucine or [$^{3}$H]leucine incorporated in the wheat germ system after the addition of mRNA from immunoprecipitated polysomes of RPC5 cells was immunoprecipitable by rabbit anti-RPC5 IgG. This value is about tenfold greater than that resulting after the addition of total polysomal poly(A) RNA and is in good agreement with the enrichment for specific IgG mRNA expected on the basis of specific and nonspecific polysome precipitation by anti-RPC5 IgG.

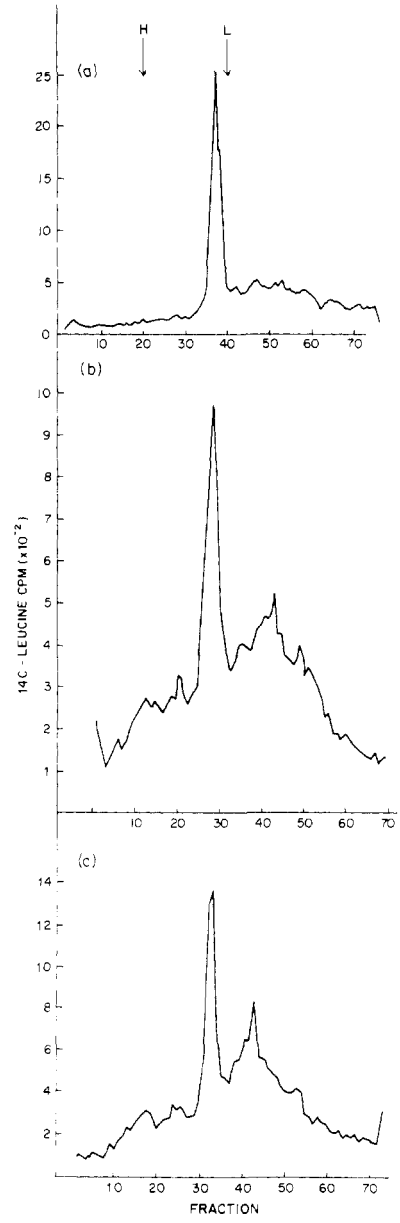


FIGURE 4: Electrophoresis of wheat germ cell free products. Reaction mixtures were prepared as described in Materials and Methods; they were subjected to electrophoresis in 10% urea–sodium dodecyl sulfate–polyacrylamide gels at 3 mA per gel for 16 h. Gels were crushed and the radioactivity in each fraction was determined. Radioactivity in proteins synthesized without added RNA (background) did not exceed 80 cpm per gel fraction. Molecular weight markers were immunoglobulin γ heavy chain (molecular weight 50 000) and κ light chain (molecular weight 25 000). (a) Products synthesized in the presence of MOPC41 poly(A) RNA; (b) products of RPC5 poly(A) RNA; (c) products of IgG mRNA from RPC5 cells.

Immunoprecipitations using antibodies specific for γ H chains alone led to the recovery of approximately 30% of the radioactivity of that found in immunoprecipitates formed with antibodies directed to both H and L chains, consistent with the known incomplete translation of larger mRNAs in the wheat germ system.

*RNA–DNA Hybridization Experiments. (a) Measurement of RNase Resistant RNA–DNA Hybrid Formation by Sephadex G-200 Column Chromatography.* Samples from hybridization reaction mixtures of $^{125}$I-labeled RNA and DNA were treated with RNase and fractionated with the aid of Sephadex G-200 columns. A typical separation of hybrid samples obtained with Sephadex G-200 is shown in Figure 5. A hybrid

TABLE II: Immunoprecipitation of Wheat Germ Cell Free Products.

| RNA | Antibody | cpm Incorporated | cpm Precipitated by Antibody | % Precipitated by Antibody |
|---|---|---|---|---|
| RPC5 poly(A) RNA | RAM IgG | 3 922 ± 78 | 387 ± 15 | 10.0 ± 0.2[a] |
| | | 17 200 ± 800 | 1 896 ± 246 | 11.0 ± 1.4[b] |
| | | 17 200 ± 800 | 107 ± 85 | 0.6 ± 0.5[c] |
| | RAMγ | 17 200 ± 800 | 777 ± 197 | 4.5 ± 1.1[a] |
| | | 17 200 ± 800 | 516 ± 28 | 3.0 ± 1.1[b] |
| | | 17 200 ± 800 | 400 ± 123 | 2.3 ± 0.7[c] |
| IgG mRNA | RAM IgG | 4 950 ± 11 | 4 146 ± 216 | 84 ± 4[a] |
| | | 15 400 ± 850 | 12 955 ± 1087 | 84.1 ± 7.0[b] |
| | | 15 400 ± 850 | 732 ± 16 | 4.7 ± 0.1[c] |
| | RAMγ | 15 400 ± 850 | 3 999 ± 185 | 25.9 ± 1.2[a] |
| | | 15 400 ± 850 | 4 601 ± 849 | 29.8 ± 5.5[b] |
| | | 15 400 ± 850 | 939 ± 129 | 6.1 ± 0.8[c] |
| Brome mosaic virus RNA | RAM IgG | 43 270 | 636 | 2.0[a] |
| None | RAM IgG | 735 ± 115 | 17 ± 11 | 3 ± 2[a] |

[a] Direct immunoprecipitation method. [b] Indirect immunoprecipitation method. [c] Indirect immunoprecipitation method with 1 mg of unlabeled RPC5 IgG protein included in the reaction mixture. For direct immunoprecipitation, an aliquot of reaction mixture was mixed with 5 μl of normal mouse serum followed by an equivalent amount of RAM IgG or RAMγ in PBS (pH 7.9)–3% Triton X-100–0.01 M EDTA. The mixture was incubated at 4 °C for 4 h. For indirect immunoprecipitation, an aliquot of the reaction mixture was mixed with RAM IgG or RAM γ in 0.1 M Tris-Cl (pH 7.6)–0.05 M NaCl–0.01 M EDTA–1% Triton X-100–1% sodium deoxycholate; after incubation at 4 °C for 18 h, an equivalent amount of goat anti-rabbit IgG serum was added and the mixture was incubated for 2 h at 4 °C. All precipitates were washed twice with PBS and the amount of material insoluble in hot $Cl_3CCOOH$ was determined. Aliquots of each reaction mixture were also precipitated with $Cl_3CCOOH$ to determine total incorporation of [$^4$C]leucine or [$^3$H]leucine into protein. The percent of total protein precipitated by anti-IgG was determined.

sample incubated to $C_0t$ 1000 was passed through Sephadex (Figure 5a). The DNA eluted in a sharp peak at fraction 7. Of the total radioactivity applied to the column, 90% eluted with the peak containing DNA and 10% trailed behind the exclusion peak, likely representing degraded RNA. After a sample taken at the beginning of the reaction was digested with RNase (Figure 5b), 4% of the radioactivity applied to the column eluted with the fraction resistant to the enzyme. After incubation to $C_0t$ 4200, 53% of the radioactivity eluted with the fraction resistant to RNase.

RNase resistant material isolated by Sephadex G-200 chromatography was analyzed further in cesium chloride gradients. The majority of the radioactivity present in samples taken at $C_0t$ 4200 banded in the density range expected for RNA-DNA hybrids. About 15% of the radioactivity banded at the densities of reassociated and single-stranded DNA indicating that the hybrids contained relatively small proportions of RNA compared with DNA; most of the radioactivity (about 85%) banded at higher densities indicating that the hybrids contained a large proportion (up to 50%) of RNA. About 10% of the radioactivity banded at densities higher than 1.80, the density expected for 1:1 RNA-DNA hybrids, possibly reflecting the iodine content of the $^{125}$I-labeled RNA.

*(b) Hybridization of Ribosomal RNA from RPC5 Cells with Mouse Liver DNA.* Ribosomal RNA obtained by cellulose fractionation of RPC5 polysomal RNA was labeled with $^{125}$I and incubated with denatured Balb/c liver nuclear DNA. The DNA/RNA radioactivity in the reaction mixture was $2.75 \times 10^5$ or about 50:1 for rDNA–RNA. Approximately 57% of the rRNA formed hybrids with DNA after incubation to a $C_0t$ of $10^3$ (Figure 6). The $C_0t_{1/2}$ of the reaction was 24. This value is in close agreement with similar determinations performed by others (Melli et al., 1971; Tonegawa et al., 1974a, b). The reiteration frequency of DNA sequences coding for rRNA was taken to be 250 (Melli et al., 1971); hybridization rates measured in all other experiments were compared with these values

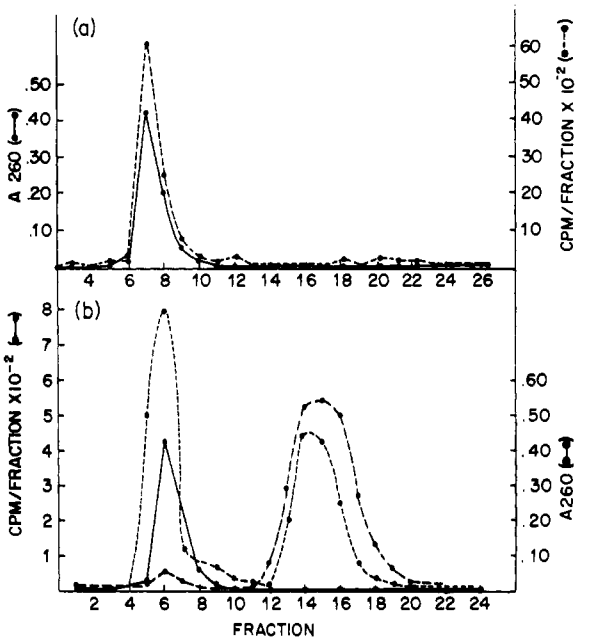


FIGURE 5: Sephadex G-200 elution patterns of RNA–DNA hybrids. Aliquots of hybridization mixtures were passed through a 1 × 40 cm Sephadex G-200 column in 0.12 M PBS. (a) A hybrid sample incubated at $C_0t$ 1000 was applied to a Sephadex G-200 column without prior RNase digestion and the $A_{260}$ and cpm per fraction were determined; (b) hybrid samples were treated with RNase as described in the text and applied to a Sephadex G-200 column. $A_{260}$ in $C_0t$ 4200 sample (●—●); radioactivity in $C_0t$ 4200 sample (●- - -●); radioactivity in zero time sample (●——●).

to estimate the reiteration frequencies of DNA sequences hybridizing to poly(A) RNAs.

*(c) Hybridization of Poly(A) RNA from Polysomes of RPC5 Cells with Liver DNA.* Polysomal poly(A) containing RNA from RPC5 cells was hybridized with sheared nuclear DNA from mouse liver at varying DNA/RNA ratios and the

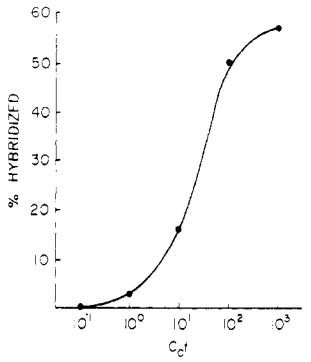


FIGURE 6: Hybridization of rRNA from RPC5 cells and liver DNA. The reaction mixture contained 550 $\mu$g of DNA (1600 $\mu$g/ml) and $2 \times 10^{-3}$ $\mu$g of RNA ($1.5 \times 10^4$ cpm) in 0.12 M PBS-0.1% sodium dodecyl sulfate. DNA/RNA = $2.75 \times 10^5$ (approximately 50:1 for rDNA:rRNA). The mixture was incubated at 70 °C; hybrid samples were divided in half. One-half of each sample was treated with 10 $\mu$g/ml RNase at 37 °C for 20 min; both halves of each sample were precipitated with $Cl_3CCOOH$. The percent hybridized was calculated as: % hybridized = [($Cl_3CCOOH$ precipitable cpm after RNase digestion)/total $Cl_3CCOOH$ precipitable cpm] − % hybridized in zero time sample. Each point is an average of two determinations.

proportion of RNA resistant to RNase was measured at various $C_0t$ values. The results (Figure 7) indicate the following: (1) The hybridization reactions for poly(A) RNA and liver DNA were indistinguishable at DNA/RNA ratios of $3 \times 10^5$, $5 \times 10^5$, and $2 \times 10^6$. The "best" curve plotted fit all of the data points obtained. (2) the $C_0t_{1/2}$ of the overall hybridization reaction was $1.5 \times 10^3$; a theoretical curve calculated for hybridization of a single species of RNA with the same $C_0t_{1/2}$ did not fit the data obtained. However, the data obtained at $C_0t$ values greater than 10 approximated a curve representing an RNA fraction comprising 75% of the total and hybridizing with a $C_0t_{1/2}$ of $3.3 \times 10^3$ (Figure 7). The remainder of the poly(A) RNA formed hybrids at lower $C_0t$ values; no single second-order curve representing hybridization of RNA to DNA sequences of a single repetition frequency could be calculated to fit this portion of the curve.

The presence of ribosomal RNA was not evident in sucrose gradient patterns of poly(A) containing RNA fractionated by adsorption to and elution from cellulose; nevertheless, a small amount of rRNA might have escaped detection. To determine whether rRNA contributed significantly to the hybridization reaction of poly(A) RNA at low $C_0t$, $^{125}$I-labeled poly(A) RNA was hybridized with liver DNA in the presence of an excess amount of unlabeled 18 and 28S rRNA. In a parallel experiment, poly(A) containing RNA was hybridized with DNA at 70 °C in similar amount but without the addition of rRNA. A 10% reduction in the total amount of poly(A) RNA forming hybrids occurred in the presence of rRNA; however, no consistent change was observed in hybridization occurring under low $C_0t$ conditions, where effective competition by contaminating rRNA would be expected to take place.

(*d*) *Hybridization of IgG mRNA and Sheared Liver DNA.* Preparations of RNA, estimated to contain more than 85% mRNA specifying IgG of RPC5 cells, were labeled with $^{125}$I and incubated with sheared and denatured liver DNA at a DNA/RNA ratio of $5 \times 10^5$. A comparison of the hybridization kinetics of IgG mRNA and DNA and total poly(A) RNA and DNA is presented in Figure 8. Approximately 55% of unfractionated poly(A) RNA hybridized with DNA after prolonged incubation and approximately 67% of IgG mRNA formed hybrids with DNA under similar conditions. At low $C_0t$ values, the rate of hybridization of DNA and poly(A)

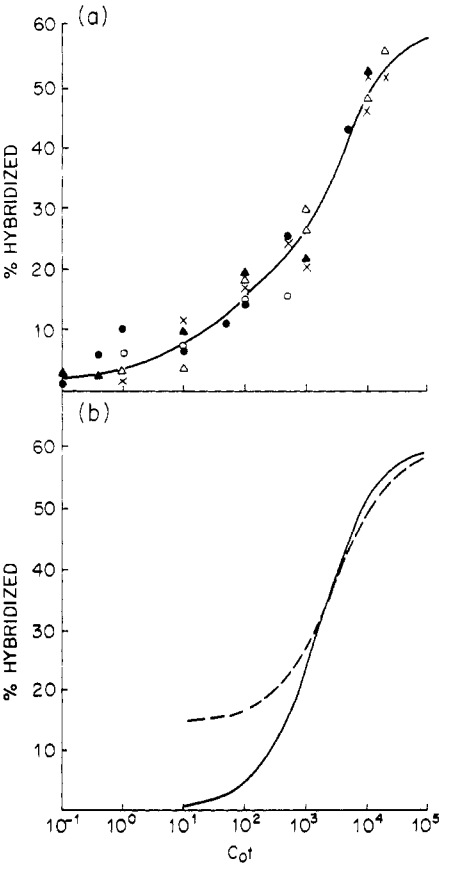


FIGURE 7: Hybridization of poly(A) RNA from RPC5 cells and liver DNA. (a) Three hybridization mixtures were used. (1) DNA input = 2500 $\mu$g; DNA concentration = 6200 $\mu$g/ml; RNA input = $8 \times 10^{-3}$ $\mu$g ($4 \times 10^4$ cpm); DNA/RNA = $3 \times 10^5$ (○, ●). (2) DNA input = 2500 $\mu$g; DNA concentration = 6200 $\mu$g/ml; RNA input = $5 \times 10^{-3}$ $\mu$g ($2.5 \times 10^4$ cpm); DNA/RNA = $5 \times 10^5$ (△, ▲). (3) DNA input = 4100 $\mu$g; DNA concentration = 6200 $\gamma$/ml; RNA input = $2 \times 10^{-3}$ $\mu$g ($1 \times 10^4$ cpm); DNA/RNA = $2 \times 10^6$ (X). Hybridization mixtures were incubated in 0.12 M PBS-0.1% sodium dodecyl sulfate (pH 6.8) or 0.24 M PBS-0.1% sodium dodecyl sulfate (pH 7.8) at 70 °C. Hybridization was measured by $Cl_3CCOOH$ precipitation or Sephadex G-200 chromatography after RNase digestion. RNA incubated with sheared *E. coli* DNA did not form hybrids above the 2% level. (b) Theoretical curves were calculated for RNA hybridizing with $C_0t_{1/2}$ of $1.5 \times 10^3$ (—) and for a 75% component hybridizing with a $C_0t_{1/2}$ of $3.3 \times 10^3$ (- - -).

RNA was as much as 100 times faster than that observed for IgG mRNA. About 75% of the poly(A) RNA hybridized with a $C_0t_{1/2}$ of $3 \times 10^3$. The data obtained for the hybridization of IgG mRNA and DNA showed good fit to a curve calculated for hybridization of a component comprising 95% of the IgG mRNA and hybridizing with a $C_0t_{1/2}$ of $4 \times 10^3$. In repeated experiments, this major component of IgG mRNA hybridized at similar rates. Compared with the hybridization rate of rRNA and mouse liver DNA measured under similar conditions, nearly all of the IgG mRNA from RPC5 cells hybridized with sequences represented in liver DNA at most five times. A small portion of IgG mRNA equal to approximately 5% of the total hybridized rapidly ($C_0t_{1/2}$ less than 10), indicating that the DNA sequences complementary to this minor fraction were repeated 500 times or more. It did not exceed 8% when hybridization reaction was performed at 60 °C. The presence of rRNA was not detected in the hybridization patterns of IgG mRNA preparationns used. In no instance did the major component of IgG mRNA from RPC5 cells hybridize with mouse liver DNA at a $C_0t_{1/2}$ of less than $4 \times 10^3$.

The capacity of poly(A) RNA from RPC5 cells to compete

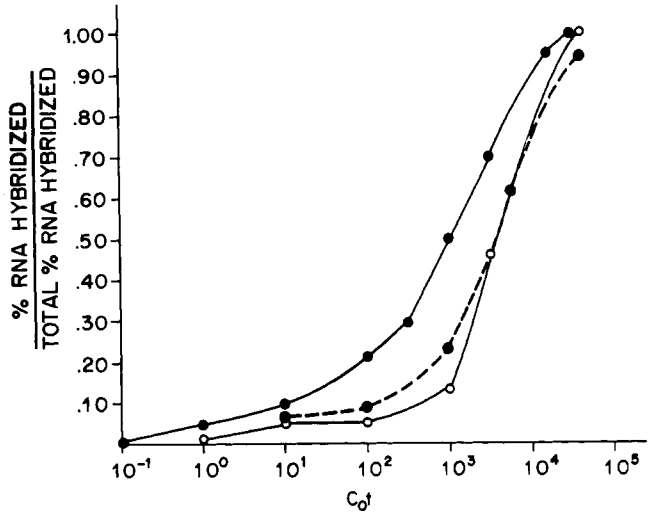


FIGURE 8: Hybridization of IgG mRNA and total poly(A) RNA with liver DNA. (●—●) Poly(A) RNA ($5 \times 10^{-3}$ μg) was hybridized with 2500 μg of DNA at a concentration of 6200 μg/ml in 0.12 M PBS–0.1% sodium dodecyl sulfate at 70 °C; DNA/RNA = $5 \times 10^5$. (○—○) IgG mRNA ($1 \times 10^{-2}$ μg) was hybridized with 5000 μg of DNA at a concentration of 4300 μg/ml in 0.12 M PBS–0.1% sodium dodecyl sulfate. Also, $1 \times 10^{-2}$ μg of IgG mRNA was hybridized with 5000 μg of DNA at a concentration of 3700 μg/ml in 0.24 M PBS–0.1% sodium dodecyl sulfate at 70 °C. Hybridization was measured by Sephadex G-200 chromatography after RNase digestion of hybrid samples. (●- - -●) A theoretical curve was calculated for a component comprising 95% of the IgG mRNA and hybridizing with a $C_0t_{1/2}$ of $4 \times 10^3$. RNA incubated without added DNA under conditions used for hybridization experiments did not form RNase resistant material.

TABLE III: Hybridization of IgG mRNA and Liver DNA in the Presence of Unlabeled RPC5 Total Poly(A) RNA.[a]

| $C_0t$ | % IgG mRNA Hybridized |
|---|---|
| $10^{-1}$ | 0 |
| $10^{0}$ | 8.8 |
| $10^{1}$ | 2.5 |
| $10^{2}$ | 3.6 |
| $10^{3}$ | 8.0 |
| $10^{4}$ | 9.0 |
| $10^{4}$ (no competitor added) | 67 |

[a] IgG mRNA ($4 \times 10^{-3}$ μg); ($2.4 \times 10^4$ cpm) was hybridized with 2000 μg of DNA at a concentration of 3100 μg/ml in 0.24 M PBS–0.1% sodium dodecyl sulfate at 60 °C; DNA/RNA = $5 \times 10^5$. Poly(A) RNA (3 μg total) was included in the reaction mixture. Samples were analyzed for hybrid content by Sephadex G-200 chromatography after RNase digestion. Results were compared with the results of a parallel experiment done under the same conditions but without poly(A) RNA added.

with IgG mRNA for sites of hybridization in liver DNA was determined. IgG mRNA was hybridized with liver DNA under conditions similar to those described above, except that unlabeled poly(A) RNA was included in the reaction mixture. The results (Table III) indicate that the proportion of IgG mRNA forming hybrids did not exceed 10% in the presence of poly(A) RNA. At the most prolonged incubation periods approximately 9% of the IIgG mRNA formed hybrids. Under similar conditions in the absence of competing RNA, approximately 67% of the IgG mRNA formed hybrids.

The stability of IgG mRNA during incubation at 70 °C in 0.12 M PBS–0.1% sodium dodecyl sulfate was determined by centrifuging aliquots of the reaction mixture in sodium dodecyl sulfate–sucrose gradients. At the beginning of the reaction and after incubations up to $C_0t$ of $10^3$, the majority of the RNA sedimented at approximately 14–18 S. Poly(A) RNA incubated under similar conditions also was not significantly degraded.

*Stability of the RNA–DNA Hybrids.* Hybrids of IgG mRNA and DNA isolated after incubation at 70 °C to $C_0t$ 10 had an average $T_m$ of 78 °C in 0.12 M PBS; hybrids formed at $C_0t$ 5000 at the same temperature had an average $T_m$ of 83 °C. In the case of total poly(A) RNA and liver DNA, the average $T_m$'s of hybrids formed at $C_0t$ 10 and at $C_0t$ 500 at 70 °C were 78 and 82 °C, respectively. The temperature range over which 20–80% of the hybrids denatured was approximately 20 °C for all hybrids examined.

## Discussion

Approximately 85% of the radioacttivity incorporated in a wheat germ cell free system in the presence of IgG mRNA was immunoprecipitated by antibodies to RPC5 IgG. This indicates that more than 85% of the translatable mRNA present, as prepared by cellulose chromatography of RNA extracted from specifically immunoprecipitated polysomes of myeloma cells, engaged in the synthesis of complete immunoglobulins, contained mRNA for both the γ H and κ L chains of IgG. This figure likely is a minimum estimate as it was not corrected for the contribution of endogenous incorporation of radioactivity into protein in the system, i.e., in the absence of added RNA, which was approximately 15% of the radioactivity incorpoorrated after the addition of IgG mRNA; nor did it account for polypeptide synthesis directed by fragments of IgG mRNA which were too small to form antigenic determinants.

Rabbit RPC5 IgG antibody was purified with respect to its antigen combining specificity by affinity chromatography using Sepharose to which RPC5 IgG was bound. The use of such antibody reduced nonspecific precipitation of polysomes resulting from adsorption of polysomes to an immunoprecipitate of larger mass. Antibodies present in the rabbit anti-RPC5 IgG preparation which might have combining specificity for non-IgG antigenic determinants present on RPC5 polysomes were also eliminated. The antibody purified by adsorption to and recovery from RPC5 IgG-Sepharose was approximately 25 times as effective in precipitating [$^3$H]leucine-labeled IgG from extracts of RPC5 cells as the crude globulin fraction of rabbit anti-RPC5 IgG serum. About 13% of the newly synthesized protein of RPC5 cells was precipitated by the optimal concentrations of both crude and purified antibody preparations. The specificity of the anti-RPC5 IgG antibody was demonstrated by the observation that only about 1% of the [$^3$H]leucine labeled protein of normmal thymus cells of Balb/c mice (not synthesizing detectable IgG) could be precipitated using this antibody.

The results of hybridization experiments using poly(A) containing RNA from *unfractionated polysomes* of immunoglobulin producing RPC5 cells revealed the presence of a major class of RNA along with an undetermined number of minor classes. Approximately 75% of the RNA formed hybrids with liver DNA with a $C_0t_{1/2}$ of $3 \times 10^3$; the remainder hybridized at lower $C_0t$ values ranging from $10^{-1}$ to $10^3$ reflecting the presence of multiple species of RNA originating from repetitive nucleotide sequences. The relative content of repetitive transcripts measured in this system was similar to those measured in mouse L cells (25% repetitive transcripts) (Greenberg and Perry, 1971), mouse fibroblasts (30% repet-

itive transcripts), rat myoblasts (40% repetitive transcripts), and HeLa cells (40% repetitive transcripts) (Spradling et al., 1974). The hybridization results using poly(A) RNA from RPC5 myeloma cells was similar to the results obtained by us using poly(A) RNAs from normal mouse liver, myeloma MOPC41 or myeloma RPC5-NP, a nonproducing myeloma derived from RPC5 (unpublished data). To estimate the proportion of rRNA present in isolates of poly(A) RNA from unfractionated polysomes of RPC5 cells, unlabeled rRNA was included as a competitor in hybridization reaction mixtures. rRNA added in a $10^4$-fold excess over labeled poly(A) RNA (about 100-fold excess over the estimated quantity of rDNA contained in the reaction mixture) failed to compete for sites of hybridization with poly(A) RNA from RPC5 cells.

The hybridization characteristics of poly(A) containing RNA obtained from polysomes of RPC5 cells immunoprecipitated before extraction with purified antibodies specific for RPC5 myeloma proteins differed significantly from the hybridization characteristics of poly(A) RNA obtained from unfractionated polysomes. Approximately 95% of the IgG mRNA we used hybridized with a $C_0t_{1/2}$ of $4 \times 10^3$; the remainder, less than 5%, hybridized with a $C_0t_{1/2}$ of less than 10. IgG mRNA failed to form detectable RNase resistant duplex structures during incubation without added DNA, and the $T_m$'s of RNA and DNA hybrids formed at 70 °C at $C_0t$ 10 (78 °C) and $C_0t$ 5000 (83 °C) indicated a high degree of sequence homology in the hybrid molecules.

The transition in the $C_0t$ curve obtained for the hybridization of IgG mRNA and DNA is more acute than would be expected for an RNA population hybridizing even to a single species of DNA. The transition between 20 and 80% completion of the reaction occurred over approximately 1.5 $C_0t$ log units. It is possible that the formation of molecular hybrids of IgG mRNA and DNA was incomplete, even at the highest $C_0t$ values measured. If significant amounts of additional hybrid formation occurred, beyond the highest $C_0t$ values we measured, then the range of $C_0t$ values over which the IgG mRNA formed hybrids would be expected to be more diverse.

The hybrids formed between IgG mRNA and DNA were heterogeneous with respect to their thermal stability. Such heterogeneity in the stability of the hybrid pairs accompanied by a relatively high $T_m$ may have results in part from hybridization of RNA with incompletely homologous DNA sequences. Hybridization between short regions of DNA and RNA which did not bind to hydroxylapatite as firmly as hybrids spanning longer regions also may have occurred.

The RNA–DNA hybridization experiments described in this report were carried out in part to determine whether nucleotide sequences were present in immunoglobulin heavy and light chain mRNAs which were complementary to repetitive DNA. Portions of immunoglobulin mRNA molecules would be expected to hybridize with repetitive DNA sequences if sufficient numbers of such sequences existed, specifying similar but nonidentical immunoglobulin variable regions of sufficient homology for hybridization to occur (e.g., within an immunoglobulin subclass). Whether large subclasses of related immunoglobulins similar to that described in humans exist in mice has not been established.

The nucleotide sequence composition of mouse $\kappa$ light chain mRNA consists of approximately 1250 nucleotides, 321 (39 percent) of which specify the amino acid sequence of the variable region (Milstein et al., 1974). While the nucleo( tide sequence of the mRNA from $\gamma$ heavy chains has not been determined with equal precision, it is likely that approximately 15% of the molecule will specify the variable region.

Approximately 95% of the IgG mRNA we used hybridized with nucleotide sequences in DNA uniquely represented; using mRNA of higher purity obtained by more specific methods we were unable to detect the presence of significant proportions of IgG mRNA forming hybrids with repetitive sequences. Hybrids of repetitive DNA and poly(A) RNA from unfractionated polysomes on the other hand were detected readily.

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# Purification and Properties of Guanosine 5′,3′-Polyphosphate Synthetase from *Bacillus brevis*†

Jose Sy* and Hugh Akers

ABSTRACT: A ribosome-independent guanosine 5′,3′-polyphosphate synthetase has been highly purified from *Bacillus brevis* (ATCC 8185). The enzyme has a molecular weight of 55 000, as measured by sucrose density gradient centrifugation. Like the ribosome-connected stringent factor of *Escherichia coli,* it catalyzes the synthesis of the guanosine 5′,3′-polyphosphates by a pyrophosphoryl transfer mechanism from adenosine triphosphate (ATP) to guanosine di- or triphosphates (GDP, GTP). It has an apparent $K_m$ of 0.14 mM for GDP and 0.77 mM for GTP, and is specific for the guanosine ribonucleotides as pyrophosphoryl acceptors. Several ATP analogues were tested for their ability to donate the pyrophosphoryl group. $Mg^{2+}$ was required as a counter ion for the nucleotide substrate; however, an excess of $Mg^{2+}$ was inhibitory. The property of the *B. brevis* enzyme is compared with the ribosome-linked enzyme of *E. coli* and an extracellular enzym excreted by several types of *Streptomyces* reported upon recently.

There is increasing evidence to support the notion that ppGpp[1] exerts a variety of effects in bacterial metabolism (Cashel and Gallant, 1969). It acts as an inhibitor of the well known stringent effect by shutting off stable RNA synthesis (Cashel, 1969; Reiness et al., 1975), and, furthermore, it inhibits key enzymes in several metabolic pathways, such as the biosynthesis of phospholipids (Merlie and Pizer, 1973), nucleotides (Gallant et al., 1971), polyamines (Hölttä et al., 1974), as well as the uptake of purine and pyrimidine (Hochstadt-Ozer and Cashel, 1972). Recently, it has been suggested that ppGpp modulates the transcriptional control of the histidine operon (Stephens et al., 1975) and other catabolic pathways (Yang et al., 1974).

The in vitro biosynthesis of ppGpp and pppGpp was found first in *Escherichia coli* as a ribosome-linked reaction which required the presence of a factor released by washing of ribosomes of stringent strains and, in addition, mRNA plus uncharged tRNA (Haseltine et al., 1972; Pederson et al., 1973; Haseltine and Block, 1973). However, it was found that, in the absence of ribosomes, the stringent factor showed low transfer activity which could be markedly increased by the addition of methanol (Sy et al., 1973). The enzyme, furthermore, was found to catalyze reverse pyrophosphoryl transfer using a high concentration of AMP as acceptor (Sy, 1974), indicating the reaction to be essentially reversible: (p)ppG + pppA ⇌ (p)pp$^{5'}$G$^{3'}$pp + pA.

Recently, we discovered in *Bacillus brevis* a guanosine 5′,3′-polyphosphate synthetase that is quite independent of the ribosomal complex (Sy, 1976). It was not stimulated by either the ribosome mRNA–uncharged tRNA complex or by methanol. Another such ribosome-independent synthetase has recently been found to be released into the extracellular fluid by a number of *Streptomyces* (Nishino and Murao, 1975; Oki et al., 1975). We report here on the large scale preparation, purification, and further characterization of the *B. brevis* synthetase, including a comparison with the other two synthetases.

## Materials and Methods

*Materials.* [$\alpha$-$^{32}$P]GTP was obtained from ICN. [$^{14}$C]GDP, [$\gamma$-$^{32}$P]ATP, and [$^{3}$H]ATP were purchased from New England Nuclear Corp. All other nucleotides were obtained from P-L Biochemicals except for ppppG which was a product of Sigma Chemical Co.

*Preparation of B. brevis Extracts. B. brevis* (ATCC 8185) was cultured on 10 g of beef extract (Difco) and 10 g of peptone (Difco) per l. At late log phase ($OD_{650}$ = 3.5–3.8) the culture was chilled, and the cells were collected by centrifugation and stored frozen. Thawed cells (107 g) were washed with 100 ml of buffer A (20 mM Tris-OAc, pH 8.1, 14 mM $Mg(OAc)_2$, 60 mM KOAc, 1 mM dithiothreitol) by centrifugation. The washed cells were resuspended in 100 ml of buffer A that contained 100 $\mu$g of electrophoretically pure DNase (Worth-

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[1] Abbreviations used are: ppGpp, guanosine 5′-diphosphate 3′-diphosphate; pppGpp, guanosine 5′-triphosphate 3′-diphosphate; ppppG, guanosine 5′-tetraphosphate; ATP, GTP, CTP, UTP, ITP, adenosine, guanosine, cytidine, uridine, and inosine 5′-triphosphates; ATPase, GTPase, adenosine and guanosine 5′-triphosphatase; dATP, dGTP, 2′-deoxyadenosine and 2′-deoxyguanosine 5′-triphosphates; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; EDTA, (ethylenedinitrito)tetraacetic acid.