References

Appella, E., & Ein, D. (1967) *Proc. Natl. Acad. Sci. U.S.A. 57,* 1449-1454.

Awdeh, Z. L., Williamson, A. R., & Askonas, B. A. (1968) *Nature (London) 219,* 66-67.

Butler, P. J. G., & Hartley, B. S. (1972) *Methods Enzymol. 25,* 191-199.

Chen, B. L., & Poljak, R. J. (1974) *Biochemistry 13,* 1295-1302.

Edman, P., & Begg, F. (1967) *Eur. J. Biochem. 1,* 80-91.

Fett, J. W., & Deutsch, H. F. (1974) *Immunochemistry 12,* 643-652.

Gross, E. (1967) *Methods Enzymol. 11,* 238-255.

Hartley, B. S. (1970) *Biochem. J. 119,* 805-822.

Hess, M., Hilschmann, N., Rivat, L., Rivat, C., & Ropartz, C. (1971) *Nature (London), New Biol. 234,* 58-61.

Humphrey, R. L., Avey, H. P., Becka, L. N., Poljak, R. J., Rossi, G., Choi, T. K. & Nisonoff, A. (1969) *J. Mol. Biol. 43,* 223-226.

Kabat, E. A., Wu, T. T., & Bilofsky, H. (1976) *Variable Regions of Immunoglobulin Chains,* Medical Computer Systems, Bolt, Beranek and Newman, Inc., Cambridge, Mass.

Lieu, T.-S., Deutsch, H. F., & Tischendorf, F. W. (1977) *Immunochemistry 14,* 429-433.

Poljak, R. J., Amzel, L. M., Avey, H. P., Becka, L. N., & Nisonoff, A. (1972) *Nature (London), New Biol. 235,* 137-140.

Poljak, R. J., Amzel, L. M., Avey, H. P., Chen, B. L., Phizackerley, R. P., & Saul, F. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70,* 3305-3310.

Poljak, R. J., Amzel, L. M., Chen, B. L., Phizackerley, R. P., & Saul, F. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71,* 3440-3444.

Rossi, G., & Nisonoff, A. (1968) *Biochem. Biophys. Res. Commun. 31,* 914-918.

Smithies, O., Gibson, D., Fanning, E., Goodfliesh, R., Gilman, J., & Ballantyne, D. (1971) *Biochemistry 10,* 4912-4921.

Summers, M., Smythers, G., & Oroszlan, S. (1973) *Anal. Biochem. 53,* 624-628.

Zimmerman, C. L., Appella, E., & Pisano, J. T. (1977) *Anal. Biochem. 77,* 569-573.

Isolation, Purification, and Properties of Mouse Heavy-Chain Immunoglobulin mRNAs†

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ABSTRACT: A procedure is described for the isolation of highly purified heavy-chain immunoglobulin mRNAs from a variety of mouse plasmacytomas (IgA, IgG, and IgM producers). The use of fresh tissue and the rapid isolation and direct extraction of membrane-bound polyribosomes were found to be essential in obtaining large quantities of undegraded heavy-chain mRNAs. The individual mRNAs were purified by two cycles of oligo(dT)-cellulose chromatography, sodium dodecyl sulfate-sucrose gradient centrifugation, and electrophoresis on 98% formamide containing polyacrylamide gels. When added to a cell-free protein-synthesizing system from wheat germ, the MPC-11 γ_{2b} and H2020 α heavy-chain mRNAs efficiently directed the synthesis of a predominant product of 55 000 molecular weight, while the synthesis of a 70 000 dalton protein in addition to other lower molecular weight polypeptides were observed with MOPC 3741 μ mRNA. All of these proteins were immunoprecipitable with class-specific heavy-chain antisera, and in the case of the γ_{2b} in vitro products good correspondence in a comparative trypsin-chymotrypsin fingerprint with in vivo labeled γ_{2b} heavy chain was observed. The γ_{2b} and α heavy-chain mRNAs possessed a chain length of ~1800 nucleotides and the μ mRNA a size of ~2150 nucleotides when examined under stringent denaturation conditions. The purities of the α, γ_{2b}, and μ mRNAs were estimated to be 60-80%, 50-70%, and 50-83%, respectively, on the basis of their hybridization rates with cDNA probes in comparison to mRNA standards of known complexity. Heavy-chain mRNAs of the same class isolated from different mouse strains (Balb/C or NZB) display no detectable sequence differences in cross hybridization experiments, even though the cDNA-mRNA hybrids are submitted to stringent S_1 nuclease digestion. These results indicate that allotypic determinants represent only a minor fraction of the heavy-chain constant region sequence in the mouse.

In order to understand the complex mechanisms responsible for the diversification and translocation of immunoglobulin (Ig) genes and for the subsequent accumulation of the Ig mRNAs which direct the synthesis of the heavy- and light-chain polypeptides, procedures for the isolation of highly purified, biologically active heavy- and light-chain mRNAs are essential. Over the past 6 years, κ and λ light-chain mRNAs have been purified, structurally characterized, and used for studies of the repertoire of mouse light-chain genes by nucleic acid hybridization techniques (see review by Kuehl, 1977) and more recently for an investigation of the mechanisms of Ig light-chain gene translocation (Hozumi and Tonegawa, 1976; Tonegawa et al., 1976, 1977).

† From the Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111. *Received December 16, 1977.* This research was supported by Research Grant BMS74-22758 to R.P.P. from the National Science Foundation, a Postdoctoral Fellowship Grant GM05287-02 to K.B.M. from the National Institutes of Health, institutional grants from the National Institutes of Health (CA-06927 and RR-05539), and an appropriation from the Commonwealth of Pennsylvania. O.V. acknowledges the support of a CONICIT fellowship from Venezuela.

0006-2960/78/0417-1723$01.00/0

However, work has not proceeded as rapidly on the heavy-chain mRNAs despite their immunological importance. This has mainly been due to their larger size, estimated to be ~1900 nucleotides (Bernardini and Tonegawa, 1974), contamination with 18S rRNA and other mRNAs (Cowan et al., 1976; Adetugbo and Milstein, 1977), and poor translational efficiency in in vitro cell-free systems (Kuehl, 1977; Green et al., 1976; Cowan and Milstein, 1973; Bedard and Huang, 1977; Sonenshein and Brawerman, 1976; Schmeckpeper et al., 1974). Consequently, only a few reports establishing the purity of heavy-chain mRNAs have appeared (Ono et al., 1977; Cowan et al., 1976), and in these instances the methodologies employed were developed for a particular type of heavy-chain mRNA. In this report, we describe methods suitable for the isolation and purification of heavy-chain mRNAs from a wide variety of plasmacytoma cells. Using these methods, we have isolated functionally competent Ig mRNAs representing three major classes of heavy-chain constant regions: IgA, IgG, and IgM. These mRNAs were transcribed into cDNA probes, which were used to estimate the purities of the mRNA preparations by kinetic complexity measurements, and to assess the extent of sequence homology between IgG and IgM mRNAs specifying different allotypic determinants.

Materials and Methods

Source of Mouse Plasmacytomas and Cell Cultures. Mouse myelomas were either maintained subcutaneously in Balb/C or NZB mice or in tissue culture. MPC-11 cells, originally supplied by Drs. D. Margulies and M. D. Scharff, were maintained at 37 °C in Dulbecco modified Eagle medium (H-21) supplemented with 10% heat-inactivated horse serum, penicillin–streptomycin, and glutamine. For the propagation of MPC-11 tumors subcutaneously in Balb/C mice, ~5×10^6 cells in 0.25 mL of cell culture medium were injected per animal (usually >7 weeks of age), and tumors generally grew to a size of 2–4 g each in 12–14 days. Other plasmacytomas from NZB mice [PC 3741 (IgMk) and PC 2880 ($IgG_{2b}k$)] and Balb/C mice [J558 (IgAλ) and H2020 (IgAλ)] were supplied by Drs. Martin Weigert and Roy Riblet. MOPC 104E (a Balb/C myeloma producing an IgMλ antibody originally developed by Dr. Michael Potter) was provided by Dr. Melvin Bosma.

Isolation of Polyadenylated RNA from Membrane-Bound Polyribosomes (MBP A^+RNA). Tumors were removed from animals (carefully avoiding any necrotic tissue and obvious bacterial infections) and immediately placed in isotonic salt solution at 0 °C. This stage of the isolation generally lasted no longer than 30–45 min, during which time about 60–80 g of tumor was dissected. The tissue was rapidly minced with scissors in a petri dish on ice and was immediately homogenized at 0–2 °C with five to six strokes of a motor-driven Teflon pestle in a Potter-Elvejhem homogenizer with a 1:2 ratio of tissue to 30% sucrose (w/v) in solution A [50 mM Tris-Cl[1] (pH 7.4), 25 mM NaCl, 5 mM magnesium acetate, 7 mM β-mercaptoethanol, 20 μg/mL poly(vinyl sulfate) and 2 μg/mL cycloheximide]. The resultant homogenate was centrifuged at 9000*g* for 10 min at 0 °C. The fatty upper layer was carefully removed with a Pasteur pipet, and the supernatant was pipetted into a chilled flask containing sodium heparin, yielding a final concentration of ~250 μg/mL. The supernatant was diluted to 0.66 M sucrose with solution A and the heparin concentration adjusted to 250 μg/mL. The membranous microsomal fraction (containing the membrane-bound polyribosomes) was obtained by centrifugation at 46 000*g* in an SS34 rotor for 20 min. The pellet was extracted at room temperature in a solution containing 1.5% sodium dodecyl sulfate ($NaDodSO_4$), 0.1 M NaCl, 10 mM Tris-Cl (pH 7.4), and 1 mM ethylenediaminetetraacetic acid (EDTA) with several strokes of a loose-fitting A pestle Dounce homogenizer and subsequently decanted into an equal volume of buffer saturated phenol/chloroform (1:1) (Perry et al., 1972). These extraction mixtures, generally consisting of a total volume of 100 mL, were agitated for 15–30 min on a rotary shaker. Phases were separated by centrifugation at 15 000*g* for 15 min, and the aqueous phase was reextracted with an additional volume of phenol/chloroform. The total membrane-bound polysomal RNA (MBP RNA) was precipitated from the last aqueous phase with 2.5 volumes of 100% ethanol. On the average, we obtained about 1.5 mg of MBP RNA per gram of tissue.

The MBP RNA was resuspended in oligo(dT)-cellulose application buffer [0.1 M NaCl, 10 mM Tris-Cl (pH 7.4), 0.1% $NaDodSO_4$, and 1 mM EDTA] to a final concentration of ~100 A_{260} units/mL and applied at room temperature to an oligo(dT)-cellulose column (Gilham, 1964) equilibrated with the same buffer. The bound RNA was eluted with 10 mM Tris-Cl (pH 7.4), 0.1% $NaDodSO_4$, and 1 mM EDTA, heated at 70 °C for 4 min, and immediately placed on ice. The sample was adjusted to 0.1 M NaCl and reapplied to the oligo(dT)-cellulose column. The absorbed MBP A^+RNA was eluted as before, adjusted to 0.1 M NaCl, and precipitated with 2.5 volumes of 100% ethanol in a siliconized tube. The final yield of MBP A^+RNA generally amounted to 1.0–1.5% of the total MBP RNA.

Fractionation of MBP A^+RNA and Purification of Ig mRNAs. (i) Sucrose Gradient Centrifugation. MBP A^+RNA (8–15 A_{260} units) was resuspended in 0.5 mL of 0.1% $NaDodSO_4$, 5.0 mM Tris-Cl (pH 7.4), and 0.5 mM EDTA, heated at 70 °C for 5 min, rapidly cooled on ice, and layered onto 12.3 mL of 5–25% (w/w) sucrose gradients in 0.1 M NaCl, 10 mM Tris-Cl (pH 7.4), 0.5% $NaDodSO_4$, 1 mM EDTA. Samples were centrifuged at 29 000 rpm in an SW40 rotor for 15 h at 22 °C, and 0.3-mL fractions were collected in polystyrene tubes (63/11, Walter Sarstedt, Inc.). Fractions were precipitated with 2.5 volumes of 100% ethanol for further analysis.

(ii) Formamide–Polyacrylamide Gel Electrophoresis. Cylindrical 3.5% polyacrylamide–0.6% bisacrylamide gels containing 98% deionized formamide were prepared essentially as described by Duesberg and Vogt (1973) with minor modifications. Fisher F-95, ACS purity grade, formamide was deionized by continuous stirring with Bio-Rad Ag 50 1-X8 mixed bed resin (5% w/v) for ~2 h at room temperature. The gels were polymerized in a solution containing 20 mM Tris-acetate (pH 7.2), 2.5 mM EDTA, and 98% deionized formamide with 2% TEMED and 0.14% ammonium persulfate at room temperature (~1½ h for complete polymerization of a 0.6 × 9.5 cm gel) and then stored at 4 °C under 0.5 mL of deionized formamide. Gels stored in this manner for up to 1 month could be used. After replacement of the deionized formamide with buffered 98% formamide [20 mM Tris-acetate (pH 7.2), 0.5 mM EDTA], the gels were preelectrophoresed at 5 mA/gel for 30 min in E buffer (Loening, 1967). Partially purified Ig mRNAs from sucrose gradients were resuspended in a 2:1 mixture of buffered formamide [2 mM Tris-acetate (pH 7.2), 0.05 mM EDTA] and glycerol plus bromophenol blue (BPB) and were applied with a micropipet directly to the top of the gels. Electrophoresis was carried out at 2.5 mA/tube until the samples entered the gels and was then

[1] Abbreviations used are: Tris-Cl, 2-amino-2-hydroxymethyl-1,3-propanediol chloride; EDTA, ethylenediaminetetraacetic acid.

continued at 5 mA/tube for 3–4 h. The BPB tracker generally left the gel in ~2 h under these conditions and an RNA the size of ~1200 nucleotides migrated halfway through the gel in about 3 h.

After the completion of electrophoresis, electrolyte was removed from the gel tubes, and the tubes were sealed with parafilm and held overnight at 4 °C. After scanning the gels at 280 nm (with no prior soaking to avoid sample loss), the RNA species (0.5–1.0 cm of gel) were excised with a syringe needle and passed through the 20 gauge needle of a 3-cm^3 syringe into 1–1.5 mL of *gel extraction buffer* [0.6 M LiCl, 20 mM sodium acetate (pH 7.5), 0.5% $NaDodSO_4$, and 1 mM EDTA] in a nylon vial. The resultant slurry was passed through the syringe three times and then shaken overnight at room temperature. The contents of the vial were centrifuged in a Brinkman–Eppendorf microfuge at top speed for 15 min. The supernatant was removed with a plastic pipet tip and the pellet reextracted twice with gel extraction buffer in the original syringe. The pooled supernatants were applied to a 0.5–1.0-mL oligo(dT)-cellulose column packed in a 5-cm^3 syringe, and the column was washed with ~20 bed volumes of gel extraction buffer. Bound RNA was eluted with 10 mM Tris-Cl, (pH 7.4), 0.1% $NaDodSO_4$, and 1 mM EDTA, and 0.3-mL fractions were collected into polystyrene tubes. A^+RNA was adjusted to 0.1 M NaCl and precipitated with 2.5 volumes of 100% ethanol. This procedure generally resulted in RNA recoveries of ≥90%.

Cell-Free Protein Synthesis. Protein synthesis assays were performed with the wheat germ cell-free system. The preparation of the extract and the assay procedure have been described elsewhere (Marcu and Dudock, 1974; Levenson and Marcu, 1976).

Preparation of Cytoplasmic and Secreted Immunoglobulins. (i) Cytoplasmic Immunoglobulin. After a 5-min preincubation in medium lacking Leu, Ile, and Val, ~1.5×10^8 MPC-11 cells at a concentration of 3×10^6 cells/mL were labeled for 2 h at 37 °C with 200 μCi of [^{3}H]Leu (20 Ci/mmol), 50 μCi of [^{14}C]Ile (312 mCi/mmol) and 15 μCi of [^{14}C]Val (260 mCi/mmol) (New England Nuclear) in spinner culture. Cells were lysed by hypotonic shock followed by five to six strokes of a tight-fitting B pestle Dounce homogenizer. The lysate was adjusted to isotonicity with KCl and centrifuged at 1000*g* to remove nuclei. The postnuclear supernatant was made 1% with NP-40 detergent (Shell Co.) and centrifuged at 12 000*g* for 20 min, and the resultant supernatant was stored at −20 °C. For the preparation of labeled heavy- and light-chain markers for $NaDodSO_4$ gel electrophoresis, an aliquot of this S12 supernatant was immunoprecipitated with IgG_{2b} κ-specific antisera as described below for the wheat germ cell-free products.

(ii) Secreted Immunoglobulin. Logarithmically growing MPC-11 cells were washed twice in culture medium containing 1/40 the normal amount of methionine and suspended in the same medium at a final concentration of 5×10^5 cells/mL. Five milliliters of this cell suspension was placed in a Petri dish together with 50 μCi of [^{3}H]methionine, and incubation was carried out at 37 °C in 5% CO_2 for 48 h. The cells were removed from the medium by centrifugation, and the immunoglobulin was precipitated from the supernatant by indirect immunoprecipitation. Heavy and light chains were prepared using $NaDodSO_4$–polyacrylamide gel electrophoresis (Birshtein et al., 1974).

Immunoprecipitations. Rabbit anti-mouse IgG_{2b} κ and sheep anti-rabbit Ig antisera were generously provided by Dr. D. Margulies and anti IgM antisera by Dr. M. Weigert. All other antisera were obtained from Meloy Laboratories, Inc. A portion of the cell-free protein synthesis mixture was incubated with the primary antibody overnight at 4 °C in 5 mM phosphate, pH 7.4, 0.15 M NaCl (PSB) containing 1% sodium deoxycholate (DOC) and 0.5% Triton X-100. This was followed by a second overnight incubation with either goat or sheep anti-rabbit antiserum. The immunoprecipitates were purified through a 1 M sucrose cushion in PBS–DOC–Triton and resuspended several times in the detergent solution with vortexing followed by centrifugation at top speed for 15 min in a Brinkman–Eppendorf microcentrifuge at room temperature. The resultant precipitate was resuspended in $NaDodSO_4$ gel solubilization buffer (Laemmli, 1970) for further analysis. The specificity of this immunoprecipitation procedure for the identification of specific heavy- and light-chain cell-free products is clearly evident from a comparison of tracks F and G of Figure 4, which illustrates the specificity of the rabbit anti IgG_{2b} κ antiserum for the MPC-11 heavy- and light-chain polypeptides.

$NaDodSO_4$ Slab Gel Electrophoresis. Cell-free products and immunoprecipitates were run on 10% acrylamide–0.8% bisacrylamide discontinuous $NaDodSO_4$ slab gels (Laemmli, 1970). The gels were either dried and directly placed on x-ray film (Kodak RP-X-omat) or treated with dimethyl sulfoxide (Me_2SO) followed by 20% 2,5-diphenyloxazole in Me_2SO, dried, and exposed to prefogged x-ray film for quantitative autoradiofluorography (Bonner and Laskey, 1974; Laskey and Mills, 1975).

Ion-Exchange Chromatography of Trypsin and Chymotrypsin Digestions. A mixture of ^{3}H- and ^{35}S-labeled proteins was subjected to proteolytic digestion by sequential additions of trypsin and chymotrypsin. The freeze-dried enzymatic digest was dissolved in 1.5 mL of 0.3 M pyridine hydrochloride, pH 1.7, and the pH was adjusted below 2.0 with glacial acetic acid (Birshtein et al., 1974). The peptides were applied to a heated (60 °C), water-jacketed column (0.9 × 23 cm), packed with a Dowex-50 sulfonated polystyrene resin (Spherix, Type XX907, Phoenix Precision Instrument Co., Philadelphia, Pa.), which was equilibrated with 0.05 M pyridine–acetic acid, pH 3.13 (Laskov and Scharff, 1970). The peptides were eluted with a gradient generated in a Varigrad (Phoenix Precision Instrument Co.), using 110 mL each of the following pyridine–acetic acid buffers: (a) 0.05 M, pH 3.13; (b) 0.10 M, pH 3.54; (c) 0.20 M, pH 4.02; (d) 0.5 M, pH 4.5; (e) 2.0 M, pH 5.0. Two-hundred fractions of 2.5 mL each were collected into glass scintillation vials. The pH was recorded and the buffer was evaporated in an oven, whereupon 0.5 mL of H_2O and 10 mL of Aquasol (New England Nuclear Corp., Boston, Mass.) were added to each vial. The samples were counted in a Beckman scintillation counter with the isosets adjusted so that the spill from the ^{3}H to the ^{35}S channel was <0.001% and the spill from the ^{35}S to the ^{3}H channel was ~17%. The data were corrected for background and spill and plotted as percent of total counts vs. fraction number using a computer program devised by Mr. David Medford.

cDNA Synthesis. cDNAs were synthesized with AMV reverse transcriptase under the following reaction conditions: 10 μg/mL oligo(dT), 40 mM NaCl, 50 mM Tris-Cl (pH 8.3), 6 mM $Mg(CH_3COO)_2$, 5 mM dithiothreitol, 50 μg/mL actinomycin D, 100 μM dGTP, 100 μM dATP, 100 μM dTTP, either 100 μM of [^{3}H]dCTP or 30 μM of [^{32}P]dCTP, 800 μg/mL bovine serum albumin (Pentax), 20 μg/mL mRNA, and 4 units of reverse transcriptase. Reactions were generally performed in 50 μL and were incubated at 37 °C for 60 min. The reaction was terminated by the addition of a tenfold excess of TES buffer [10 mM Tris-Cl (pH 7.4), 2 mM EDTA, 0.1% $NaDodSO_4$]. Phenol red was added and the mRNA hydro-

TABLE I: Protein Synthetic Activities of Formamide Gel Purified Ig mRNAs.

	A Incorp[b] (cpm)		B Stimulation		C pmol incorp		D mol of amino acid incorp/mol of mRNA[c]		E Efficiency of translation[d]		F Immunoprecip (%)[e]	
mRNA[a]	[^{35}S]Met	[^{3}H]Leu	[^{35}S]Met	[^{3}H]Leu	[^{35}S]Met	[^{3}H]Leu	[^{35}S]Met	[^{3}H]Leu	[^{35}S]Met	[^{3}H]Leu	[^{35}S]Met	[^{3}H]Leu
	3.0×10^4	1.7×10^4			0.037	0.11					~1.0	~1.0
κ(11)	1.2×10^6		40×		1.48		2.47		0.65		40	
γ_{2b}(11)	2.5×10^5	3.14×10^5	8.3×	18×	0.27	2.8	0.41	4.3	0.12	0.2	50	42
α(2020)	7.5×10^5	1.35×10^6	25×	79×	0.90	9.4	2.25	23.4	0.45	1.0	55 (5)[f]	55
μ(3741)	2.8×10^5		9.3×		0.31		0.38		0.094		20	

[a] Assays were performed in 25 μL with nonsaturating amounts of the Ig mRNAs (0.25 μg for κ (11), γ_{2b}, and α and 0.62 μg for μ). [b] Hot Cl_3AcOH-insoluble material. The cell-free products were labeled with either [^{35}S]methionine or [^{3}H]leucine. [c] Assuming the following approximate molecular weights of Ig mRNAs obtained from formamide gel electrophoresis: κ (11) (420 000), α and γ_{2b} (630 000), μ (752 500). Counting efficiencies for ^{35}S and ^{3}H were 90 and 30%, respectively. [d] Moles of protein synthesized/mole of specific Ig sequence: computed from column D, the purities of the specific Ig mRNAs shown in Table II and the number of methionines and leucines in the specific proteins [5 Met for κ (11) (Smith, 1973); a minimum of 7 Met and 40 Leu for γ_{2b} (11) (Birshtein, B., personal communication); 10 Met and 47 Leu for α_{2020} (Weigert, M., personal communication; McKean, D. and Marcu, K., unpublished results); 8 Met and ~59 Leu for μ_{3741} (Weigert, M., personal communication)]. [e] Performed with class-specific antisera. [f] Level of immunoprecipitation with γ_{2b} specific antiserum.

lyzed by incubation in 0.3 N NaOH for 60 min at 50 °C. The incubation was neutralized with concentrated HCl and 50–100 μg of yeast RNA carrier was added. The reaction was extracted with an equal volume of phenol/chloroform (1:1) and the phenol/chloroform phase extracted once with TES buffer containing 0.1 M NaCl. Phases were separated by centrifugation in a microfuge, and the aqueous phases were pooled and submitted to Sephadex G-50 chromatography in TE buffer [10 mM Tris-Cl (pH 7.4), 2 mM EDTA]. The cDNA was monitored by counting aliquots of the column eluent in a scintillant composed of 0.5 mL of H_2O and 5.0 mL of Formula 963 (New England Nuclear Corp.). The void volume fractions were pooled in a siliconized tube and adjusted to 0.1 M NaCl, and after the addition of 50 μg of yeast RNA carrier the cDNA was precipitated with 3 volumes of 100% ethanol.

cDNA–mRNA Hybridizations. Hybridizations were performed under paraffin oil in a polypropylene tube in a total volume of 50 μL. Reactions were incubated at 70 °C with at least a 20-fold mass ratio of mRNA to cDNA in a solution containing 0.6 M NaCl, 40 mM Tris-Cl (pH 7.2), 2 mM EDTA, 1 mg/mL yeast RNA, and 0.2 μg/mL poly(A). The samples were incubated for 1.5 min at 100 °C in the absence of salt prior to initiating the hybridization reaction. Reactions were terminated by the addition of 0.5 mL of S_1 nuclease buffer [30 mM sodium acetate (pH 4.5), 10 μg/mL Sigma Type I calf thymus DNA (denatured at 100 °C for 10 min followed by quick cooling at 0 °C), 0.3 M NaCl, and 3 mM $ZnSO_4$] and subsequently divided into four equal portions. Two portions were incubated for 1 h at 50 °C with 10 units of partially purified S_1 nuclease (Sutton, 1971). [^{14}C]Thymidine-labeled native DNA was found to be totally resistant to S_1 nuclease digestion under these conditions. After the addition of 80 μg of Sigma Type II calf thymus DNA to all four portions, the samples were precipitated with 1 mL of 10% Cl_3-AcOH on ice. The precipitates were collected onto Whatman GF/C filters and washed with five 2-mL portions of 5% Cl_3AcOH followed by a wash with 95% ethanol. The filters were dried at 70 °C and counted in 5 mL of Liquifluor (New England Nuclear Corp.) in toluene. The percentage of S_1-resistant counts was plotted as a function of the product of the molar concentration of RNA nucleotides and incubation time in seconds (C_rt) multiplied by a factor of 5 to correct the monovalent cation concentration to standard conditions (Britten and Smith, 1970).

Purification of Ig mRNA Specific cDNA Probes. Preparative hybridization of immunoglobulin cDNAs was performed as described above at an mRNA/cDNA ratio of ~10:1 in a total volume of 100–300 μL. Hybridization was carried out to a C_rt of 5×10^{-2}, at which only the probe corresponding to the abundant mRNA species would be hybridized. The reaction was terminated on ice, and a portion of concentrated S_1 nuclease buffer (lacking salt) was added to a final concentration of 1×. After the addition of 20 units of S_1 nuclease, the samples were incubated at 50 °C for 60 min and the mRNA in hybrid was subsequently hydrolyzed by incubating in 0.3 N NaOH at 50 °C for 60 min. Following neutralization with HCl and phenol–chloroform extraction as described above, the cDNA was purified by Sephadex G-50 chromatography. In the case of heavy-chain mRNAs, the recovery of cDNA with this procedure was generally ~60% of the cDNA input, in agreement with the level of hybridization of the original cDNA preparation at a C_rt of 5×10^{-2}. Following the addition of NaCl to 0.1 M and 50 μg of yeast RNA carrier, the purified cDNA was precipitated with 3 volumes of 100% ethanol. Following this protocol, the size of the cDNA preparations was generally 500–600 nucleotides as judged by analysis on alkaline sucrose gradients.

Alkaline Sucrose Gradient Analysis of cDNAs. cDNAs were sized by centrifugation in an SW50.1 rotor at 33 000 rpm for 16 h at 22 °C in linear 4.8-mL 5–20% (w/w) alkaline sucrose gradients containing 0.1 M NaOH and 0.9 M NaCl. Samples were applied in 25 mM EDTA, pH 8.0. *Hae*III and *Hin*dIII digests of polyoma [^{3}H]DNA, generously supplied by Dr. Jesse Summers, served as DNA markers.

Results

(a) Purification and Cell-Free Translation of Three Major Classes of Heavy-Chain Immunoglobulin mRNAs. (i) IgG mRNA. Following sucrose gradient centrifugation of total MBP A$^+$RNA from MPC-11 tumors (an IgG_{2b} κ producer), two major peaks are observed to sediment at about 16.5 and 13.5 S, as shown in Figure 1A. As seen in Figure 1B, the 16.5S peak contains an mRNA activity encoding a polypeptide of 55 000 daltons which comigrates with authentic γ_{2b} heavy chain, and the13.5S component codes for a 28 000 molecular weight protein corresponding to the κ light-chain precursor (Milstein et al., 1972; Swan et al., 1972; Mach et al., 1973; Schechter, 1973).

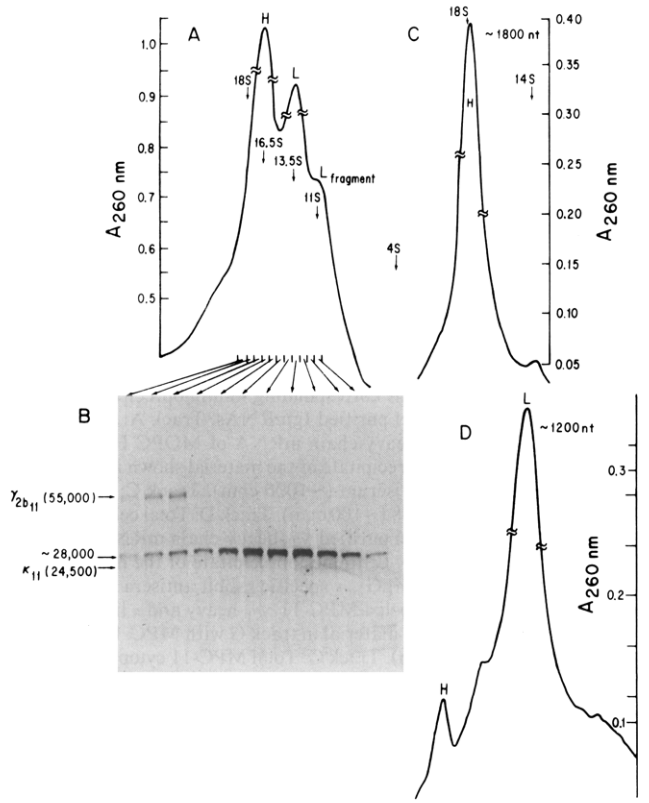

FIGURE 1: Identification and purification of the γ_{2b} heavy- and κ light-chain mRNAs of MPC-11 cells. (A) NaDodSO$_4$–sucrose gradient profile of 10 A_{260} units of the MBP A$^+$RNA from MPC-11 cells. The positions of 18S RNA and 4S RNA markers, run in a parallel gradient, are indicated by arrows. (B) Consecutive fractions of the sucrose gradient were precipitated with ethanol and resuspended in H_2O. Approximately 0.3 μg of RNA in each fraction was translated in a wheat germ cell-free system using [^{35}S]methionine. A portion of the cell-free products (25 000–60 000 cpm) was applied to a 10% discontinuous polyacrylamide NaDodSO$_4$ slab gel and run at 25 mA for 2.5 h. The gel was dried and exposed to x-ray film for 1 day. The positions of migration of in vivo labeled MPC-11 γ_{2b} and κ immunoglobulins are indicated to the left of the gel. (C) Approximately 25 μg of the RNA component migrating at 16.5 S in the sucrose gradient (panel A) was electrophoresed on a cylindrical 3.5% acrylamide–0.6% bisacrylamide gel containing 98% formamide, and the gel was scanned at 260 nm. (D) Approximately 25 μg of the 13.5S RNA species (panel A) was electrophoresed on a formamide–polyacrylamide gel as in panel C.

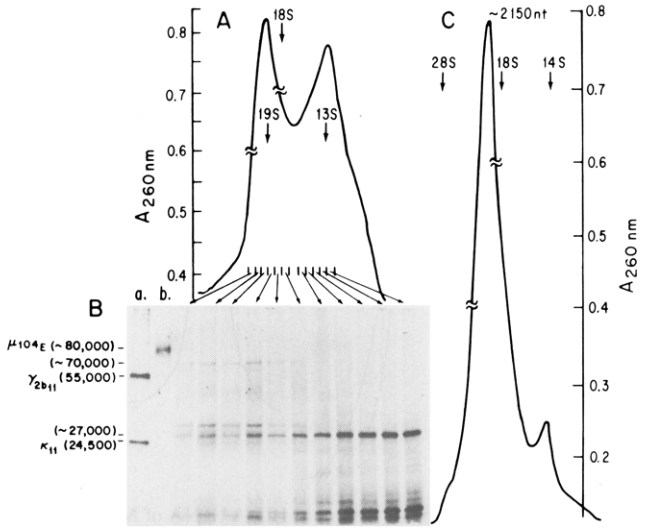

FIGURE 2: Identification and purification of the μ heavy chain mRNA of MOPC 3741. (A) NaDodSO$_4$–sucrose gradient fractionation of ~12 A_{260} units of the MBP A$^+$RNA of MOPC 3741 (an IgMk producer). The positions of migration of 18S and 4S RNA markers from a separate gradient are indicated by arrows. (B) A NaDodSO$_4$ slab gel analysis of the cell-free products directed by sucrose gradient fractions in panel A. 4 × 10^4 cpm [^{35}S]methionine-labeled in vitro products were applied per track. (a) ~9000 cpm of ^{3}H- and ^{14}C-labeled MPC-11 γ_{2b} and κ polypeptide markers; (b) 1800 cpm of purified ^{125}I-labeled MOPC 104E μ heavy chain. (C) Formamide–polyacrylamide gel electrophoresis of the 19S A$^+$RNA species in panel A. Ribosomal RNA markers were run on a parallel gel.

Further fractionation of the 16.5S peak on formamide–polyacrylamide gels yields an RNA species of ~1800 nucleotides (Figure 1C), which codes for the γ_{2b} heavy chain as demonstrated by a detailed analysis of the cell-free translation products. A high proportion (42–50%) of the cell-free products of the γ_{2b} mRNA are precipitable with class-specific antiserum (Table I). When the immunoprecipitated product was submitted to tryptic–chymotryptic fingerprint analysis together with authentic MPC-11 γ_{2b} heavy-chain immunoglobulin, a good correspondence was observed (Figure 5). Minor qualitative differences in the digestion profiles might be attributable to possible amino-terminal precursor sequences amongst the immunoprecipitated cell-free products (Jilka and Pestka, 1977) or to the inability of the wheat germ system to form the glycosylated polypeptides, which are characteristic of the in vivo synthesized immunoglobulins. Since the major cell-free product is observed to comigrate with authentic MPC-11 γ_{2b} heavy chain on NaDodSO$_4$–polyacrylamide slab gels (Figure 4, tracks D–F), such properties would presumably exert either compensatory or very minor effects on the electrophoretic mobility of the polypeptide chain.

The κ_{11} light-chain mRNA migrates with a size of about 1200 nucleotides on formamide gels (Figure 1D) and appears to be ca. five times more efficient in cell-free translation than the γ_{2b} mRNA (Table I). Even though the formamide gel step appears to achieve a complete separation of the heavy- and light-chain mRNAs, a band comigrating with the κ_{11} light-chain precursor was observed amongst the cell-free products directed by gel purified γ_{2b} mRNA (Figure 4, tracks D and E). This cell-free product was conclusively demonstrated to be the light chain precursor in experiments (to be reported elsewhere) in which the translation of heavy chain mRNA preparations was selectively arrested (Paterson et al., 1977) with cloned sequences of κ_{11} and γ_{2b} cDNA.

(*ii*) *IgM mRNA.* The MBP A$^+$RNA of MOPC 3741 cells exhibits some significantly different characteristics from those of the MBP A$^+$RNA derived from the MPC-11 tumor. In the sucrose gradient profile, a 19S RNA component replaces the 16.5S RNA species (Figure 2A). In the cell-free translation assays a 70 000-dalton protein band in addition to several other lower molecular weight products appear in place of the 55 000-dalton polypeptide chain (Figure 2B). A κ light-chain mRNA species and its corresponding precursor protein are also present and possess essentially the same mobilities as observed for the MPC-11 tumor. The RNA species coding for the 70 000-dalton polypeptide migrated as a sharp peak on formamide gels with a size of ~2150 nucleotides (Figure 2C). Following the gel elution of this RNA species and its cell-free translation, about 20% of the cell-free products was immunoprecipitable with anti-MOPC 104$_E$ antiserum (IgM class specific) as shown in Table I. Since the sequence purity of the μ chain mRNA is comparable to other heavy-chain mRNAs (see below), this relatively low level of immunoprecipitation may be attributed to the higher level of early chain termination observed for the μ mRNA in comparison to the κ_{11}, γ_{2b}, and α mRNAs (Figures 1B and 4).

(*iii*) *IgA mRNA.* Additional evidence indicating that a major portion of the MBP A$^+$RNA sedimenting in the

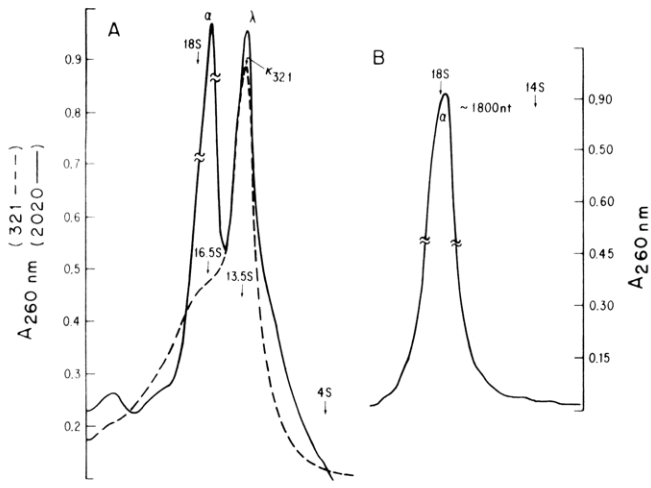

FIGURE 3: A comparison of the total MBP A$^+$RNA of MOPC H2020 (an IgA producer) and MOPC 321 (a κ light-chain producer). (A) NaDodSO$_4$–sucrose gradient analyses of the MBP A$^+$RNAs of MOPC H2020 and MOPC 321 tumors. (B) Purification of the α heavy-chain mRNA of H2020. Formamide–polyacrylamide gel electrophoresis of the 16S A$^+$RNA species in panel A. Ribosomal RNA markers were run on a parallel gel.

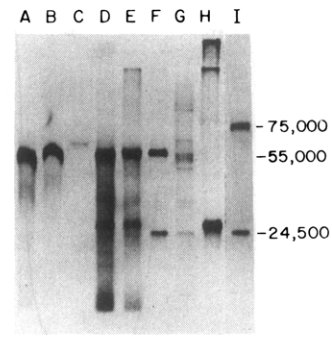

FIGURE 4: Analysis on a 10% polyacrylamide–NaDodSO$_4$ slab gel of cell-free products and their corresponding immunoprecipitates directed by selected formamide gel purified IgmRNAs. Track A: Total cell-free products directed by α heavy-chain mRNA of MOPC H2020 (~1000 cpm). Track B: Immunoprecipitate of the material shown in track A with a class-specific rabbit antiserum (~1000 cpm). Track C: ^{125}I-labeled α heavy chain of MOPC J558 (~100 cpm). Track D: Total cell-free products directed by formamide gel purified γ_{2b} heavy-chain mRNA of MPC-11 cells (~3000 cpm). Track E: Immunoprecipitate of the material shown in track D with MPC-11 IgG$_{2b}$ κ specific rabbit antisera (~3000 cpm). Track F: ^{3}H- and ^{14}C-labeled MPC-11 γ_{2b} heavy and κ light chains: an immunoprecipitate of the material in track G with MPC-11 IgG$_{2b}$ κ specific antiserum (~750 cpm). Track G: Total MPC-11 cytoplasmic proteins labeled in vivo with [^{3}H]Leu, [^{14}C]Val, and [^{14}C]Ile (~10 000 cpm). Track H: Immunoprecipitation with rabbit anti-mouse κ antisera of cell-free products directed by κ_{11} mRNA (~2000 cpm). Track I: ^{125}I-labeled MOPC 3741 μ and κ polypeptides (~2000 cpm). The gel was treated with Me$_2$SO and PPO, dried, and exposed to Kodak XR-5 film for ~5 days.

16.5–19S size range is heavy-chain immunoglobulin mRNA was provided by a comparison of the total MBP A$^+$RNA isolated from H2020 cells [which produce both heavy and light chains (IgAλ)] with MOPC 321 cells [which secrete only κ light chains (Potter, 1967)]. As shown in Figure 3A, the 16.5S component, which is so prominent in the profiles of H2020 A$^+$RNA, is essentially absent in the MOPC 321 plasmacytoma. Upon formamide gel electrophoresis (Figure 3B) essentially all of the material in the 16.5S peak migrates as a single component of about 1800 nucleotides. The A$^+$RNA eluted from the gel efficiently directed the synthesis of an α heavy chain in the wheat germ system as demonstrated by immunoprecipitation with IgA-specific antisera (Table I). The cell-free products migrated as a single component of 55 000 daltons on NaDodSO$_4$–polyacrylamide slab gels with a slightly faster mobility than an authentic α heavy-chain marker (Figure 4, tracks A–C). This difference may be attributable to the level of in vivo glycosylation of the α protein. The α heavy-chain cell-free product comigrates with the predominant γ_{2b} product as do their corresponding mRNAs on formamide gels (~1800 nucleotides). The cell-free product directed by α(H2020) mRNA has recently been shown to contain a leucine-rich amino-terminal precursor sequence of 19 residues (McKean and Marcu, unpublished results).

(*b*) *Purity of Heavy-Chain mRNA Preparations.* The purities of the individual heavy-chain mRNAs were assayed by hybridization to their respective cDNA probes. It has been demonstrated by numerous investigators that the C_rt at which one-half of the cDNA is hybridized under conditions of mRNA excess is a measure of the nucleotide sequence complexity of the RNA preparation. The $C_rt_{1/2}$ values of two mRNA preparations can be related by considering the size of the RNA driver and the length of the cDNA tracer (Birnstiel et al., 1972; Wetmur and Davidson, 1968). In these studies, the complexities of the heavy-chain mRNAs were compared to those of two standards of known purity: (a) an equal mixture of α and β human globin mRNA assumed to be 100% pure and (b) formamide gel purified κ_{11} mRNA, determined to have a chemical purity of ~75% by 5′ terminal sequence analysis (Marcu et al., 1978). A hybridization $C_rt_{1/2}$ of 1.6×10^{-3} (corrected to standard salt conditions) was obtained for the globin mRNA in agreement with values reported by numerous other investigators (Ross et al., 1973, 1974; Stavnezer et al., 1974; Honjo et al., 1976; Hereford and Rosbash, 1977). However, the κ_{11} light-chain mRNA hybridized with a $C_rt_{1/2}$ value of 1.3×10^{-3} (Figure 6A) which was significantly lower than expected. Assuming a combined size for the α and β human globin mRNAs of about 1400 nucleotides (Proudfoot et al., 1977; Tolstoshev et al., 1977) and taking 1200 nucleotides (Figure 1D) for the κ_{11} mRNA, the expected $C_rt_{1/2}$ value for a κ_{11} mRNA preparation that is 75% pure would be 1.8×10^{-3}. These results demonstrate that the sole use of globin mRNA as a complexity standard in our case would have resulted in an overestimation of mRNA purity. Consequently, both the κ_{11} and the globin mRNAs were used as standards in the following analyses to obtain minimum and maximum estimates for heavy-chain mRNA purities.

Hybridization curves for gel-purified κ_{321} and λ_{2020} light-chain mRNAs are depicted in Figure 6B. The $C_rt_{1/2}$ values are 9.5×10^{-4} for both mRNAs, indicating a sequence purity of close to 100% in comparison to either the κ_{11} or globin standards. No cross-hybridization between these mRNA preparations was observed, indicating that these plasmacytomas express solely κ or solely λ genes.

The complexities of the individual gel-purified heavy-chain mRNA preparations are shown in Figure 6C, D. Hybridization curves for α and γ_{2b} heavy-chain mRNAs to their initial cDNA preparations both indicate the presence of abundant RNA species amounting to about 60% of the mRNA population. An additional 20% of the γ_{2b} cDNA was observed in hybrid at C_rt values greater than 10, indicating the presence of a highly complex population of contaminating species (data not shown). In contrast, cDNA preparations from the globin mRNA or from κ_{321} generally hybridize to over 90% in the region of abundant mRNA sequences ($<5 \times 10^{-2}$ C_rt), indicating a higher purity of abundant sequences than the heavy-chain mRNA preparations. In order to remove the high-complexity contamination of the heavy-chain cDNA probes

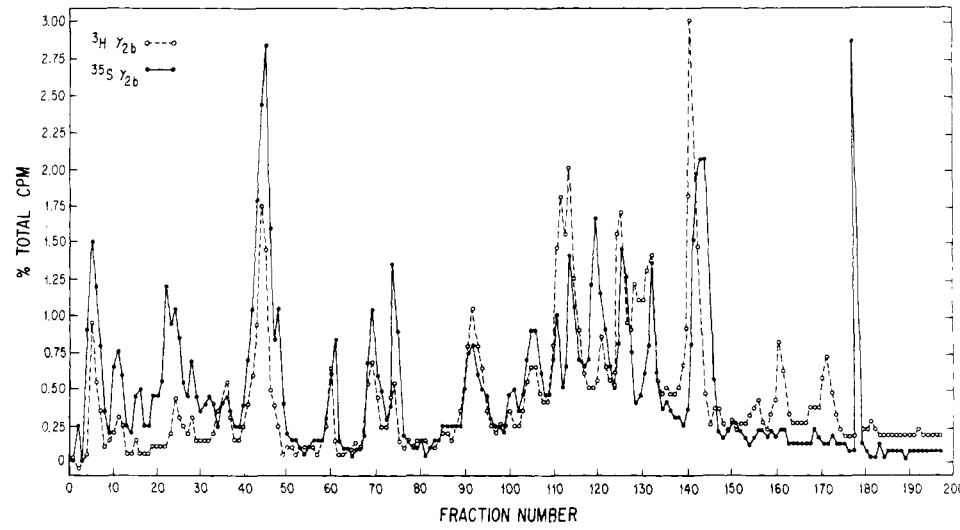

FIGURE 5: A comparative trypsin–chymotrypsin fingerprint analysis via ion-exchange chromatography on Dowex 50 of [^{3}H]methionine-labeled in vivo MPC-11 γ_{2b} heavy chain and [^{35}S]Met-labeled immunoprecipitated cell-free products directed by formamide gel purified MPC-11 γ_{2b} mRNA. Peptides resulting from sequential trypsin and chymotrypsin digestions were analyzed by ion-exchange chromatography on Dowex as described in detail under Methods. The data were plotted as a function of percent of total counts vs. fraction number and were also corrected for a 17% spillover of ^{35}S into ^{3}H. Approximately 16 000 cpm of ^{35}S cell-free products was combined with ~84 000 cpm of $^3H\gamma_{2b}$ marker for the analysis. The differences observed in the ratios of particular ^{3}H- and ^{35}S-labeled polypeptides may be a reflection of early chain-termination products occurring in variable yields. Minor differences such as the shift of the ^{35}S material in fractions 142–144 may simply result from a deamidation resulting in one additional charge. The sharp one-fraction peak of ^{35}S material at the end of the profile (fraction no. 178) is most probably artifactual and was not observed in other analyses.

and thereby obtain a more accurate estimate of the purity of the abundant mRNA species, we employed a purification step in which the unhybridized, high-complexity cDNA was digested with S_1 nuclease after an initial hybridization to a C_rt of 5×10^{-2} in mRNA excess (mRNA/cDNA, 10:1). The hybridized cDNAs were separated from the RNA, purified by Sephadex G-50 chromatography, and rehybridized to their respective heavy-chain mRNAs as shown in Figure 6D. Upon hybridization to the original mRNA preparations, all of these recycled cDNAs generally reach a plateau of hybridization ≥90% with no change in the original hybridization kinetics. The complexities of all three heavy-chain mRNAs fall in essentially the same range, yielding $C_rt_{1/2}$ values of $2.5–3.4 \times 10^{-3}$, which indicate sequence purities of 50–83% (see Table II).

The levels of purity of all the light- or heavy-chain mRNA preparations were clearly higher than the levels of immunoprecipitation attained with their in vitro synthesized polypeptides (compare data of Tables I and II). This may either reflect the efficiency of this latter procedure and/or the presence of early chain-termination products which may lack specific antigenic determinants.

Cross-hybridization experiments were performed with κ cDNA and gel purified γ_{2b} and μ mRNAs at mRNA/cDNA ratios of 400:1 (Figure 6D). Both experiments indicate a contamination level of less than one light-chain sequence per 1000 heavy-chain mRNA molecules. This clearly demonstrates the absence of light-chain mRNA aggregates in the heavy-chain mRNA preparations. Cross-hybridization of γ_{2b} cDNA with κ_{11} light-chain mRNA indicated a low level of degraded heavy-chain sequences in the light-chain mRNA preparation. From the $C_rt_{1/2}$ value of this reaction, we estimate that about 7% of the mass of light-chain mRNA preparation could consist of degraded heavy-chain sequences.

Given the scarcity of light-chain sequences in the heavy-chain mRNA preparations, the detection of κ_{11} light-chain precursor among the cell-free translation products of γ_{2b} mRNA (Figure 4) would seem to indicate that the translation efficiency of light-chain mRNA is at least two orders of magnitude greater than that of the heavy-chain mRNA under these conditions. However, since such a large difference was not indicated by our estimates of translation efficiency (Table I), we can offer no simple explanation of this observation at the present time.

TABLE II: Purity of Heavy-Chain mRNAs.

mRNA	Approx. length (nt)[a]	$C_rt_{1/2}$	Purity (%)
κ_{11}	1200	1.3×10^{-3}	~75[d]
α & β globin	1380[b]	1.6×10^{-3}	100
κ_{321}	1200	9.5×10^{-4}	~100[e]
λ_{2020}	1200	9.5×10^{-4}	~100[e]
α_{2020}	1800	2.5×10^{-3}	60–83[f]
γ_{2b}	1800	3.0×10^{-3}	49–70 (46)[d]
μ_{3741}	2150	3.4×10^{-3}[c]	51–84[f]

[a]Determined by formamide gel electrophoresis as shown in Figures 1C,D, 2C, and 3B: nt, nucleotides. [b]Proudfoot et al. (1977); Tolstoshev et al. (1977). [c]Observed $C_rt_{1/2}$ value of 4.3×10^{-3} (Figure 6D) was decreased by a factor of 1.25 due to the smaller size of μ cDNA used for the C_rt analysis (~350 nucleotides on alkaline sucrose gradients compared to ~550 nucleotides for the κ_{11} and globin mRNA standards) according to Wetmur and Davidson (1968). [d]Purity observed by determination of 5′ terminal sequence of $^{32}PO_4$-labeled, formamide gel purified κ and γ_{2b} mRNAs (Marcu et al., 1978). [e]Estimated from $C_rt_{1/2}$ of κ_{11} mRNA. [f]Determined by using the $C_rt_{1/2}$ values of the κ_{11} and α and β human globin mRNAs as minimum and maximum standards for mRNA purities respectively:

$$\text{purity of heavy chain mRNA (\%)} = 100\left[\frac{\left(\frac{\text{length of H mRNA (nt)}}{\text{length of complexity std (nt)}}\right) \times (C_rt_{1/2} \text{ of std}) \text{ (fractional purity of std)}}{C_rt_{1/2} \text{ of H mRNA}}\right]$$

(c) *Recovery of Heavy-Chain mRNA Sequences.* The iso-

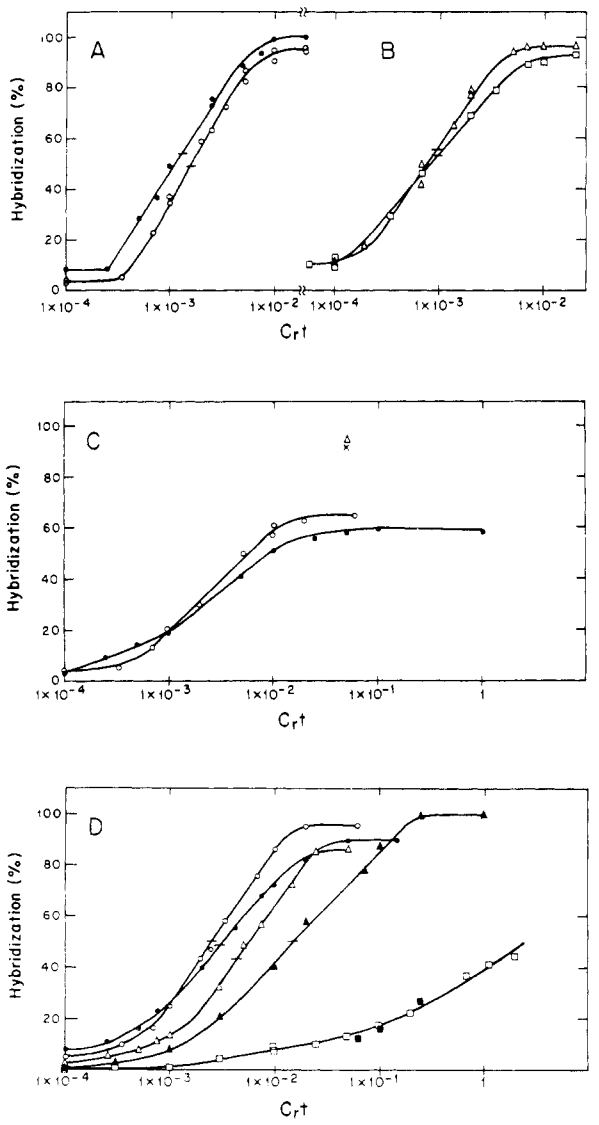

FIGURE 6: Kinetics of hybridization of specific Ig cDNAs with Ig mRNAs. (A) Hybridization of κ_{11} cDNA with formamide gel purified κ_{11} mRNA (●-●) and a mixture of human α and β cDNAs with their mRNAs (○-○). The κ_{11} cDNA was prepared from formamide gel purified κ_{11} mRNA and purified by a prehybridization to κ_{11} mRNA at a C_rt of 5×10^{-2}. The α and β human globin mRNAs (a generous gift of Dr. Elias Schwartz) were prepared by oligo(dT)-cellulose chromatography of human reticulocyte polysomal RNA followed by two NaDodSO$_4$-sucrose gradients, resulting in a homogeneous 10S A$^+$RNA which efficiently directed the cell-free synthesis of α and β polypeptides as monitored by carboxymethylcellulose chromatography. Formamide gel electrophoresis has indicated equal quantities of the α and β mRNAs (Schwartz, E., personal communication). (B) Kinetics of hybridization of formamide gel purified κ_{321} (▲-▲) and λ H2020 (□-□) mRNAs with their respective purified cDNAs. (C) Kinetics of hybridization of formamide gel purified α H2020 (○-○) and γ_{2b} MPC-11 (●-●) heavy-chain mRNAs with their respective unpurified cDNA preparations. The level of hybridization beyond the initial plateau was found to increase significantly only at C_rt values higher than 10. The levels of hybridization observed with human α and β globin (△) and κ_{321} (×) mRNAs with their respective unpurified cDNA preparations are indicated at a C_rt of 5×10^{-2}. (D) Kinetics of hybridization of formamide gel purified heavy-chain mRNAs with their respective purified cDNAs: α (○-○), γ_{2b} (●-●), and μ (△-△). Hybridization of γ_{2b} (MPC-11) cDNA with formamide gel purified κ_{11} mRNA (▲-▲). Hybridization of κ_{11} cDNA with a 400/1 excess of either γ_{2b} mRNA (□-□) or μ 3741 mRNA (■-■), both purified from formamide gels. Unless otherwise noted, all hybridizations were performed with a 20/1 excess of mRNA/cDNA. The levels of S_1 nuclease resistance for all points were determined with ~3500 cpm of [^{3}H]cDNA (10^7 cpm/μg) or (for μ 3741) [^{32}P]cDNA (4×10^8 cpm/μg). As determined by alkaline sucrose gradient analysis, all cDNAs were 550–600 nucleotides long, except the 3741 [^{32}P]cDNA which was ~350 nucleotides in length.

lation of the MPC-11 γ_{2b} heavy-chain mRNA was followed by hybridization of purified cDNA probe to the mRNA at various stages of its purification (Table III). Greater than 90% of the heavy-chain mRNA sequences was recovered in the MBP A$^+$RNA, resulting in a purification factor of about 2.4-fold. Additional factors of approximately two each are obtained by the sucrose gradient fractionation and the formamide gel electrophoresis. The best final recovery of heavy-chain mRNA was obtained by proceeding directly to the formamide gel step after a single sucrose gradient.

(*d*) *Cross-Hybridization of Heavy-Chain mRNAs of Different Classes and Allotypes.* Purified heavy-chain cDNA probes were hybridized in mRNA excess to heavy-chain mRNA sequences representing a variety of classes and allotypes (Table IV). Considering that oligo(dT) was used as a primer in the cDNA synthesis, the size of the cDNA probes (about 600 nucleotides) is such that these hybridizations may be considered to measure sequence homologies predominately of the constant and 3′ noncoding regions of the mRNAs. For these studies, three additional heavy-chain mRNAs (α_{J558}, $\gamma_{2b_{2880}}$ and μ_{104E}) were partially purified. The results clearly demonstrate the high level of specificity of the purified cDNA probes, since the γ_{2b}, α, and μ cDNAs hybridize only with mRNAs of the same class of heavy chain. These plasmacytomas express only one class of heavy-chain mRNA, and, in agreement with the in vitro translation date, the mRNA species identified as purified heavy-chain mRNAs are not contaminated with high-abundance mRNA species common to mouse plasmacytoma cells. The essentially complete S_1 resistance of hybrids containing heavy-chain mRNAs of the different allotypes (Balb/C vs. NZB mice) suggests that constant-region allotypic differences, at least those of the Balb/C-NZB type (Herzenberg et al., 1968; Mage et al., 1973; Warner et al., 1977), consist of minor base changes scattered throughout the 3′ terminal portion of the mRNA sequence. In addition, it would appear that the 3′ noncoding sequences of heavy-chain mRNAs of the same class irrespective of allotype are highly homologous.

Discussion

The mechanisms of diversification and expression of heavy-chain immunoglobulin genes appear to be processes of considerable complexity. Six major heavy-chain constant regions have been identified in the mouse and all have been found to be closely linked genetically (Herzenberg et al., 1968; Mage et al., 1973). The variable regions of mouse heavy chains would appear to be coded by multiple germ line genes (Barstad et al., 1974; Weigert and Riblet, 1977). In addition, the process of heavy-chain gene expression has been proven to be a sequential one with the initial response resulting in IgM synthesis followed by a switch to IgA or IgG expression with strong evidence for the maintenance of the original variable region (Nisonoff et al., 1975). Similar switching phenomena have also been observed by mutagenesis of cell culture lines in vitro, resulting in a switch of IgG$_{2b}$ to IgG$_{2a}$ synthesis (Kuehl, 1977). In order to fully understand these phenomena at the molecular level, specific probes for the variable and constant regions of a variety of heavy chains are essential.

In this report, we have described a convenient protocol which is suitable for the isolation and purification of intact heavy-chain mRNAs representing any class of heavy-chain immunoglobulin. The essential factors in obtaining high yields of biologically active heavy-chain mRNAs were as follows:

(a) The rapid isolation of membrane-bound polyribosomes was routinely performed with fresh tissue. The use of frozen

TABLE III: Purification and Recovery of γ_{2b} Heavy-Chain mRNA from MPC-11 Tumors.

Purif step	Yield of RNA (μg)[a]	$C_rt_{1/2}$[c]	Purif factor	Yield of γ_{2b} mRNA[d] μg	%
Total A^+RNA	3600[b]	2.4×10^{-2}	1×	225	100
MBP A^+RNA	1440[b]	1.0×10^{-2}	2.4×	216	96
Sucrose gradient	280	6.0×10^{-3}	4×	70	32.4
Sucrose gradient rerun of peak fractions	140	4.0×10^{-3}	6×	53	23.5
Formamide gel after 1st sucrose gradient	119	3.0×10^{-3}	8×	60	26.6
Formamide gel after 2nd sucrose gradient	60	3.0×10^{-3}	8×	30	13.3

[a]Obtained from 80 g of tumors. [b]~1.2% of total RNA. [c]cDNA probe was synthesized from formamide gel purified mRNA and further purified by S_1 nuclease digestion of cDNA-mRNA hybrids formed at a C_rt of 5×10^{-2}. [d]Assuming pure γ_{2b} mRNA has a $C_rt_{1/2}$ of 1.5×10^{-3} (see Table II for minimum purity estimate of γ_{2b} mRNA).

tumors resulted in the loss of heavy-chain mRNA activity, even though functional light-chain mRNA was still observed (O. Valbuena, unpublished observations).

(b) In order to prevent the loss of material due to RNase activity, a variety of precautions were taken during the extraction procedure. A 30% sucrose solution containing cycloheximide and poly(vinyl sulfate) was included in the initial homogenization step performed at 0 °C, and sodium heparin (250 μg/mL) was immediately added to the postnuclear supernatant (to prevent nuclear lysis). The microsomal fraction containing the membrane-bound polysomes was rapidly extracted with 1.5% $NaDodSO_4$ and phenol-chloroform. After the dissection of the tumors, this entire procedure generally lasted no longer than 1–1.5 h. Significantly lower yields of intact heavy-chain mRNAs were obtained when the microsomal pellet was treated with detergents (NP-40, Triton or deoxycholate) and submitted to a lengthy ultracentrifugation in order to pellet the released polysomes.

(c) Heating of the MBP A^+RNA before a second pass over oligo(dT)-cellulose was found to be critical for the removal of high levels of contaminating 18S rRNA. The efficiency of in vitro translation of sucrose gradient purified heavy-chain mRNA was observed to substantially increase following this treatment. In addition, DEAE-Sephadex chromatography of an RNase T_2 digestion of formamide gel purified, $^{32}PO_4$-labeled γ_{2b} mRNA of MPC-11 cells has indicated a negligible level of 2′-O-methylated dinucleotides (Marcu et al., 1978), a diagnostic feature of rRNAs (Perry, 1976).

(d) Finally, electrophoresis and elution of the specific mRNAs from formamide containing polyacrylamide gels resulted in heavy-chain mRNA purities of 50–83% and the removal of all but negligible amounts of contaminating light-chain mRNAs.

Using these procedures, we have succeeded in isolating and characterizing six heavy-chain mRNAs of different classes and allotypes. IgG and IgA heavy-chain mRNAs both directed the in vitro synthesis of immunoprecipitable polypeptides of 55 000 molecular weight. On the basis of their size on 98% formamide gels (~1800 nucleotides) and amino acid compositions (~430 residues each), these mRNAs would appear to consist of 300–400 nucleotides of noncoding sequences, assuming an average poly(A) sequence length of 150–200 nucleotides. The μ heavy-chain mRNA encoded a major immunoprecipitable in vitro product of ~70 000 daltons. This mRNA migrated with a size of ~2150 nucleotides on denaturing gels, and from its amino acid content (~550 residues) also appears to contain 300–400 nucleotides of noncoding sequences. Consequently, all classes of heavy-chain mRNAs would appear to possess approximately the same noncoding sequence content as observed for light-chain mRNAs (Cowan et al., 1976; Brownlee et al., 1973; Kuehl, 1977). These results suggest the maintenance of a fairly constant size of noncoding sequences in the organization of all immunoglobulin mRNAs independent of overall mRNA size and coding potential.

TABLE IV: Cross-Hybridization of Heavy-Chain mRNAs of Different Classes and Allotypes.

cDNA[a]	mRNA[b]	Hybridization (%)[c]
γ_{2b} (MPC-11)	γ_{2b} (MPC-11)	88.0
	γ_{2b} (2880)	89.8
	α (H2020)	2.3
α (H2020)	α (H2020)	95.3
	α (J558)	93.3
	γ_{2b} (MPC-11)	2.2
μ (3741)	μ (3741)	85.6
	μ (104E)	81.9
	γ_{2b} (MPC-11)	1.4
	γ_{2b} (2880)	1.0
	α (H2020)	2.0
	α (J558)	2.1

[a]cDNAs were synthesized from formamide gel purified mRNAs and subsequently purified by hybridization to their respective mRNAs to a C_rt of 5×10^{-2} followed by S_1 nuclease digestion of unhybridized cDNA. The γ_{2b} and α cDNAs have a mean size of ~600 nucleotides and the μ cDNA ~500 nucleotides on alkaline sucrose gradients. [b]All mRNA preparations were purified through either two sucrose gradient steps [γ_{2b} (11 and 2880); α (2020 and J558)], one sucrose gradient followed by a formamide gel (μ 3741), or one sucrose gradient (μ_{104E}) and all were hybridized to a C_rt of 1×10^{-1} at mRNA/cDNA ratios of 100–200:1. [c]Values are corrected for self annealing of cDNA probes: 2.7% (γ_{2b}), 3% (α), and 1.7% (μ).

Cross-hybridization studies performed with specific heavy-chain constant-region cDNA probes (~600 nucleotides) and heavy-chain mRNAs of different allotypes (Balb/c vs. NZB) were unable to detect any nucleotide sequence differences, even with stringent S_1 nuclease assays. We have obtained similar high levels of cross-hybridization when a series of mRNAs coding for various κ proteins, which exhibit up to 15% difference in amino acid sequence throughout their variable regions, was annealed with a full-length cDNA probe for one of the mRNAs (Marcu et al., 1977). Thus, nucleotide sequence differences that reflect as much as a 15% difference in amino acid sequence might not be detected by our hybridization assay. On the other hand, only negligible hybridization was observed between cDNA probes for the constant regions

of mouse heavy chains and excess human placental DNA (Valbuena et al., 1977), in spite of the fact that the amino acid sequences of mouse and human heavy chains are at least 60% homologous in the constant regions (Fougereau et al., 1976). Thus, the amino acid sequences associated with allotype specificity must certainly constitute less than 40% of the constant region in order for the high levels of cross-hybridization to have been observed.

In conclusion, allotypic differences would appear to represent a relatively minor fraction of the heavy-chain constant-region nucleotide sequence, implying that the amino acid substitutions resulting in the observed repertoire of mouse heavy-chain constant-region allotypes (Herzenberg et al., 1968; Mage et al., 1973; Warner et al., 1977) may be of a simple nature consisting of a limited number of sequence alterations. In order to determine if the observed phenomenon is the rule and not the exception for the mouse immune system, a larger sample size consisting of more divergent strains of mice must be investigated.

Acknowledgments

We gratefully acknowledge the help of Dr. Barbara Birshtein and Mr. Richard Campbell of the Department of Cell Biology, Albert Einstein College of Medicine in New York for performing the proteolytic analysis of the MPC-11 γ_{2b} mRNA directed cell-free products and Ms. Joselina Gatmaitan of this institute for performing amino acid base compositions of heavy-chain polypeptides. In addition, we thank Drs. Melvin Bosma, Martin Weigert, Roy Riblet, Robert Marks, Martin Julius, and David Margulies for supplying us with mouse plasmacytomas, ^{125}I-labeled, purified heavy- and light-chain polypeptides, and class-specific immunoglobulin antisera. The MOPC 104E tumor was originally developed by Dr. Michael Potter. We are indebted to Dr. Michael Chirigos of the Office of Program Resources and Logistics, Viral Cancer Program, National Institutes of Health, and Dr. James Beard for supplying AMV reverse transcriptase.

References

Adetugbo, K., and Milstein, C. (1977), *J. Mol. Biol. 115,* 75.

Barstad, P., Farnsworth, V., Weigert, M., Cohen, M., and Hood, L. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 4096.

Bedard, D. L., and Huang, R. C. C. (1977), *J. Biol. Chem. 252,* 2592.

Bernardini, A., and Tonegawa, S. (1974), *FEBS Lett. 41,* 73.

Birnstiel, M. L., Sells, B. H., and Purdon, I. F. (1972), *J. Mol. Biol. 63,* 21.

Birshtein, B. K., Preud'homme, T. L., and Scharff, M. D. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 3478.

Bonner, W. M., and Laskey, R. A. (1974), *Eur. J. Biochem. 46,* 83.

Britten, R. J., and Smith, J. (1970), *Carnegie Inst. Wash. Yearb. 68,* 378.

Brownlee, G. G., Cartwright, E. M., Cowan, N. J., Jarvis, J. M., and Milstein, C. (1973), *Nature (London), New Biol. 244,* 236.

Cowan, N. J., and Milstein, C. (1973), *Eur. J. Biochem. 36,* 1.

Cowan, N. J., Secher, D. S., and Milstein, C. (1976), *Eur. J. Biochem. 61,* 355.

Duesberg, P. H., and Vogt, P. K. (1973), *J. Virol. 12,* 594.

Eisen, H. N. (1974), Immunology, New York, N.Y., Harper & Row, p 446.

Fougereau, M., Bourgois, A., dePreval, C., Serra, J. R., and Schiff, C. (1976), *Ann. Immunol. (Paris) 127,* 41.

Gilham, P. T. (1964), *J. Am. Chem. Soc. 86,* 4982.

Green, M., Zehavi-Willner, T., Graven, P. N., McInnes, J., and Pestka, S. (1976), *Arch. Biochem. Biophys. 172,* 74.

Hereford, L. M., and Rosbash, M. (1977), *Cell 10,* 453.

Herzenberg, L. A., McDevitt, H. O., and Herzenberg, L. A. (1968), *Annu. Rev. Genet. 2,* 209.

Honjo, T., Swan, D., Nau, M., Norman, B., Packman, S., Polsky, F., and Leder, P. (1976), *Biochemistry 15,* 2775.

Hozumi, N., and Tonegawa, S. (1976), *Proc. Natl. Acad. Sci. U.S.A. 73,* 3628.

Jilka, R. L., and Pestka, S. (1977), *Proc. Natl. Acad. Sci. U.S.A. 74,* 5692.

Kuehl, W. M. (1977), *Curr. Top. Microbiol. Immunol.* (in press).

Laskey, R. A., and Mills, A. D. (1975), *Eur. J. Biochem. 46,* 83.

Laskov, R., and Scharff, M. D. (1970), *J. Exp. Med. 131,* 515.

Levenson, R. G., and Marcu, K. B. (1976), *Cell 9,* 311.

Loening, U. E. (1967), *Biochem. J. 102,* 251.

Mach, B., Faust, C., and Vassalli, P. (1973), *Proc. Natl. Acad. Sci. U.S.A., 70,* 451.

Mage, R., Lieberman, R., Potter, M., and Terry, W. D. (1973), in The Antigens, Vol. 1, Sela, M., Ed., New York, N.Y., Academic Press, p 299.

Marcu, K., and Dudock, B. (1974), *Nucleic Acids Res. 1,* 1385.

Marcu, K., Schibler, U., and Perry, R. (1978), *J. Mol. Biol.* (in press).

Marcu, K., Valbuena, O., Weigert, M., and Perry, R. (1977), *J. Cell Biol. 75,* 385a.

Milstein, C., Brownlee, G. G., Harrison, T. M., and Mathews, M. B. (1972), *Nature (London), New Biol. 239,* 117.

Nisonoff, A., Hopper, J. E., and Spring, S. B. (1975), in The Antibody Molecule, Dixon, F. J., Jr., and Kunkel, H. G., Ed., New York, N.Y., Academic Press, pp 477–481.

Ono, M., Kondo, T., Kawakami, M., and Honjo, T. (1977), *J. Biochem. (Tokyo) 81,* 949.

Paterson, B. M., Roberts, B. E., and Kuff, E. L. (1977), *Proc. Natl. Acad. Sci. U.S.A. 74,* 4370.

Perry, R. P. (1976), *Annu. Rev. Biochem. 45,* 605.

Perry, R. P., LaTorre, J., Kelley, D. E., and Greenberg, J. R. (1972), *Biochim. Biophys. Acta 262,* 220.

Potter, M. (1967), *Methods Cancer Res. 2,* 105.

Proudfoot, N. J., Gillam, S., Smith, M., and Longley, J. I. (1977), *Cell 11,* 807.

Ross, J., Gielen, J., Packman, S., Ikawa, Y., and Leder, P. (1974), *J. Mol. Biol. 87,* 697.

Schechter, I. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 2256.

Schmeckpeper, B. J., Cory, S., and Adams, J. M. (1974), *Mol. Biol. Rep. 1,* 355.

Smith, G. P. (1973), *Science 181,* 941.

Sonenshein, G. E., and Brawerman, G. (1976), *Biochemistry 15,* 5501.

Stavnezer, J., Huang, R. C. C., Stavnezer, E. and Bishop, J. M. (1974), *J. Mol. Biol. 88,* 43.

Sutton, W. D. (1971), *Biochim. Biophys. Acta 240,* 522.

Swan, D., Aviv, H., and Leder, P. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 1967.

Tolstoshev, P., Williamson, R., Eskdale, J., Verdier, G., Godet, J., Nigon, V., Trabuchet, G., and Benabadji, M. (1977), *Eur. J. Biochem. 78,* 161.

Tonegawa, S., Brack, C., Hozumi, N., and Schuller, R. (1977), *Proc. Natl. Acad. Sci. U.S.A., 74,* 3518.

Tonegawa, S., Hozumi, N., Matthyssens, G., and Schuller, R. (1976), *Cold Spring Harbor Symp. Quant. Biol. 41,* 877.
Valbuena, O., Marcu, K., Croce, C., Huebner, K., Weigert, M., and Perry, R. (1977), *J. Cell Biol. 75,* 390a.
Warner, N. L., Goding, J. W., Gutman, G. A., Warr, G. W., Herzenberg, L. A., Osborne, B. A., Van der Loo, W., Black, S. J., and Loken, M. R. (1977), *Nature (London) 265,* 447.
Weigert, M., and Riblet, R. (1976), *Cold Spring Harbor Symp. Quant. Biol. 41,* 837.
Wetmur, J. G., and Davidson, N. (1968), *J. Mol. Biol. 31,* 349.

Molecular Studies of Subspecificity Differences among Phosphorylcholine-Binding Mouse Myeloma Antibodies Using ^{31}P Nuclear Magnetic Resonance†

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ABSTRACT: We have determined the pH dependencies of the binding affinities of the mouse myeloma immunoglobulins M603, W3207, and M167 for the haptens phosphorylcholine (PC) and L-α-glycerophosphorylcholine (GPC). These affinities are generally maximal near neutral pH with the exception of the binding of PC by M167 which is strongest at pH 5.5. These data have helped to clarify the nature and relative importance of the ionic interactions between hapten and antibody. ^{31}P nuclear magnetic resonance (NMR) techniques were used to probe the influence of pH on the microenvironment of the phosphate group of several haptens when these were bound to M603, W3207, and M167. The phosphate subsites of M603 and W3207 are both electropositive and also show other similarities; that of M167 has a net electronegative character. The two hydrogen bonds known to be formed between M603 and the phosphate oxygens of PC are also involved in binding GPC and are essentially unaffected by pH in the region 3–9. Studies with the hapten 3-trimethylamino-1-propanol phosphate (TMAPP) show that the binding cavity of M167 is substantially wider than those of M603 and W3207. These results lead to a detailed molecular model of the pH dependent binding of PC and related haptens to these three antibodies; they further indicate the roles of various amino acid residues in defining the differing ligand specificities of these antibodies.

A group of mouse myeloma immunoglobulins which specifically bind phosphorylcholine, PC[1] (Potter, 1972; Rudikoff et al., 1972; Potter & Lieberman, 1970), serves as a convenient system for studying structure–function relationships between similar, homogeneous antibodies. In this group, the primary structures of the heavy chains and of portions of the light chains from six different proteins are now known (Hood et al., 1975, 1976). The three-dimensional structure of the Fab′ fragment of one of these proteins, M603, as well as its complex with PC (Segal et al., 1974) has allowed direct visualization of the interactions between hapten and specific residues in the antigen combining site of the immunoglobulin which define the antibody specificity.

The high degree of sequence homology among the heavy chain residues of immunoglobulins specific for phosphorylcholine in both mouse and man (Padlan et al., 1976; Riesen et al., 1976) and the observation that most of the residues which interact with hapten in M603 are located in the heavy chain have allowed some rationalization of the molecular origins of the varying binding properties of these proteins (Padlan et al., 1976; Goetze & Richards, 1977a). These facts also suggest that a single general structure may define PC specificity in several immunoglobulins and this work is an attempt to understand how, or if, this structure can serve as an example of the ability of the immune response to create a binding site optimally complimentary to a class of antigenic determinants. We have, therefore, examined the detailed molecular interactions responsible for the high affinity of these proteins for PC and related ligands and how these affinities are modulated by changes in a small number of crucial amino acids within this group of immunoglobulins. Such information may yield insight into the question of how immunoglobulins with subtly differing binding specificities can be created with a minimum number of changes in amino acids (mutations) and, thereby, help us to understand the origins of the broad diversity and exquisite specificity of the immune response.

In this work we have used ^{31}P NMR to probe the molecular details of the environment of the phosphate group of several haptens when bound to three of these myeloma proteins (M603, W3207, and M167). Several factors proved fortuitous for these experiments: (i) the phosphate binding sites in these proteins appear (by analogy to M603) to be formed exclusively by residues from the heavy chains whose complete sequences are known; (ii) the various known subspecificities of these proteins (Leon & Young, 1971) most likely result from differences in the phosphate binding subsites; (iii) ^{31}P NMR is an especially useful technique for not only can one use ^{31}P chemical shift information to probe the microenvironment of

† Contribution No. 5655 from the Church Laboratory of Chemical Biology, Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, California 91125. *Received August 30, 1977.* This work was supported by the President's Fund of the California Institute of Technology and the National Institutes of Health (Grant GM-16424).

[1] Abbreviations used: NMR, nuclear magnetic resonance; PC, phosphorylcholine; GPC, L-α-glycerophosphorylcholine; NPPC, *p*-nitrophenylphosphorylcholine; TMAPP, 3-trimethylamino-1-propanol phosphate.

0006-2960/78/0417-1733$01.00/0