Murine Myeloma Immunoglobulin Heavy-Chain mRNA. Isolation, Partial Purification, and Characterization of γ_1, γ_{2a}, γ_{2b}, γ_3, μ, and α Heavy-Chain mRNAs†

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ABSTRACT: Seven mouse immunoglobulin heavy-chain mRNAs have been partially purified from several myeloma tumors. Each of the individual, partially purified γ_1, γ_{2a}, γ_{2b}, γ_3, or α heavy-chain mRNA migrated as a 17S species on sucrose velocity sedimentation gradients. However, these heavy-chain mRNAs migrated slightly slower than 18S ribosomal RNA, by use of denaturing acrylamide or agarose gel analyses. Each different heavy-chain mRNA has a molecular weight of 750 000, equivalent to 2200 nucleotides. The partially purified μ heavy-chain mRNA migrated as a 20S species on sucrose gradients and ran significantly slower than 18S rRNA on denaturing formamide polyacrylamide gels, with a molecular weight equal to 950 000, or 2800 nucleotides. This RNA fraction also contained a small and distinctly separate 18S RNA moiety—not coding for detectable μ heavy chain. The 17S or 20S mRNA fractions coded for the cell-free syntheses of several detectable proteins in the expected size range of the various authentic heavy chains. In all cases, only one size class of cell-free synthesized protein was serologically reactive with the respective monospecific antiserum. These immunoprecipitated proteins accounted for as much as two-thirds of the cell-free synthesized, [^{35}S]methionine-labeled product. All in vitro generated γ heavy chains have an apparent molecular weight of 52 000, as determined by denaturing sodium dodecyl sulfate slab gel analyses. This compares with an apparent molecular weight of 57 000 for the authentically secreted γ_1 heavy chain of MOPC-31C and 52 000 for each of the other authentically secreted γ_1, γ_{2a}, γ_{2b}, and γ_3 heavy chains studied here, as determined in the same slab gel. The α heavy chain 17S mRNA fraction coded for the in vitro synthesis of only one major serologically reactive protein component with an estimated molecular weight of 56 000. A minor one (<5%) also was detected with an estimated molecular weight of 48 000. These compare with an estimated molecular weight of 54 000 for the in vivo authentically secreted α heavy chain. The 20S μ heavy-chain mRNA fraction coded for the synthesis of only one major serologically reactive protein component with an estimated molecular weight of 64 000. This is significantly smaller than the in vivo authentically secreted homologous μ heavy chain, estimated at 70 000 daltons. A small amount (<5%) of the immunoprecipitated product appeared to have nearly the same molecular weight as the in vivo μ heavy chain. Both these IgM and IgA, from MOPC-104E and J 558, respectively, have the same naturally occurring idiotype of all immunized normal BALB/c mice, suggesting very similar heavy-chain variable regions. Tryptic peptide analyses revealed significant homologies between the authentically secreted heavy chains and their respective cell-free synthesized, immunoprecipitated products, verifying the identity of that particular heavy-chain mRNA. Kinetic complexity analyses between the mRNA and their respective complementary DNA revealed RNA fractions which were from 40 to 75% homogeneous, depending upon the particular isotype of heavy-chain mRNA studied. Models for the mature cytoplasmic form of all murine γ, α, and μ heavy-chain mRNA are presented. Only about 60% of the nucleotide sequence of any subclass of RNA is directly responsible for encoding the structural protein. This means as few as 600–700 nucleotides are unaccounted for in both location and function for all murine γ and α heavy-chain isotypes, while a minimum of 800 nucleotides of the μ heavy-chain isotype fit this situation. Therefore, any cDNA probe used to measure any parameters of the constant region of any γ or α heavy-chain gene must be significantly longer than 700 bases to ensure that at least part of that heavy-chain constant region coding segment is included. Similarly, the minimum length of cDNA to be used as probe for the constant region of the μ heavy-chain gene must be significantly larger than 800 bases.

Since the original reports on the isolation, purification, and characterization of mouse immunoglobulin (Ig)[1] light (L) chain mRNA have appeared (Stavnezer & Huang, 1971; Milstein et al., 1972; Swan et al., 1972; Mach et al., 1973), it has been the desire of many investigators to pursue similar studies with mouse Ig heavy (H) chain mRNA. Furthermore, there is an even greater interest in the H-chain mRNA, as opposed to L-chain mRNA, because of the greater number of questions which could be investigated by use of the H-chain mRNA. For example, many details of the immune system could now be directly approached, allowing more precise analyses of the organization, expression, and regulation of antibody H-chain genes. These include studies of possible RNA or DNA rearrangements, which in part may regulate H-chain gene expression according to the so-called "switch" hypothesis (Nossal et al., 1964; Cooper et al., 1972). In fact, the hypothesis itself could be tested with various C_H (H-chain constant region) probes and a specific V_H (H-chain variable region) probe, thus determining the order and linkage of C_H

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[1] Abbreviations used: Ig, immunoglobulin; L, H, C_H, and V_H, light chains, heavy chains, and constant region and variable region of immunoglobulin heavy chains, respectively; mRNA, messenger RNA; cDNA, DNA complementary to mRNA; rRNA, ribosomal RNA; tRNA, transfer RNA; C_rt, product of RNA concentration (moles of nucleotides per liter) and time (seconds); Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetate; A_{260} unit, 1.0 unit of absorbance obtained following the passage of light at 260 nm through a 1-cm path; BSA, bovine serum albumin; RNase A, ribonuclease A; TPCK, L-(tosylamido-2-phenyl)ethyl chloromethyl ketone; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid; NRS, normal rabbit serum.

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to V_H genes, thereby noting any relationship to presumptive C_H gene switching. Studies of the potential DNA spacer regions between C_H genes or domains should also prove to be very important. In addition, various aspects of the ontogeny of the immune response and the mechanism of antibody diversity could be investigated with these probes.

While a great deal of progress has been made with Ig L-chain mRNA, only a few preliminary H-chain mRNA studies have appeared so far. Consequently, there has been an obvious paucity of data for Ig H-chain mRNA (Cowan & Milstein, 1973; Stevens & Williamson, 1973; Cowan et al., 1976; Green et al., 1976; Bedard & Huang, 1977; Ono et al., 1977). This results in a significant lack of details regarding both the purification of the H-chain mRNA and, even more critically, a thorough characterization of its biological and chemical properties. Furthermore, of utmost and critical importance for any meaningful molecular genetic approach to key questions in immunology is an assessment of the purity of the mRNA probe, unless one uses recombinant DNA technology to generate absolutely pure probes. Such information is presently lacking or, at best, very sketchy. In fact, there is only one report thus far for a complete γ_1 H-chain mRNA (Ono et al., 1977) and one study on a γ_1 H-chain mRNA fragment (Cowan et al., 1976), which estimates the purity of the mouse γ_1 H-chain Ig mRNA. Especially notable is that the complete γ_1 H-chain mRNA was prepared by immunoprecipitation techniques (Ono et al., 1977). But even in this particularly important case, the technical details are rather limited on the isolation and purification of the γ_1 H-chain mRNA. This can lead to difficulties in evaluating the efficiency and practicality of one method vs. other potential isolation methods of H-chain mRNA. Also, this presents a handicap to the other investigators trying to repeat such experiments to obtain specific probes for their own investigations (Stevens & Williamson, 1975). For these reasons, we have undertaken these very carefully detailed studies, presenting extensive purification and characterization data which should allow other investigators to successfully prepare the Ig H-chain mRNA of any class or subclass. Thus far, these exhaustive studies not only represent the most extensive and comprehensive to date on the isolation, partial purification, and characterization of a single Ig H-chain mRNA, but also present similar, thorough details for *all* classes and subclasses of mouse Ig H-chain mRNA, except the δ and ϵ classes.

While the H-chain mRNA is not 100% homogeneous, it is of sufficiently high homogeneity to allow it to be used as a probe for certain types of studies of the immune system or immune response (cf. Faust et al., 1974; Farace et al., 1976). Furthermore, it can be used as a template for the synthesis of complementary DNA (cDNA), as has been done in the case of Ig L-chain mRNA (Aviv et al., 1973; Faust et al., 1973, 1974; Honjo et al., 1974). This cDNA could then be used to prepare a virtually homogeneous fraction of cDNA specific for a given H-chain by utilizing the C_rt hybridization technique as has also been done with Ig L-chain cDNA (Honjo et al., 1976a,b). Alternatively, with the appropriate vector, it could also be cloned to homogeneity by use of recombinant DNA technology (Rougeon et al., 1975; Maniatis et al., 1976; Rabbits, 1976).

Experimental Procedures

Preparation of Polysomes and RNA Fractions. The myeloma tumors, MOPC-21, MOPC-31C, MOPC-195S, FLOPC-21, MOPC-104E, and J 558, were obtained from Dr. M. Potter through Litton Bionetics. MOPC-173 was obtained from Dr. David Swan as a generous gift of Dr. P. Leder. These were maintained in BALB/c mice by serial subcutaneous transplantation (Mach et al., 1973). Sterile precautions were taken throughout all procedures involving RNA. Membrane-bound polysomes were prepared from microsomes (Mach et al., 1973). Fifty to one hundred units of heparin/mL was used with the microsome and polysome preparations as a ribonuclease inhibitor. Total polysomal RNA was extracted using a Proteinase K procedure (Mach et al., 1973; Faust et al., 1973). In addition, 0.1 mM cycloheximide was used to minimize polysome runoff during the preparations (Honjo et al., 1974). RNA was extracted from these polysomes by use of a phenol–chloroform–isoamyl alcohol mixture (25:24:1), by employment of two successive extractions (Mach et al., 1973; Faust et al., 1973).

Purification of mRNA. The preparation of the poly(dT)-cellulose column and its optimization have been described previously (Gilham, 1964; Faust et al., 1973). Because the optimum operating conditions vary from one cellulose batch to another, these conditions here are different from those previously reported (Faust et al., 1973).

Total polysomal RNA was applied to a poly(dT)-cellulose column (Faust et al., 1973) with the following modifications. The RNA was dissolved to a concentration of 20–30 A_{260} units/mL in H_2O and adjusted with a high-salt buffer to contain 500 mM NaCl, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 0.2% sodium dodecyl sulfate. This RNA mixture was applied directly to a poly(dT)-cellulose column equilibrated with the same buffer and maintained at 25 °C. Following application of the sample, the column was washed with one column volume of the high-salt buffer, followed with the appropriate amount of intermediate-salt buffer, containing 200 mM NaCl, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, and no sodium dodecyl sulfate, until the A_{260} of the eluate was reduced to 0.05 A_{260} unit/mL. The bound RNA, previously shown to be poly(A) rich (Brownlee et al., 1973; Faust et al., 1973), was eluted with sterile water and is called Peak II RNA (Faust et al., 1973). The poly(A)-rich RNA was concentrated by the addition of two volumes of ethanol and one-tenth volume of 2 M sodium acetate, pH 5. The Peak II RNA was redissolved in water at a concentration of 4–10 A_{260} units/mL in 2 mM EDTA, heat denatured at 65 °C for 10 min to eliminate aggregation, adjusted to the high-salt buffer final concentration, and applied to the poly(dT)-cellulose column for a second pass to eliminate most contaminating rRNA. The significant contamination by rRNA in this fraction (approximately 50%) has been verified previously (Faust et al., 1973). The poly(A)-rich RNA was then eluted with the same series of washing steps described above for the first poly(dT)-cellulose chromatography pass. The Peak II RNA obtained in this manner was subjected to two successive sucrose gradient fractionations, always heat denaturing (65 °C for 10 min) the RNA sample before each run as described above. The sucrose gradient runs were typically done by use of an IEC B-60 ultracentrifuge and the IEC SB 283 rotor with 13-mL 5–20% sucrose gradients containing 10 mM sodium acetate, pH 5, run at 41 000 rpm and 2 °C for 17–19 h. The sample load varied from 0.3 to 0.5 mL (6–12 A_{260} units/gradient) and contained 10 mM sodium acetate and 2 mM EDTA. Gradient fractions were collected on the basis of time. The amounts of RNA in the individual fractions were determined by absorbance at 260 nm, by use of the conversion 1 A_{260} unit equals 40 μg of RNA. The RNA in each fraction was concentrated by ethanol–salt precipitation as described earlier, redissolved in sterile water at a concentration of 1 mg/mL, and stored at −20 °C. Mouse globin mRNA was

prepared as described (Mach et al., 1973). The RNA obtained in this fashion was then assayed for biological activity in a cell-free translational system.

Cell-Free Translation of Myeloma mRNA. The preparation of the rabbit reticulocyte lysate has been described (Lingrel, 1972), the lysis being carried out in 4 mM $MgCl_2$. In addition, 1.0 mL of the mRNA-dependent reticulocyte cell-free system (Pelham & Jackson, 1976) contained the following components listed as final amounts or concentrations: 800 μL of reticulocyte lysate, 3.2 mM $MgCl_2$, 50 μg/mL of creatinine phosphokinase, 13.4 mM creatinine phosphate, 100 mM KCl, 19 unlabeled amino acids at 50 μM each, 24 μM hemin, 1.0 mM $CaCl_2$, 25 μg/mL of micrococcal nuclease stored at −20 °C in 50% glycerol, and 20 mM Tris-HCl at pH 8.2. The above was preincubated for 15 min at 20 °C, chilled on ice, and then made 2.9 mM final concentration with 0.1 M EGTA to inactivate the nuclease. Forty microliters of this preincubated lysate was used per 50-μL assay, reducing all the above concentrations accordingly in the assay. The label was either [^{35}S]methionine, 400–1100 Ci/mmol (New England Nuclear or Amersham), or [^{3}H]leucine at 79 Ci/mmol from New England Nuclear. Typically 1–5 μCi was used per 50-μL assay. RNA fractions were tested: (1) 4 μg of Peak II-dT_1 fraction; (2) 2 μg of Peak II-dT_2 fraction; and (3) 1 μg of sucrose gradient 1 or sucrose gradient 2 fraction. The assay was incubated for 1–3 h at 30 °C, followed by incubation at room temperature with 5 μg of RNase A for 30 min, and analyzed on the sodium dodecyl sulfate acrylamide gel (12.5%) system of Laemmli (1970). A 5-μL sample of this mixture was utilized for determining total Cl_3CCOOH-precipitable radioactivity to estimate amino acid incorporation. Routinely a 5-μL sample of this 50-μL assay was used for the acrylamide gel assays. Gels were stained with Coomassie blue and radioactive protein products were located by autoradiography (Mach et al., 1973) or fluorography (Bonner & Laskey, 1974), by use of Kodak XR-5 X-Omat R film.

Immunochemical Procedures. Monospecific rabbit antimouse Ig H-chain antisera were obtained from Litton Bionetics. Specific anti-H-chain activity was confirmed by us via Ouchterlony double immunodiffusion techniques. Rabbit anti-BSA antiserum was a generous gift of Dr. Marvin B. Rittenberg.

Cell-free incubation mixtures of the reticulocyte lysate were diluted with an equal volume of immunoprecipitation buffer (1 M NaCl, 0.1% sodium dodecyl sulfate, 1% Triton X-100, 0.5% sodium deoxycholate, 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 0.02% NaN_3). The diluted sample was centrifuged in an IEC A-321 rotor at 40000 rpm ($145000g_{max}$) for 1 h at 2 °C and the supernatant saved. An aliquot was taken, mixed with 50 μg of carrier BSA, treated with Cl_3CCOOH, and collected on Whatman GF/C filters to determine the total radioactivity in the supernatant. Another aliquot was used for immunoprecipitation. To this aliquot (100–200 μL) was added 5 μL of the monospecific rabbit antimouse H-chain antiserum, normal rabbit serum, or rabbit anti-BSA antiserum and reacted for 1–12 h at 4 °C. This amount of antiserum is in excess of the antigen under these conditions. After the primary antiserum reacted, 25 μL of a 10% suspension of *Staphylococcus aureus* (Pansorbin, Calbiochem; Kessler, 1975) was added and allowed to react another 1–4 h at 4 °C. The bacteria were stored at −20 °C in 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 0.5% sodium deoxycholate, 0.1 mg/mL of BSA, and 0.02% NaN_3 and thawed immediately before use. After the reaction, the bacterial suspension was centrifuged for 5 min in an Eppendorf microfuge in the 1.5-mL conical tubes (12000*g*) at room temperature or 2 °C. The supernatant was saved for further analyses and the pellet washed three times with 1.0 mL of immunoprecipitation buffer. A 200-μL cushion, containing 1 M sucrose, 100 mM NaCl, 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1% Triton X-100, 0.02% NaN_3, was layered under the last wash. The bacterial pellet was resuspended in 35 μL of electrophoresis sample buffer (Laemmli, 1970), heated at 95 °C for 10 min, and centrifuged for 5 min at 12000*g*. The supernatant was removed, an aliquot was taken for total radioactivity determination with 50 μg of BSA carrier following Cl_3CCOOH precipitation, and the remainder was used for acrylamide gel analyses and tryptic fingerprint analyses. BSA was not used as a constituent of the immunoprecipitation buffer, since it was found to reduce the efficiency of the reaction with no noticeable effect on reduction in nonspecific binding (Kray & Faust, unpublished observations).

Tryptic Peptide Mapping. Labeled radioactive, secreted Ig was prepared from tumor fragment incubations (Mach et al, 1973). Ig H and L chains were separated by sodium dodecyl sulfate gel electrophoresis as above. The radioactive proteins located by autoradiography of dried gels were eluted electrophoretically into a dialysis bag with a buffer containing 6.0 g of Tris base and 28.8 g of glycine/L with 0.1% sodium dodecyl sulfate. The proteins were then precipitated from solution by addition of an equal volume of Cl_3CCOOH and allowed to stand for 1–2 h before centrifugation at 20000*g* for 15 min or directly lyophilized. The precipitate was washed twice with 10% Cl_3CCOOH and once with 0.1 N HCl and 90% acetone, followed by two absolute acetone washes to remove any stain and Cl_3CCOOH, and then air-dried. The protein solutions were then subjected to a performic acid oxidation according to Hirs (1967) for a period of 3 h on ice. The twice lyophilized–oxidized proteins were digested twice successively at 37 °C by use of 10 μg of Worthington TPCK-treated trypsin (20:1 substrate–enzyme) with each digestion all contained in 1.0 mL of 50 mM NH_4HCO_3, pH 8.0. The first digestion was overnight and the second digestion was 4–6 h. In the case of tryptic peptide analyses of cell-free products the procedure of Elder et al. (1977) was used following serological precipitation, sodium dodecyl sulfate gel electrophoresis, and autoradiography.

The tryptic peptide digest (2–20 μL) was applied to a 20 × 20 cm Brinkman MN 300 0.25-mm thin-layer cellulose plate, followed by ascending chromatography with the following chromatography buffer: butanol–pyridine–acetic acid–H_2O in the ratio 8:7:1:4 (v/v). On completion of the chromatography, the plates were dried and electrophoresed in the orthogonal direction at 400 V for a period of 120–150 min by use of a pyridine–acetate buffer system (5 mL of pyridine, 50 mL of glacial acetic acid, 945 mL of water, pH 3.5). Marker dye was acid fuschin, which runs in the opposite direction as the peptides. They were then developed with ninhydrin and autoradiographed with Kodak XR-5 film, by use of fluorography (Randerath, 1970).

RNA Gel Electrophoresis. Various RNA samples were analyzed by use of denaturing 98% formamide–polyacrylamide gels (Staynov et al., 1972) with modifications (Honjo et al., 1976b). Eleven centimeter, 3% gels were cast in 0.5 cm (inside diameter) by 12.5 cm quartz tubes. This permitted scanning with a Beckman Acta CIII at 280 nm to ascertain the position of the RNA during electrophoresis. The gel concentrations were as indicated and always contained *N,N'*-methylenebis(acrylamide) as 15% of the total acrylamide indicated. Spectrograde formamide from Eastman Kodak was used and was deionized with 5 g of Bio-Rad mixed-bed resin (AG

501-X8) per 100 mL of formamide used. Deionization took place over a period of 4 h. The typical 3% acrylamide gel contained the following components: 39.8 mL of deionized formamide, 180 mg of *N,N'*-methylenebis(acrylamide), 1.2 g of acrylamide monomer, 55.2 mg of NaH_2PO_4, and 56.8 mg of Na_2HPO_4. Polymerization was then initiated with 200 μL of freshly prepared 25% ammonium persulfate and 80 μL of *N,N,N',N'*-tetramethylethylenediamine. Although polymerization usually occurred in 0.5 h, gels were allowed to stand overnight for maximally uniform polymerization before use. We then prepared the gel samples by dissolving the RNA in a solution consisting of 99% formamide containing 2 mM EDTA, followed by heat denaturation for 10 min at 65 °C. The RNA sample was then adjusted to the following final buffer conditions with a concentrated stock solution: 15% glycerol, 20 mM sodium barbital, 0.0025% bromphenol blue, 2.0 mM EDTA, and 75% formamide. The RNA sample was layered under about 200 μL of sample buffer containing no glycerol. This ensured the RNA sample was always in denaturing conditions. Electrophoresis was run at a constant voltage of 32 V for about 16 h until the dye marker reached the bottom. Following electrophoresis, the gels were scanned at 280 nm, removed from the quartz tubes, stained with Stains-All (Eastman Kodak), destained with sterile H_2O, and scanned again at 580 nm. The scan at 280 nm served to assure that the electrophoresis was complete and the peaks were in the approximate locations expected. But the scans at 280 nm were generally not useful for detailed analyses, because of the background obtained in the gel, as well as the significant gradient of refractive index at the lower end of the gel in contact with the electrophoresis solution, resulting from diffusion of the formamide into the electrophoresis buffer. For these reasons a scan of 580 nm was relied upon primarily to obtain details on the RNA molecular components distributed in a given RNA sample analyzed.

Denaturing agarose gels (1.5%) with 2.2 M formaldehyde (Lehrach et al., 1977) or 10 mM methylmercuric hydroxide (Bailey & Davidson, 1976; Lehrach et al., 1977) were run essentially as described except RNA samples were layered under 100 μL of their respective sample's buffers as described above for the 98% formamide gels. Electrophoresis was run at 30 V (1.0 mA per gel) for 16 h with formaldehyde–agarose gels and with methylmercuric hydroxide agarose gels—both run at room temperature. The bromphenol blue ran out of the bottom of the gel. In all three above cases, the reservoir buffers were recirculated during electrophoresis. Gels were scanned at 260 nm or stained with 0.01% methylene blue and scanned at 650 nm with a Beckman Acta CIII spectrophotometer equipped with an automatic 15-cm gel scanner. Methylmercuric hydroxide was obtained as a 1.0 M aqueous stock from Alfa Division, Ventron Corporation, Danvers, MA.

Mouse RNA molecular weight standards used were the following: 28S rRNA (1 600 000), 18S rRNA (700 000), 14S MOPC-41 κ L-chain mRNA (420 000), 14S RPC-20 λ L-chain mRNA (400 000), 10S β-globin (220 000), 10S α-globin (200 000), 5.8S rRNA (48 000), 5S rRNA (40 000), and 4S tRNA (25 000).

H-Chain cDNA Synthesis. Enzymatic synthesis of H-chain cDNA is based on procedures previously worked out for Ig L chain (Diggelmann et al., 1973; Faust et al., 1973). More extensive details of these syntheses and characterizations will be published later (Faust, Moore, and Heim, unpublished results). Briefly, a 50-μL reaction contained the following: 80 mM Tris-HCl, pH 8.2; 10 mM $MgCl_2$; 4 mM $Na_4P_2O_7 \cdot 10H_2O$ (Kacian & Myers, 1976); 0.2 mM dATP; 0.2 mM dGTP; 0.2 mM dCTP; 0.2 mM 0.4 mCi [^{3}H]dTTP (Schwartz/Mann, 20 Ci/mmol); 4 mM dithioerythritol; 100 μg/mL of actinomycin D; 0.2% Nonidet P-40; 0.1–0.2 μg of oligo(dT) 12–18; 1 μg of H-chain mRNA from peak of sucrose gradient 2; 1 mg/mL of BSA; and 160 units/mL of avian myeloblastosis virus RNA-dependent DNA polymerase. The reaction was incubated at 40 °C for 3–6 h and stopped by the addition of sodium dodecyl sulfate to 1% and EDTA to 10 mM, and the product was hydrolyzed in 0.3 N NaOH at room temperature overnight. The neutralized DNA was then passed over a Sephadex G-75 column; the void volume was collected and was ethanol precipitated with carrier salmon sperm DNA and sized on a linear alkaline sucrose gradient (Faust et al., 1973) or analyzed on denaturing 98% formamide–5% polyacrylamide gels as described above for the RNA analyses. The cDNA preparations were typically polydisperse in size, ranging from 200 bases to full-length product with a specific activity estimated to be about 10^7 cpm/μg. Hybridizations were as described (Faust et al., 1973).

Translational Assays of Gel Fractionated RNA. Both H-chain mRNAs were obtained from 98% formamide–polyacrylamide gels for cell-free synthesis studies as follows. We cut the gels into 1-mM slices, pooling two adjacent slices per fraction in 0.5 mL of 1 M NaCl containing 1 mM EDTA and 0.1% sodium dodecyl sulfate, and incubated the slices overnight at room temperature. The supernatants were collected after centrifugation, 10 μg of yeast carrier tRNA was added per fraction, twice precipitated with 0.2 M sodium acetate, pH 5, and two volumes of ethanol overnight. The RNA was redissolved to a final volume of 10 μL containing 10 μCi of [^{35}S]methionine and tested in a 50-μL cell-free synthesizing system.

Results

All mouse H-chain mRNA have been isolated according to the general scheme presented in the flow diagram of Figure 1. This procedure is based on that developed for Ig L-chain mRNA (Mach et al., 1973) and contains only those modifications listed under Experimental Procedures. By use of these procedures, it has been shown that a poly(A)-rich RNA fraction is obtained following poly(dT)-cellulose chromatography of mouse myeloma RNA (Faust et al., 1973). Generally, this procedure is applicable to any Ig synthesizing tumor of the mouse, as evidenced by an investigation of 19 different heavy-chain synthesizing and secreting tumors (Faust, unpublished results). Although there was some variation from one tumor to the next, the results were nevertheless fairly constant for a given tumor (see Table I). Typically, the yield of poly(A)-rich RNA is in the range of 2–3% of the total polysomal RNA applied to the column, following the first pass through the poly(dT)-cellulose column. Furthermore, it has been previously shown by one of us (Faust et al., 1973) during a detailed performance characterization of the poly(dT)-cellulose column that a significant amount of rRNA contaminates this poly(A)-rich RNA fraction. This contamination amounts to about 1% of the total input of polysomal RNA. Therefore approximately 50% of the poly(A)-rich RNA fraction, in fact, is expected to be contaminating rRNA (cf. Faust et al., 1973). This is also suggested here by a second pass of the poly(A)-rich RNA fraction through the poly(dT)-cellulose column (Table I). That is, approximately one-half of the repassed poly(A)-rich RNA fraction was bound. But the critical point here is that the poly(A)-rich RNA fraction must be heat denatured before the second pass through the poly(dT)-cellulose column in order to reduce or minimize aggregation and presumptive mRNA–18S rRNA

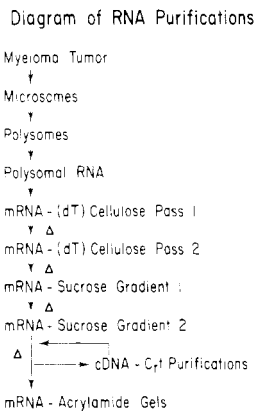

FIGURE 1: Flow diagram of H-chain mRNA isolation, including cDNA C_rt enrichment (see Experimental Procedures). (Δ) 65 °C for 10 min.

complexes (data not shown). This observation is in direct contrast with results obtained using an α H-chain secreting myeloma tumor, MOPC-315, in which no heat denaturation steps were used and no difference was found in the amount of RNA bound on two successive passes (Bedard & Huang, 1977).

In order to more fully understand and characterize the nature of the RNA moieties existing in the various wash fractions eluted from the poly(dT)-cellulose column during these two passes, denaturing formamide gel analyses were performed on these RNA fractions. Examples of these results are presented in Figures 2A,B. As can be seen from the diagrams, the total polysomal RNA applied to the column consists of predominantly 28S, 18S, and 5S rRNA as expected. Also the 500 mM NaCl wash fraction and 200 mM NaCl wash fraction from the first pass through the poly(dT)-cellulose column showed these rRNA components as the major RNA species present. In contrast, the poly(A)-rich RNA fraction (Peak II RNA; Faust et al., 1973), eluted with water after both the first (Figure 2A) and second (Figure 2B) poly(dT)-cellulose chromatography passes, showed a rather polydisperse population of RNA molecules, consistent with many presumptive messengers of different sizes being present within this RNA population. However, it will be noted that, with the first poly(dT)-cellulose pass of MOPC-31C RNA (Figure 2A), the Peak II fraction containing the poly(A)-rich RNA also contained two prominent peaks, 18 S and 28 S, above the polydisperse background. These are presumably rRNA. These 18S and 28S peaks are reduced and, in most cases, cannot be seen in the Peak II fraction following the second poly(dT)-cellulose passage. Instead, they appear in the 500 mM NaCl wash fraction and the 200 mM NaCl wash fraction of the second poly(dT)-cellulose pass (Figure 2B). This is typical of all myelomas investigated (Faust, unpublished results). A third poly(dT)-cellulose chromatography step proved to be of no additional value, since virtually all the RNA of the Peak II fraction rechromatographed as poly(A)-rich RNA, i.e., Peak II fraction, with no significantly detectable amounts of RNA in either wash fraction. Upon rechromatography, none of the RNA of any of the wash fractions from any passage through the poly(dT)-cellulose column exhibited any significant binding properties expected from that of poly(A)-rich RNA (Faust, unpublished results). The various RNA fractions obtained from the poly(dT)-cellulose column were tested for biological activity in a cell-free assay system, the mRNA-dependent, reticulocyte lysate system (Pelham & Jackson, 1976), prior to any further purification. This was necessary in order to fully assess the RNA fractionation

Table I: γ_1, γ_{2a}, γ_{2b}, γ_3, α, and μ H-Chain mRNA Purification Yields[a]

RNA purification stage	Ig subclass:	γ_1		γ_1		γ_{2a}		γ_{2b}		γ_3		α		μ	
	tumor:	MOPC-31C		MOPC-21		MOPC-173		MOPC-195S		FLOPC-21		J 558		MOPC-104E	
	yield:	A_{260}	(%)	A_{260}	(%)	A_{260}	(%)	A_{260}	(%)	A_{260}	(%)	A_{260}	(%)	A_{260}	(%)
polysomes		8745		6965		13905		6250		19920		3920		9750	
total polysomal RNA		6031	(100)	5318	(100)	10300	(100)	4312	(100)	14435	(100)	2799	(100)	7616	(100)
poly(dT)-cellulose pass 1		176.0	(2.92)	105.9	(1.99)	344.2	(3.34)	96.5	(2.24)	382.0	(2.65)	69.8	(2.49)	194.8	(2.56)
poly(dT)-cellulose pass 2		92.6	(1.54)	68.0	(1.28)	167.6	(1.63)	68.0	(1.58)	203.4	(1.41)	29.5	(1.05)	92.6	(1.22)
sucrose gradient 1		15.35	(0.25)	6.80	(0.13)	34.85	(0.34)	14.25	(0.33)	42.9	(0.30)	14.57	(0.52)	14.1	(0.19)
sucrose gradient 2		7.10	(0.12)	2.15	(0.040)	10.45	(0.10)	4.95	(0.11)	15.55	(0.11)	6.80	(0.24)	2.45	(0.032)

[a] See Experimental Procedures and Figure 1. The final yield at the sucrose gradient 2 stage of purification includes three sucrose gradient fractions per isotype—the peak fraction, giving maximum serological reactivity (see Tables II and III), and the single adjacent lighter and heavier RNA fractions, except in the cases of MOPC-21 and -104E which include only the two highest yield fractions. The maximum serologically reactive fraction of each isotype was used for cDNA synthesis, kinetic complexity analyses (Table IV), and mRNA molecular weight determinations (Figure 10 and supplementary material). A_{260} is an absorbance unit equal to 40 μg of RNA.

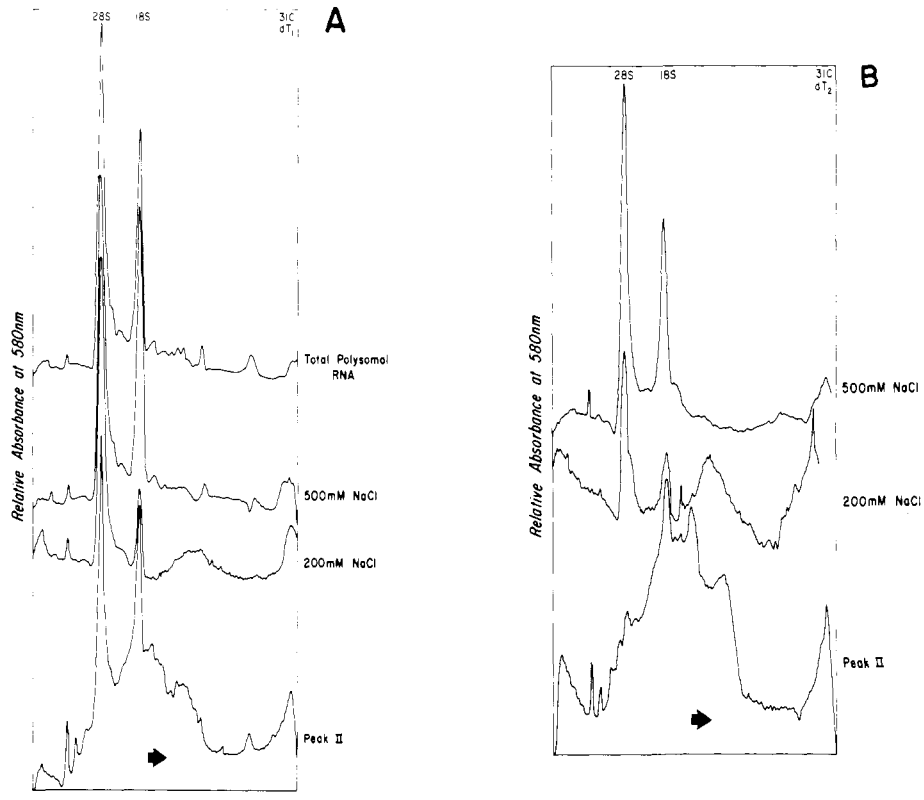

FIGURE 2: Ninety-eight percent formamide gel analyses of RNA fractions. (A) MOPC-31C poly(dT)-cellulose pass 1; (B) poly(dT)-cellulose pass 2. The abscissa is distance migrated (see Experimental Procedures).

procedure with poly(dT)-cellulose column. The results of these particular assays are not presented. But significant incorporation of radioactive amino acids into protein in the presence of added mRNA from the Peak II RNA fractions occurred, which could be immunoprecipitated with monospecific antiserum. Very limited incorporation of radiolabel into protein occurred with any of the wash fractions (Faust, unpublished results).

Partial Purification of H-Chain mRNA and Characterization of the Protein Products. Since it was established above that the poly(A)-rich RNA fraction from the poly(dT)-cellulose column contained γ H-chain-like mRNA from MOPC-31C, as well as the H-chain-like mRNA for the other isotypes from their respective tumors, it was decided to further fractionate the H-chain mRNA by physicochemical methods, by use of sucrose gradient velocity sedimentation. Typical results of such experiments for H-chain mRNA are presented in Figures 3–9. Similarly detailed results for the partial purification and characterization of the H-chain mRNA for the γ_{2a}, γ_{2b}, and γ_3 subclasses are presented in the supplementary material (see the paragraph concerning supplementary material at the end of this paper). As can be seen from the first sucrose gradient, and as was to be expected from the acrylamide gel assay of the Peak II RNA fraction, there is a fairly broad, polydisperse population of RNA molecules present. When the RNA fractions from the first sucrose gradient were assayed for biological activity, it is noted that there exists a fairly broad population of products synthesized in response to the input mRNA of each fraction, when looking at the total radioactivity incorporated into protein (Figures 3 and 7). The major peak of radioactive incorporation was about 13–14 S, with another one about 17 S for all γ and α H chains studied, or about 20 S for the μ H chain. This is consistent with the size range of protein products produced, as revealed by autoradiography of sodium dodecyl sulfate gel

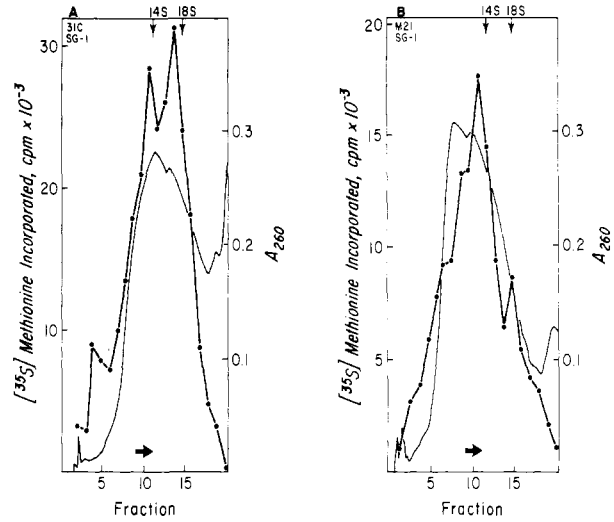

FIGURE 3: Sucrose gradient 1 A_{260} profile of RNA from Peak II of poly(dT)-cellulose pass 2 (solid tracing) and [^{35}S]methionine labeled total protein (●–●) synthesized in a cell-free system in response to this RNA (see Experimental Procedures). (A) MOPC-31C; (B) MOPC-21.

analyses (Figures 4 and 8). It will be also noted that there sometimes exists an endogenous [^{35}S]methionine-labeled protein about 47 000 daltons, which can be detected in all gradient fractions. As previously cited (Pelham & Jackson, 1976), this radioactive band is labeled independent of any protein synthesis or mRNA activity. While its nature is unknown, we have subsequently discovered that it may be completely eliminated if the reticulocyte lysate is thawed very slowly (about 2 h) on ice prior to the nuclease preincubation step (Heim & Faust, unpublished observations).

Autoradiography of the various protein products synthesized from each fraction revealed a very prominent series of distinct

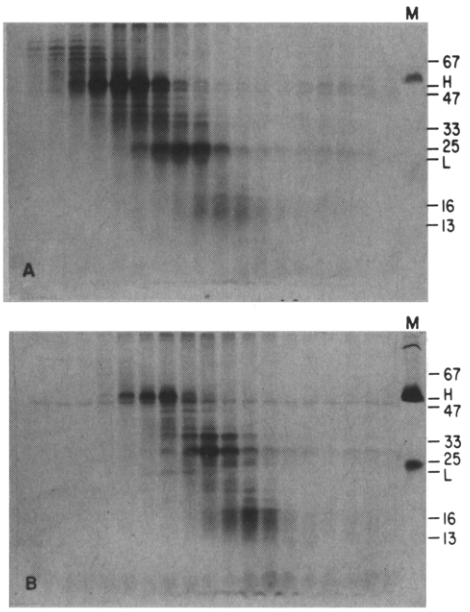

FIGURE 4: Polyacrylamide gel analyses of in vitro synthesized products from Figure 3. (A) MOPC-31C; (B) MOPC-21; see Experimental Procedures. Marker (M) is the homologous in vivo secreted IgG_1 H chain and L chain. H shows the reference position of the authentic MOPC-21 γ_1 H chain, and L shows the reference position of the authentic MOPC-21 κ L chain. MOPC-21 H chains and L chains are used for standard reference throughout these studies.

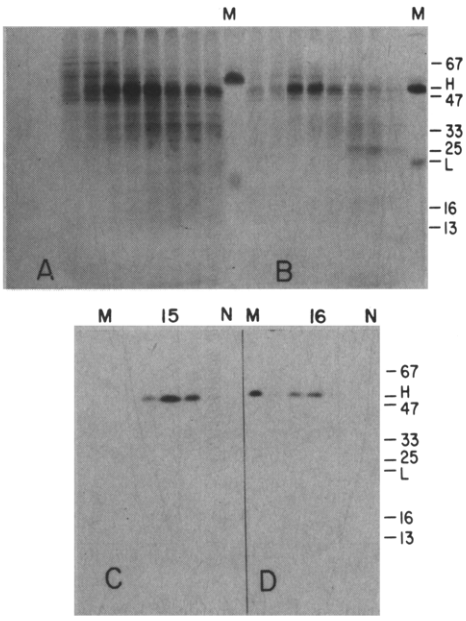

FIGURE 5: Polyacrylamide gel analyses of in vitro synthesized products from sucrose gradient 2. (A) MOPC-31C, total proteins synthesized; (B) MOPC-21, total proteins synthesized; (C) MOPC-31C, immunoprecipitated γ_1 H-chain related proteins, fractions 13–17; (D) MOPC-21, immunoprecipitated γ_1 H-chain related proteins, fractions 14–18; N, NRS control for (C) and (D) (see also Table II and Experimental Procedures). M, H, and L are as in Figure 4.

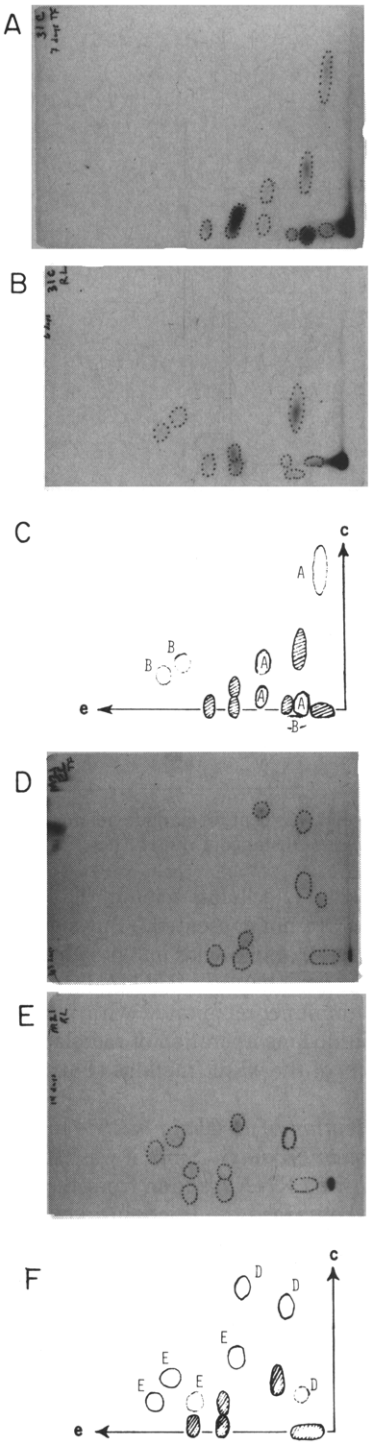

FIGURE 6: Two dimensional tryptic peptide analysis of in vivo and in vitro synthesized γ_1 H chain: (A) MOPC-31C, in vivo protein; (B) MOPC-31C, in vitro product; (C) comparative interpretation of (A) and (B); (D) MOPC-21, in vivo protein; (E) MOPC-21, in vitro product; (F) comparative interpretation of (D) and (E) (see Experimental Procedures). The electrophoretic dimension is *e* and the chromatography dimension is *c*. Hatched areas of homologies are indicated in comparisons.

proteins synthesized with molecular weights similar to that of H chain, i.e., from 48 000 to 70 000 daltons compared with 57 000 daltons for authentic MOPC-31C γ_1 H chain, 52 000 daltons for the other authentic γ_1, γ_{2a}, γ_{2b}, and γ_3 H chains, 54 000 daltons for authentic α H chains, or 70 000 daltons for authentic μ H chains. Furthermore, one of these cell-free synthesized species was known to be serologically related in each case to the respective H chain, as noted above (Faust, unpublished results). So, these H-chain-like mRNA containing fractions were pooled and rerun on a second sucrose gradient for further purification. These results are given in the supplementary material, together with the results of the total radioactivity incorporated into protein in the cell-free translational assays. The autoradiographic data of these particular assays are shown in Figures 5 and 9.

Again, an array of proteins of similar size to H chain are synthesized in the cell-free system in response to the added

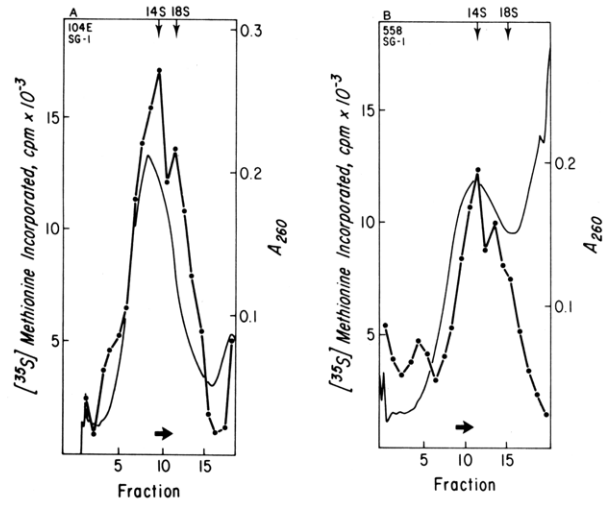

FIGURE 7: Sucrose gradient 1 A_{260} profile of RNA from Peak II of poly(dT)-cellulose pass 2 (solid tracing) and [^{35}S]methionine-labeled total protein (●–●) synthesized in a cell-free system in response to this RNA (see Experimental Procedures). (A) MOPC-104E; (B) J 558.

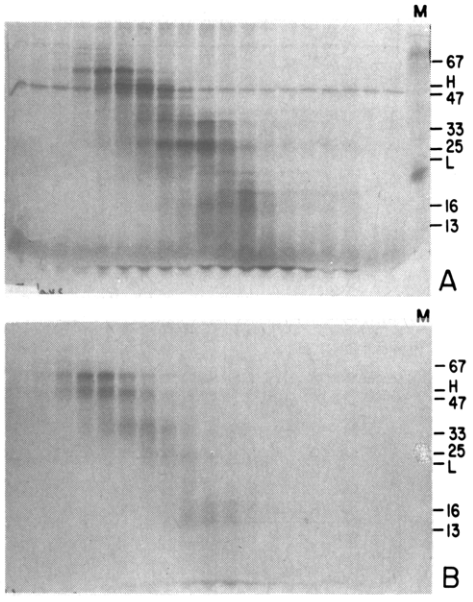

FIGURE 8: Polyacrylamide gel analyses of in vitro synthesized products from Figure 7 (see Experimental Procedures). Marker (M) is the in vivo secreted IgM μ H chain and L chain or IgA α H chain and L chain. H shows the reference position of the authentic MOPC-21 γ_1 H chain and L shows the reference position of the authentic MOPC-21 κ L chain. (A) MOPC-104E and (B) J 558.

RNA from the second sucrose gradient stage of mRNA purification (Figures 5 and 9). When these total proteins are reacted with the respective monospecific rabbit antimouse H-chain antiserum, only a single size class of in vitro synthesized, serologically reactive protein can be detected (Figures 5 and 9). These have a molecular weight of 52 000 for all γ H-chain subclasses, 56 000 for the α H chain, and 64 000 for the μ H chain. A minor band (<5%) of serologically reactive protein is observed in the case of both the α and μ H chains studied here (Figure 9). These minor bands were 48 000 daltons in the case of α H chain and 70 000 daltons in the case of the μ H chain. They were not observed for any other α or μ H chains studied by us (Faust, Moore, and Heim, unpublished observations). In all cases, the amounts of serologically reactive, cell-free product range from about one-third to two-thirds of the total [^{35}S]methionine product synthesized,

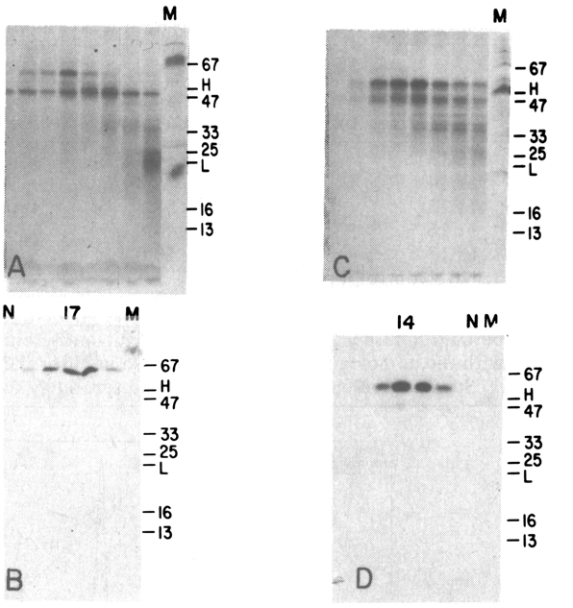

FIGURE 9: Polyacrylamide gel analyses of in vitro synthesized products from sucrose gradient 2. (A) MOPC-104E, total proteins synthesized; (B) immunoprecipitated μ H-chain related proteins, fractions 15–19; (C) J 558, total proteins synthesized; and (D) immunoprecipitated α H-chain related proteins, fractions 12–16 (see Table II and Experimental Procedures). M, H, and L as in Figure 4.

Table II: Serologically Reactive γ_1 H-Chain-like Proteins in the mRNA Programmed Cell-Free System[a]

sucrose gradient 2 RNA fraction[b]	total dpm of [^{35}S]methionine-labeled protein	immunoprecipitated dpm of [^{35}S]methionine-labeled protein	% γ_1 H-chain-like protein
MOPC-31C			
Fr 13	10 847	2 188	20.2
Fr 14	21 934	10 525	48.0
Fr 15	34 130	22 187	65.0
Fr 16	29 885	12 779	42.8
Fr 17	24 439	7 999	32.7
NRS control (MOPC-31C)			
Fr 15	29 248	494	1.7
MOPC-21			
Fr 14	19 532	5 087	26.0
Fr 15	15 534	10 424	67.1
Fr 16	19 103	12 389	64.8
Fr 17	21 453	4 639	21.6
Fr 18	27 642	2 749	9.9
NRS control (MOPC-21)			
Fr 16	26 053	368	1.4

[a] RNA fractions are those derived from the sucrose gradient 2 stage of purification as illustrated in Figures 5 and 6 (see Experimental Procedures). Monospecific rabbit antimouse γ_1 H chain, BSA-anti-BSA (data not shown), or NRS was used. [b] Fr, fraction.

depending upon the isotype, suggesting a enriched H-chain mRNA fraction in these regions of the gradient (Tables II and III). Tryptic peptide analyses of the cell-free translational products which are serologically related to the respective authentic H chain reveal a significant degree of similarity with the authentically secreted H chain, confirming the identity of the respective H-chain mRNA (Figure 6 and supplementary material).

Characterization of H-Chain mRNA. Two peaks of protein

Table III: Serologically Reactive γ_{2a}, γ_{2b}, γ_3, α, or μ H-Chain-like Proteins in the mRNA Programmed Cell-Free System[a]

sucrose gradient 2 RNA fraction[b]	% H-chain-like protein				
	γ_{2a}, MOPC-173	γ_{2b}, MOPC-195S	γ_3, FLOPC-21	α, J 558 (Fr)	μ, MOPC-104E (Fr)
Fr 13	10.4	5.4	40.7	29.7 (12)	16.8 (15)
Fr 14	35.8	30.2	49.9	50.9 (13)	19.8 (16)
Fr 15	58.8	36.6	69.7	51.7 (14)	35.9 (17)
Fr 16	44.6	28.0	56.3	53.8 (15)	37.0 (18)
Fr 17	10.0	7.1	18.1	38.2 (16)	18.0 (19)
NRS control, Fr 15	1.1	0.9	1.0	0.9 (14)	1.7 (17)

[a] RNA fractions are those derived from the sucrose gradient 2 stage of purification as illustrated in the supplementary material. Monospecific rabbit antimouse γ_{2a}, γ_{2b}, γ_3, α, or μ H-chain antiserum or NRS was used. Fraction 15, or the one showing maximum serological reactivity with the homologous antiserum, was also used for the NRS control. Input levels of radioactivity were comparable to those shown in Table II. Sucrose gradient 2 fractions are listed separately for the α and μ H chain. [b] Fr, fraction.

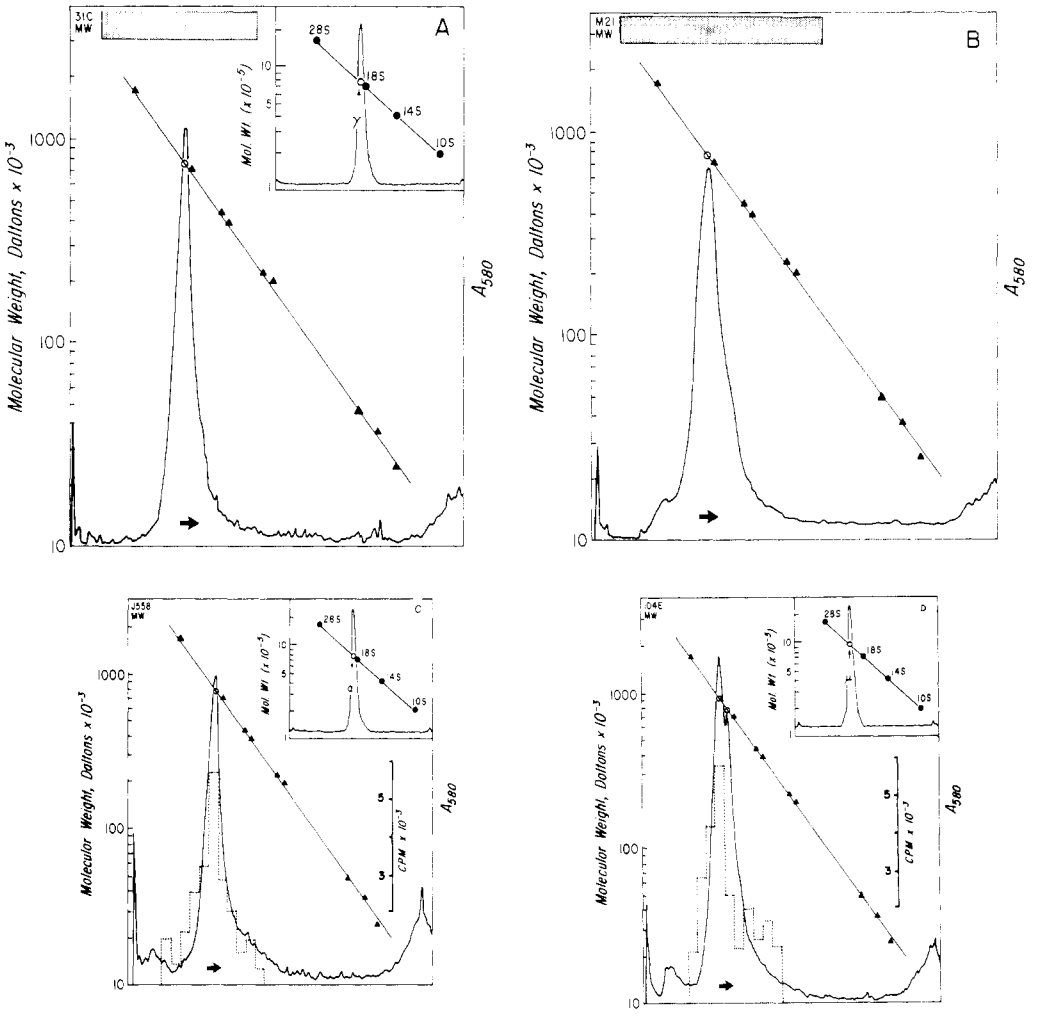

FIGURE 10: Molecular weight determination of γ_1, α, or μ H-chain mRNA (O) from the H-chain peak of sucrose gradient 2. (A) MOPC-31C; (B) MOPC-21; (C) J 558; (D) MOPC-104E. Mouse RNA markers (▲) are in descending order: 28S rRNA, 18S rRNA, MOPC-41 κ L-chain mRNA, RPC-20 λ L-chain mRNA, 10S β-globin, 10S α-globin, 5.8S rRNA, 5S rRNA, and 4S tRNA (see Experimental Procedures). Insets are photos of actual stained RNA of gradient fractions 15 and a molecular weight determination of H-chain mRNA in 1.5% agarose gels containing 10 mM methylmercuric hydroxide. Mouse RNA markers are 28S rRNA, 18S rRNA, 14S κ L-chain mRNA, and 10S globin mRNA. The globin mRNA was not resolved into two components on this gel system but ran as a single broad peak. [^{35}S]Methionine-labeled, immunoprecipitable product from mRNA derived from elution of gel slices is shown (···).

synthetic activity are observed for all the Ig RNA in the regions of 14 S and 17 S, or 20 S, of the first sucrose gradient (Figures 3 and 7 and supplementary material). The 14 S is known to contain κ or λ L-chain mRNA (Faust, unpublished results) and the 17S fraction contains γ or α H-chain mRNA, while the 20S fraction contains μ H-chain mRNA. When the autoradiographs of these cell-free translational products and their tryptic peptide maps are examined, it is clear that these 17S or 20S peak RNA fractions do indeed code for the major amount of serologically related H chain (Figures 5 and 9, Tables II and III, and supplementary material).

Each partially purified H-chain mRNA from the peak of the second sucrose gradient migrated as a single band in polyacrylamide gels in 98% formamide (Figure 10 and supplementary material). The molecular weight of each peak of γ and α H-chain mRNA is estimated to be about 750000 or

about 2200 bases. These mRNAs, corresponding to 17 S in size, migrate slower than 18S rRNA in the gels. These results were validated by denaturing agarose gel analyses, by use of either formaldehyde or methylmercuric hydroxide (Figure 10 and Moore, unpublished results). By use of the same methods, the μ H-chain mRNA was shown to run significantly slower than 18S rRNA on denaturing gels, with an apparent molecular weight of 950 000, which is equivalent to about 2800 nucleotides (Figure 10). A minor peak at 750 000 daltons is also noted. Its significance is unknown; however, it is probably not functional, biologically active μ H-chain mRNA, since the major amount of serologically reactive protein generated from this mRNA fraction appears to be associated with the predominant 950 000-dalton RNA peak (Figure 10). In addition, a preliminary investigation involving four other mouse myelomas producing IgM reveals only the 950 000-dalton component being present and also coding for the serologically reactive μ H-chain-like product (Faust, unpublished observations). The final yield of RNA, containing maximum H-chain mRNA activity from these sucrose gradient 2 fractions, represents about 0.10% of the total input polysomal RNA (Table I). This stands out more favorably than the 0.0085% yield of γ_1 H-chain mRNA isolated by immunoprecipitation methods (Ono et al., 1977). The purity of the H-chain mRNA is estimated to range from 40 to 75% by kinetic complexity analysis, depending upon the isotype studied (Table IV). This compares reasonably with a purity of about 90% for the γ_1 H-chain mRNA prepared by immunochemical methods (Ono et al., 1977).

Table IV: Determination of Kinetic Complexity of H-Chain mRNA[a]

cDNA template mRNA	analytical complexity[b] (daltons)	$C_rt \times 10^3$ mol s L^{-1}	kinetic complexity[c] (daltons)	% homogeneity
mouse globin	400 000	3.5	kinetic std	100
MOPC-31C (γ_1)	750 000	10	1 150 000	~65
MOPC-21 (γ_1)	750 000	12	1 375 000	~55
MOPC-173 (γ_{2a})	750 000	11	1 257 000	~60
MOPC-195S (γ_{2b})	750 000	16	1 875 000	~40
FLOPC-21 (γ_3)	750 000	8.75	1 000 000	~75
J 558 (α)	750 000	9.5	1 085 000	~70
MOPC 104E (μ)	950 000	20.8	2 375 000	~40

[a] Hybridizations were done at 41 °C in the presence of 8 mM Tris-HCl, pH 7.5, 120 mM NaCl, 1 mM EDTA, 0.1% sodium dodecyl sulfate, 50% formamide (see also Experimental Procedures). Routinely 60–80% of all [^{3}H]cDNA hybridized back to the same mRNA fraction from the sucrose gradient 2, which was used as template. Only about 2% hybridization was observed in the presence of poly(A) or in a self-annealing control. [b] The analytical complexity (size) is measured on 98% formamide gels and for mouse globin is the sum of both the α chain and β chain (about 200 000 daltons each). [c] The kinetic complexity was measured as a function of the $C_rt_{1/2}$ according to a previous method for mouse κ L chain (Faust et al., 1974).

Discussion

Properties of the γ, μ, and α H-Chain mRNA. This report is part of a comprehensive effort to define at the molecular level the H-chain Ig multigene complex of the mouse, by use of the corresponding H-chain mRNAs and their respective cDNAs as molecular probes. The purpose of these studies was to describe the isolation, partial purification, and characterization of the mouse Ig H-chain mRNA. These results confirm and exhaustively expand previous preliminary investigations of γ_1 and α H-chain mRNA and represent the first such reports for the other three mouse γ H-chain subclasses, i.e., γ_{2a}, γ_{2b}, γ_3, and μ. Each partially purified H-chain mRMA migrated as a 17S or 20S component on sucrose velocity sedimentation gradients. Each appeared to be larger than 18S rRNA on denaturing polyacrylamide gels and has an estimated molecular weight of 750 000 or 950 000, depending on the isotype. Denaturing agarose gels confirm these results. This corresponds to a nucleotide chain length of about 2200 or 2800 bases, respectively—about twice the size of the mouse Ig L-chain mRNA (Milstein et al., 1972; Mach et al., 1973).

Although this estimate is about 10% larger than previous estimates for γ_1 H-chain mRNA (Cowan & Milstein, 1973; Ono et al., 1977), it is believed to be accurate for the following reasons: (1) all the molecular weight markers fell on a straight line, regardless of guanosine–cytidine content (Figure 10); (2) while the absolute positions of these markers varied slightly, but proportionally, from run to run, the slope of the curve did not change even through different runs over more than a year; (3) although the markers proportionately ran faster, the slope did not change when the electrophoresis was run at 55 °C instead of room temperature (Moore and Faust, unpublished observations), contrary to observations of a previous report (Spohr et al., 1976), suggesting that in our case all RNA samples were equally and, presumably, completely unfolded, perhaps the results of our preliminary heat denaturation step at 65 °C for 10 min in only 99% formamide and 2 mM EDTA prior to initiating electrophoresis; (4) when the γ_1 H-chain mRNA and 18S rRNA were run in the same gel, the γ_1 H-chain mRNA always ran consistently slower (Moore and Faust, unpublished observations), a phenomenon observed only with low-voltage gradients, long run times, and high-porosity gels and discussed by others for RNA (Lehrach et al., 1977) and for DNA (McDonnell et al., 1977); in this connection it should further be noted that Lehrach et al. (1977) obtained a linear molecular weight curve up to 2.2×10^6 in 3.2% polyacrylamide gels with 98% formamide at room temperature or 58 °C, comparable to our results in 3.0% polyacrylamide gels and 98% formamide at room temperature or 55 °C; and (5) these results were substantiated with denaturing agarose gels, by employment of formaldehyde or methylmercuric hydroxide. However, denaturing acrylamide gels with 98% formamide was the method of choice for analytical runs because of their superior resolution. Finally, the size estimates were identical for all the subclasses of mouse γ H-chain mRNA or α H-chain mRNA studied.

Since the coding capacity for authentic γ_1 H chain requires about 1350 bases for about 440 amino acids (Adetugbo et al., 1975) and up to 200 bases are expected to be required for the poly(A) tail [presumed to be present based on its binding to the poly(dT)-cellulose column] located at the 3′ end of the mRNA (Brawerman, 1974), approximately 700–800 bases remain unaccounted for in both location and function within this mRNA. This is twice that of previous estimates (Cowan et al., 1976; Ono et al., 1977). So, similar to the mouse Ig L-chain mRNA, only about 60% of the length of the H-chain mRNA is directly responsible for encoding the structural protein. Furthermore, if the γ H chain is synthesized as a putative precursor, similar to that of the L chain (Milstein et al., 1972; Swan et al., 1972; Mach et al., 1973), then approximately another 50 bases or more would be expected to be involved, thereby reducing to about 600 or 700 the unassigned number of bases. A recent paper appearing for mouse Ig α H chain supports this speculation, showing an additional sequence of 18 amino acids located on the amino terminus of the mature α H chain (Jilka & Pestka, 1977).

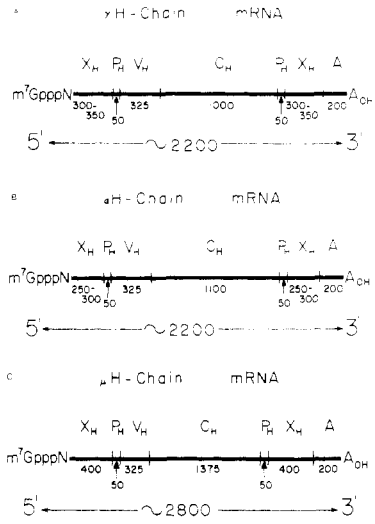

FIGURE 11: Schematic diagram of possible sequence arrangement in (A) γ_1, γ_{2a}, γ_{2b}, or γ_3 H-chain mRNA; (B) α H-chain mRNA; and (C) μ H-chain mRNA; based on data presented here and elsewhere (Jilka & Pestka, 1977). Numbers refer to lengths in bases. A, poly(A) sequence; V_H, variable region sequence; C_H, constant region sequence; $P_H{}^{5'}$ and $P_H{}^{3'}$, putative precursor sequence at the 5' end and/or 3' end; $X_H{}^{5'}$ and $X_H{}^{3'}$, untranslated sequence at the 5' end and/or 3' end; and m^7GpppN, methylated guanosine cap found on most mammalian mRNA and specifically on mouse Ig L chain of MPC-11 (Adams & Cory, 1975).

Nothing is known of the disposition of the carboxy terminus. However, a similar amino acid sequence length could be predicted for the carboxy terminus of γ H chain in those cases where the IgG is a cell surface membrane component. This would be required to serve as a membrane anchor. In such a case, the arrangement of nucleotide sequences in any mature cytoplasmic γ H-chain mRNA should be similar to that illustrated in Figure 11.

In this particular model, the unaccounted 600–700 bases have been evenly assigned to a 5' region, $X_H{}^{5'}$, and a 3' region, $X_H{}^{3'}$. But in the extreme, they could be all in the 5' region or 3' region. For reasons put forth earlier in the case of mouse Ig L-chain mRNA (Cowan & Milstein, 1973; Faust et al., 1974; Kohler & Milstein, 1975), it is not expected that any of these unassigned bases would be found between the C_H and V_H coding regions of the mature cytoplasmic γ H-chain mRNA nor between the V_H and putative γ H-chain precursor, P_H, coding regions. So the γ H chain is most likely synthesized from a single contiguous mRNA. This argument also holds for the μ and α H-chain mRNA discussed below. However, such is probably not the case for newly transcribed H-chain mRNA nor for various RNA precursors to the mature cytoplasmic H-chain mRNA as is suggested for L chain (Gilmore-Herbert & Wall, 1978).

The model for the mature cytoplasmic form of the α H-chain mRNA is similar to that of γ H-chain mRNA (Figure 11), since both mRNA are the same size. The difference is reflected in the increased amount of coding length required for the larger α H-chain protein (~475 amino acids; Jaffe et al., 1971; Lui et al., 1976), leaving somewhat fewer undefined bases. A model can be constructed also for the 2800 base length of the μ H-chain mature cytoplasmic mRNA (Figure 11). The main features of this model are as follows. The structural coding portions of the μ H-chain mRNA require about 1700 bases for 575 amino acids, estimated from human μ H chain (Putnam et al., 1973), up to 200 bases for the poly(A) tail on the 3' end and up to 100 bases for a putative precursor on the amino and/or carboxyl termini of the μ H chain. The extra carboxy terminal piece is again a likely possibility, since it is anticipated that a membrane anchor would be required to retain the IgM or IgA as an integral part of the membrane. This would distinguish membrane Ig from secreted Ig. That still leaves approximately 800 bases unassigned with respect to function and location, which have been evenly distributed between the 5' region and the 3' region in this mRNA.

Since the δ H-chain (Leslie et al., 1971) and the ϵ H-chain (Kochwa et al., 1971) proteins appear to be about the same size as the μ H-chain proteins, that is, they appear to contain four C_H domains in addition to the V_H domain, we would predict that their mature cytoplasmic mRNA would also be about 950000 daltons.

A point of special interest with this particular α H chain focuses on its V_H region. The V_H region of the IgA of J 558 has served to define a normal BALB/c idiotype and, consequently, a V_H gene (Blomberg et al., 1972). This is apparently the same idiotype of the μ H chain of MOPC-104E. Therefore, the utilization of these two H-chain classes should allow the preparation of specific C_H and, more importantly, V_H probes to study the structure, function, organization, and regulation of V_H genes in the mouse relative to themselves and to the multigene C_H family. The potential for studying the so-called switch mechanism (Cooper et al., 1972) of sequential C_H gene activation is especially promising with the availability of such highly and rigorously defined probes as these.

Properties of the Cell-Free Synthesized H Chain. The cell-free synthesized, serologically reactive γ_1 H-chain product of both MOPC-31C and MOPC-21 migrated as a 52000-dalton protein on sodium dodecyl sulfate–polyacrylamide gels. This is smaller than authentic MOPC-31C γ_1 H chain (57000 daltons) and equivalent to the authentic γ_1 H chain of MOPC-21. This is in direct contrast to one report which claimed the cell-free synthesis of two major γ_1 H-chain proteins, 54000 and 56000 daltons, and several smaller ones from MOPC-31C mRNA (Ono et al., 1977). These proteins were derived from a wheat germ cell-free system, and a precursor–product relationship was suggested but no data were provided. The report on MOPC-21 published earlier suggested that the cell-free synthesized γ_1 H chain was about the same size as the authentic γ_1 H chain (Cowan & Milstein, 1973).

The significance of the identical sizes of the cell-free synthesized γ_1 H-chain products of MOPC-31C and MOPC-21 which we found remains unclear, as well as the differences in sizes of the two authentically secreted γ_1 H chains. But these observations may be the result of several different features. For example, a previous report suggests the reticulocyte cell-free system cannot glycosylate any proteins synthesized in it (Cowan & Milstein, 1973). Therefore, the cell-free synthesized γ_1 H chain of both MOPC-31C and MOPC-21 is probably not glycosylated. If this is so, then at least MOPC-21 γ_1 H chain is larger than that expected for a carbohydrate-free γ_1 H chain, suggesting a putative precursor, as has been found for α H chain (Jilka & Pestka, 1977). A similar situation would then be expected for that of MOPC-31C, since its cell-free product is the same size as that of MOPC-21. It is possible that the authentic MOPC-31C γ_1 H chain probably has a higher level of glycosylation than that of MOPC-21. Alternatively, a processing defect may exist in the case of MOPC-31C, such that the fully glycosylated precursor of the in vivo secreted γ_1 H chain is not cleaved to the authentic γ_1 H chain upon secretion. In the case of the other γ H chain subclasses—γ_{2a}, γ_{2b}, and γ_3—the cell-free synthesized, serological reactive γ H-chain product migrated as a 52000-dalton protein on sodium dodecyl

sulfate–polyacrylamide gels, equivalent to the homologous authentic γ H chain. This is analogous to the γ_1 H chain of MOPC-21 and suggests a γ H-chain precursor for the other subclasses.

The single major immunochemically reactive cell-free α H-chain-like product generated here has an apparent molecular weight of 56 000, compared with 54 000 for the in vivo secreted α H chain of the IgA of J 558. These differences may be the result of several factors discussed above. However, the major likelihood is that this cell-free α H-chain-like product is synthesized as a putative precursor, since this is the case for one α H chain, MOPC-315, already documented in the literature (Jilka & Pestka, 1977). In addition, we find that the cell-free synthesized α H chain of MOPC-315 is also 56 000 daltons compared with 54 000 daltons for the authentic α H chain (Faust et al., unpublished observations). The possibility of a putative precursor for the μ H chain must also be considered. But no conclusions can be drawn, since the major serologically reactive cell-free, presumably carbohydrate-free (Cowan & Milstein, 1973) protein is smaller than the in vivo μ H chain. However, the in vivo MOPC-104E μ H chain is expected to contain at least 10% carbohydrate (Milstein et al., 1975), which is normal for IgM, possibly accounting for this observed size difference.

Tryptic peptide comparisons of authentically secreted H chains revealed similar patterns with their homologous cell-free products. The lack of complete homology may be the result of (1) peptide difference of V_H regions; (2) presumed putative precursors; (3) differences in the level of glycosylation; and (4) contaminating impurities at relatively low levels (see Tables II and III).

Purity and Yield of the H-Chain mRNA. It is estimated from the kinetic complexity analyses that the H-chain mRNA purified from myeloma tumors, as described here, is about 40–75% homogeneous. This is in agreement with a previous report, by use of a similar purification scheme (Cowan et al., 1976). Also, this degree of purification reasonably compares with that estimated for the recently published immunoprecipitation method of purification of MOPC-31C γ_1 H-chain mRNA (Ono et al., 1977). Their estimate was 97% homogeneity by kinetic complexity analyses, and because of certain basic errors inherent in these types of measurements it is reasonable to assume the purity of their mRNA preparation is likely around 90%. Therefore, as expected the immunochemical procedure provides a purer γ_1 H-chain mRNA fraction than does a standard physicochemical purification procedure.

However, the immunochemical purification procedure appears to be a very inefficient method for obtaining γ_1 H-chain mRNA. That is, Ono et al. (1977) recovered 0.0085% of their input total polysomal RNA of MOPC-31C as specific γ_1 H-chain mRNA, which is about 90% pure. In contrast, we have recovered 0.12% of our input polysomal RNA of MOPC-31C as specific γ_1 H-chain mRNA, which is about 60% pure. Therefore, when taking into account the 1.5-fold difference in purity and amounts of RNA recovered, the physicochemical methods described here result in at least a 10-fold higher recovery of the same specific γ_1 H-chain mRNA. The same logic applies to the other H-chain isotypes based on the results reported here.

So, even though a higher homogeneity may be obtained by use of immunochemical methods, neither method yields completely pure γ_1 H-chain mRNA. Moreover, for many types of experiments involving the use of the H-chain mRNA as a probe, complete purity is not required. An example of such a situation has already been presented for mouse Ig L chain (Faust et al., 1974; Farace et al., 1976). In addition, if a completely pure (>99%) probe were required, this could only be achieved from H-chain mRNA prepared by either above method through limited back-hybridization with the appropriate cDNA or through the newly developed cloning procedures of recombinant DNA technology (Rougeon et al., 1975; Maniatis et al., 1976; Rabbits, 1976).

At this point a caution must be issued. The untranslated protion of all subclasses of the γ H-chain mRNA is 600–700 bases, exclusive of poly(A), and their function and location are presently unknown. Similarly, that of α and μ H-chain mRNA are about 500 and 800 bases, respectively. Therefore, all cDNA initiated conventionally with oligo(dT) will start at the poly(A) tail on the 3′ end of the H-chain mRNA and could contain from 500 to 800 of these untranslated bases before including any portion of the H-chain structural coding region. So, any cDNA probes used for the purpose of assessing C_H gene function must be significantly longer than 500–800 bases to include at least part of the C_H structural gene and essentially full length to ensure inclusion of the V_H structural gene. Otherwise, it is conceivable in light of the new segmented gene data for both rabbit (Jefferies & Flavell, 1977) and mouse β-globin (Tilghman et al., 1977), and more appropriately mouse λ L chain (Tonegawa et al., 1978), that, while untranslated C_H gene will be measured, C_H structural gene segments will not. Finally, it must be pointed out tnat certain questions concerning comparisons of these γ H-chain subclasses cannot yet be answered. For example, while each γ H-chain mature cytoplasmic mRNA and primary translational product are the same size, their gene templates or primary transcriptional products may not be equal. However, these questions could now be pursued with cloned H-chain probes in a manner already used for preliminary κ L-chain studies (Gilmore-Hebert & Wall, 1978).

After submission of this manuscript for publication, a report appeared describing the isolation, partial purification, and characterization of a γ_{2b}, μ, and α H-chain mRNA from mouse myelomas (Marcu et al., 1978). Our results are at variance with some of theirs. Firstly, we obtained similar degrees of mRNA purification with only sucrose gradient fractionation. Secondly, rapid isolation and extraction procedures were not essential, since some of our polysome preparations were stored at −20 °C for over 2 years before extraction. The yields in both cases are about 0.1%. Thirdly, we find only one major serologically reactive cell-free product for all isotypes studied.

Finally, there are significant differences in reported H-chain mRNA sizes. We report lengths for γ H-chain mRNA (γ_1, γ_{2a}, γ_{2b}, including unpublished data on MPC-11, and γ_3), μ H-chain mRNA, and α H-chain mRNA of 2200, 2800, and 2200 bases, respectively. These results were obtained with three different denaturing gel systems. They are significantly larger than the values reported by Marcu et al. (1978) for only a γ_{2b} H-chain mRNA, μ H-chain mRNA, and α H-chain mRNA—1800, 2150, and 1800 bases, respectively. These latter values must be viewed with reservation, since the γ H chain (~440 amino acids) requires about 1600 bases total for presumed precursor, coding sequence and a maximum of 200 bases for the poly(A) sequence, leaving only about 200 unaccounted bases in their model. Similarly, the α H chain (~475 amino acids) requires about 1700 bases, leaving only about 100 unaccounted bases, and most significantly, the μ H chain (~575 amino acids) requires about 2000 bases, leaving only about 150 unaccounted bases—all according to the models of Marcu et al. (1978). These models may be untenable, since they leave so little noncoding sequence [only

5–10%, excluding poly(A)] compared with what is known of other mRNA in general. For example, the mRNA coding for α- and β-globin (Gould & Hamlyn, 1973), Ig κ and λ L chain (Mach et al., 1973; Farace et al., 1976), and also ovalbumin, conalbumin, ovomucoid, and lysozyme (Buell et al., 1978) all have about 30–40% of their length as noncoding sequence. Our data for all the γ H-chain mRNA subclasses, μ H-chain mRNA, and α H-chain mRNA are consistent with these other previous observations. Therefore, while all classes of H-chain mRNA contain the same percentage of coding sequences, they do not contain the same amount (number of bases) of noncoding sequences.

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Supplementary Material Available

Supplemental Figures 1–29 which show experimental detailed results for the γ_1, γ_{2a}, γ_{2b}, γ_3, α, and μ H-chain mRNA showing sucrose gradient profiles, cell-free translational assays, acrylamide gel analyses of total cell-free synthesized protein products and those serologically reactive with their respective antiserum, tryptic peptide maps, and H-chain mRNA molecular weight determinations (29 pages). Ordering information is given on any current masthead page.

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Critical Micelle Concentrations of Gangliosides†

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ABSTRACT: The micellar properties of mixed, bovine gangliosides and purified galactosyl-*N*-acetylgalactosaminyl(*N*-acetylneuraminyl)galactosylglucosylceramide were studied by gel filtration, equilibrium dialysis, and band and boundary centrifugation in sucrose gradients. The dissociation of micelles is very slow (days) in water and required us to approach equilibrium by association of monomers rather than by the dissociation of micelles. The gangliosides were therefore first converted into very low molecular weight aggregates (1–3 molecules) by dissolving them in Me_2SO. Galactosyl-*N*-acetylgalactosaminyl(*N*-acetylneuraminyl)galactosylglucosylceramide was then diluted into aqueous sucrose gradients and sedimented by the boundary centrifugation technique. This gave a sedimenting micelle and a nonsedimenting monomer concentration of (1–2) × 10^{-10} M (or less) which corresponds to the critical micelle concentration value. The mixed gangliosides revealed two micellar sizes (i.e., 10 and 4.5 S), the slower sedimenting species being formed from the larger one with time (days). The critical micelle concentration of the mixed gangliosides was found to be approximately 10^{-8} M by gel filtration, equilibrium dialysis, and band centrifugation.

Gangliosides were originally described as acylsphingosyl oligosaccharides containing sialic acid (Klenk, 1942). It was later shown that higher order gangliosides (G_{M1}, G_{D1a}, G_{D1b}, G_{T1}, etc.)[1] have a common basic structure, i.e., *N*-acylsphingosineglucosegalactose-*N*-acetylgalactosaminegalactose, with one or more molecules of *N*-acetylneuraminic acid (Svennerholm, 1962). G_{M2} and G_{M3} are biosynthetic precursors with deletions at the terminal galactose and galactosaminegalactose residues, respectively.

Gangliosides have been implicated in the binding or function of many biologically important molecules, i.e., cholera toxin (Sattler et al., 1977), tetanus toxin (Helting et al., 1977), botulinum toxin (Haberman & Heller, 1975), thyrotropin (Mullin et al., 1976), human chorionic gonadotropin (Lee et al., 1976), luteinizing hormone (Lee et al., 1977), serotonin (Ochoa & Bangham, 1976), interferon (Vengris et al., 1976), bilirubin (Kahan et al., 1968), and wheat germ agglutinin (Redwood & Polefka, 1976).

Table I: Literature Values for the cmc of Gangliosides

cmc (M)	ganglioside	technique	reference
1×10^{-4}	mixed	surface tension	Gammack (1963)
1×10^{-5}	mixed	conductance	Howard & Burton (1964)
1×10^{-4}	G_{M1}	neuraminidase	Rauvala (1976)
7.5×10^{-5}	G_{M2}	triiodide method	Yohe & Rosenberg (1972)
8.5×10^{-5}	G_{M1}	triiodide method	Yohe & Rosenberg (1972)
9.5×10^{-5}	G_{D1a}	triiodide method	Yohe & Rosenberg (1972)
1×10^{-4}	G_{T1}	triiodide method	Yohe & Rosenberg (1972)

The interpretation of studies involving gangliosides depends on a knowledge of their state of aggregation since the observed biological effects occur, in many cases, in the concentration range of the reported cmc values, i.e., 10^{-4}–10^{-5} M (see Table I). Unfortunately, none of the cmc values was obtained by use of a technique which directly measured either the size or the molecular weight of the gangliosides. It has also been observed that at concentrations below these values purified preparations of G_{M1} and G_{M2} were unable to cross a dialysis membrane (Kanfer & Spielvogel, 1973; Sattler et al., 1977). In an attempt to resolve this apparent contradiction, we undertook a study of the molecular behavior of typical gangliosides.

Materials and Methods

Mixed, bovine brain gangliosides were purchased from ICN Pharmaceuticals. The composition was measured by thin-layer chromatography (TLC) on silica gel plates and is shown in Figures 1 and 2. Purified G_{M1} was either purchased from Supelco or obtained by a purification of the mixed gangliosides

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[1] Abbreviations used: cmc, critical micelle concentration; Me_2SO, dimethyl sulfoxide; ganglioside nomenclature is according to Svennerholm (1964)—G_{M1}, galactosyl-*N*-acetylgalactosaminyl(*N*-acetylneuraminyl)galactosylglucosylceramide; G_{D1a}, *N*-acetylneuraminylgalactosyl-*N*-acetylgalactosaminyl(*N*-acetylneuraminyl)galactosylglucosylcerimide; G_{D1b}, galactosyl-*N*-acetylgalactosaminyl(*N*-acetylneuraminyl-*N*-acetylneuraminyl)galactosylglucosylcerimide; G_{T1}, *N*-acetylneuraminylgalactosyl-*N*-acetylgalactosaminyl(*N*-acetylneuraminyl-*N*-acetylneuraminyl)galactosylglucosylceramide; G_{M2}, *N*-acetylgalactosaminyl(*N*-acetylneuraminyl)galactosylglucosylcerimide; G_{M3}, *N*-acetylneuraminylgalactosylglucosylcerimide.