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Purification and Properties of Biologically Active Messenger RNA for a Myeloma Light Chain

(poly(A)-mRNA/oligo d(T)–cellulose/monocistronic 13S mRNA)

D. SWAN, H. AVIV, AND P. LEDER

Laboratory of Molecular Genetics, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20014

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ABSTRACT **A cell-free system derived from Krebs II ascites tumor has been used to assay biologically active mRNA for myeloma (MOPC-41) light chain during its purification by oligothymidylate–cellulose chromatography and sucrose gradient centrifugation. The purified mRNA directs the synthesis of a product that yields tryptic peptides corresponding to those derived from authentic myeloma protein and that forms a specific immunoprecipitate with antibody directed against the MOPC-41 protein. The fact that the light-chain mRNA anneals to oligothymidylic acid–cellulose suggests that it, like several other eukaryotic mRNAs, contains a region rich in adenylic acid residues. The most active fractions of light-chain mRNA, representing about 0.1% of the RNA originally extracted from membrane-bound myeloma polysomes, sediment as a discrete peak with an $s_{20,w}$ of about 13, roughly corresponding to an RNA molecule containing 850 bases. The results suggest that the light-chain mRNA is monocistronic and that it contains about 200 more bases than would be necessary to encode the variable and constant regions of a single light-chain molecule.**

Two contrasting theories have been proposed to account for the diversity and specificity of antibody molecules (1–8). The germ-line hypothesis, which demands that information for the synthesis of different antibody molecules preexists in germ-line cells, cannot be distinguished from the somatic mutation hypothesis, which holds that the immune response develops as the result of modification of genetic information during development. To decide between these two theories requires a technique that could quantitate antibody genes during cellular commitment to antibody synthesis. We have, therefore, set out to isolate an immunoglobulin mRNA that might prove useful in a sensitive hybridization assay for antibody genes, as well as for other purposes.

Our initial attempts have been directed toward purifying a mouse kappa-chain mRNA derived from myeloma tumor MOPC-41 (9). Stavnezer and Huang have recently translated this mRNA in a reticulocyte cell-free system (10). Our strategy has been to use the ascites tumor cell-free system that is dependent on tRNA (11) to assay biologically active light-chain mRNA, and to use oligo(dT)–cellulose chromatography (12) and sucrose gradient centrifugation as initial steps in its purification. In previous work, we have shown that this approach can be used successfully for the isolation of poly(A)-rich rabbit globin mRNA (12). This mRNA, in turn, can be converted into its highly labeled DNA complement with RNA-directed DNA polymerase of avian myeloblastosis virus (13–15). In the present work, we show that the ascites tumor cell-free system provides a sensitive and convenient assay for the translation of light-chain mRNA, that light-chain mRNA is retained by oligo(dT)–cellulose, suggesting that it is a poly(A)-rich mRNA, and that biologically active light-chain mRNA sediments during sucrose gradient centrifugation as a discrete peak, of $s_{20,w}$ about 13, roughly corresponding to a molecule containing about 200 more bases than are necessary to encode both the variable and the constant regions of an immunoglobulin light chain.

MATERIALS AND METHODS

The sources of many of the reagents used in this study have been indicated (11, 12).

Preparation of Myeloma Polysomes and Myeloma mRNA. Myeloma tumors, the generous gift of Dr. M. Potter, were grown and harvested according to standard procedures (9), and were frozen and stored in liquid N_2 before processing. The microsomal fraction was obtained according to a modified procedure (16). 70 g of dissected, nonnecrotic tumor was suspended in 170 ml of buffer A containing 0.05 M Tris·HCl (pH 7.7)–0.025 M KCl–5 mM $MgCl_2$–7 mM 2-mercaptoethanol–0.88 M sucrose, disrupted for 30 sec in a chilled Waring Blendor at medium speed, and further disrupted with eight strokes in a loose-fitting, Teflon–glass, motor-driven homogenizer. The homogenate was centrifuged at 20,000 × *g* for 20 min; the supernatant was diluted to 0.62 M sucrose with buffer A minus sucrose, and layered over a discontinuous sucrose gradient composed of 5 ml of buffer A containing 2.0 M sucrose and 7 ml of buffer A containing 1.5 M sucrose. After centrifugation at 50,000 rpm for 5 hr in a Beckman 60 Ti rotor, the membrane-bound polysomes, which had concentrated between the 2.0 and 1.5 M sucrose layers, were harvested, the RNA extracted, and purified with oligo(dT)–cellulose chromatography as described (12).

Oligo(dT)–Cellulose Chromatography (12). 1000 A_{260} units of crude myeloma polysomal RNA dissolved in application buffer containing 0.01 M Tris·HCl (pH 7.5)–0.5 M KCl were applied to a 10 ml (about 2.5 g, dry weight) oligo(dT)–cellulose column previously equilibrated with the same buffer. The unadsorbed material was eluted by continued washing with the application buffer. The material retained by the column was eluted with buffer containing 0.01 M Tris·HCl (pH 7.5). The material eluted in this way was immediately precipitated by the addition of CH_3COONa and two volumes of ethanol as described (12).

Cell-Free Protein Synthesizing Systems and Assays. Ribosomes and ribosome-free supernatant were obtained by

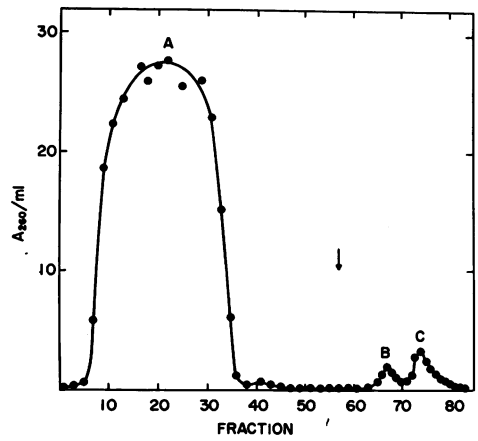

FIG. 1. Chromatography of MOPC-41 polysomal RNA on oligo(dT)-cellulose. 1-ml fractions were collected. *Arrow:* start of elution with 0.01 M Tris·HCl.

centrifugation of incubated ascites tumor cell extracts (11) over a 7-ml layer of 1.25 M sucrose containing 0.03 M Tris·HCl (pH 7.5)–0.125 M KCl–5 mM magnesium acetate–7 mM 2-mercaptoethanol for 12 hr at 30,000 rpm in a Spinco No. 30 rotor. Myeloma mRNA was assayed in 60 μl reaction mixtures that contained 30 mM Tris·HCl (pH 7.5), 3.2 mM magnesium acetate, 76 mM of KCl, 7 mM 2-mercaptoethanol, 1 mM of ATP, 0.1 mM of GTP, 0.6 mM of CTP, 10 mM of creatine phosphate, 8 μg of creatine kinase, 40 μM (each) of nonradioactive amino acids, 5 μM of radioactive amino acid of indicated high specific activity ([^{3}H]leucine or [^{14}C]-leucine, [^{14}C]valine, [^{14}C]lysine, [^{14}C]serine, and [^{14}C]alanine, as indicated), 0.2 A_{260} units of $tRNA_{rabbit}$, 0.12 A_{260} units of ribosomes, 0.12 mg of protein in ribosome-free supernatant, and mRNA, as indicated. The endogenous activity of myeloma polysomes was studied with ascites supernatant by substitution of an equal amount of membrane-bound myeloma polysomes for ascites ribosomes and exogenous mRNA. Reaction mixtures, were assayed as described (12). [^{14}C]-Leucine-labeled MOPC-41 light chain used as a standard for tryptic peptide analysis was synthesized in excised tumor (17) and purified (18). The standard was homogeneous as judged by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Rabbit globin mRNA, used as a control in the immunoprecipitin assay, was prepared as described (12).

Immunoprecipitin Assay for MOPC-41 Protein. [^{3}H]Leucine-labeled MOPC-41 protein and rabbit globin were synthesized in standard reaction mixtures and treated with 5 μg of ribonuclease. A 10-μl aliquot was removed, precipitated, and counted as above. To the remaining 0.05 ml were added 2 μg of nonradioactive MOPC-41 protein, 1 mg of bovine serum albumin, Triton X-100 to a final concentration of 3%, and 0.28 mg of a fraction of goat anti-MOPC-41 serum precipitated with 45%-saturated $(NH_4)_2SO_4$ (generously provided by Dr. I. Schechter); the reaction mixture was brought to 0.2 ml with buffer containing 0.01 M sodium phosphate (pH 7.0)–0.14 M NaCl. Incubation was for 2 hr at 37° and was continued overnight at 4°. Tubes were centrifuged for 15 min at 10,000 × *g*. The precipitates were washed twice with the above buffer solution containing 3% Triton X-100, then dissolved in 0.2 ml 0.1 M KOH, precipitated with 2 ml of 10% Cl_3CCOOH, filtered, and counted.

Sodium Dodecyl Sulfate–2-Mercaptoethanol–Polyacrylamide Gel Electrophoresis. The products of reaction mixtures containing [^{14}C]leucine, [^{14}C]valine, [^{14}C]lysine, [^{14}C]serine, and [^{14}C]alanine were prepared and electrophoresed at 200 V for 5 hr in a slab containing a linear, 7–28% gradient of polyacrylamide, according to procedures identical to those described (19, 20). The dried slabs were exposed to x-ray film for 36 hr and developed.

Sucrose Gradient Sedimentation. About 15 A_{260} units of oligo(dT)-purified RNA were layered on a 5–20% sucrose gradient containing 0.01 M Tris·HCl (pH 7.5)–0.1 M NaCl–1 mM EDTA in a Beckman SW 27 rotor carrying no. 1044 adapter buckets and centrifuged at 27,000 rpm for 17 hr. Fractions were collected, and their absorbance at 260 nm was determined. The ability of 3-μl aliquots to direct protein synthesis in the ascites tumor cell-free system was determined as above.

Tryptic Peptide Analysis. Double reaction mixtures containing [^{3}H]leucine were incubated at 37° for 120 min, after which time 10 μg of pancreatic ribonuclease was added and the incubation was continued for 20 min. Carrier MOPC-41 light chain (1 mg) and [^{14}C]leucine-labeled MOPC-41 light chain were added, the proteins were reduced, aminoethylated, and digested with trypsin at an enzyme–substrate ratio 1:50 (21). The digested reaction mixtures were lyophilized and dissolved in the appropriate starting buffers. The tryptic peptides were analyzed by cation-exchange chromatography on Technicon type P cation exchange resin, and eluted with pyridine acetate (12). The peptides were also analyzed by anion-exchange chromatography on a 0.6 × 25-cm Dowex 1-X2 column, which was eluted with a pyridine acetate gradient between pH 8.0 and 3.0 (E. Appella, personal communication). Each chamber of the multigradient contained 80 ml of the following solutions: chambers 1, 2, 3; 3% pyridine, chambers 4,5; 0.1 M pyridine–0.05 M acetic acid, chambers

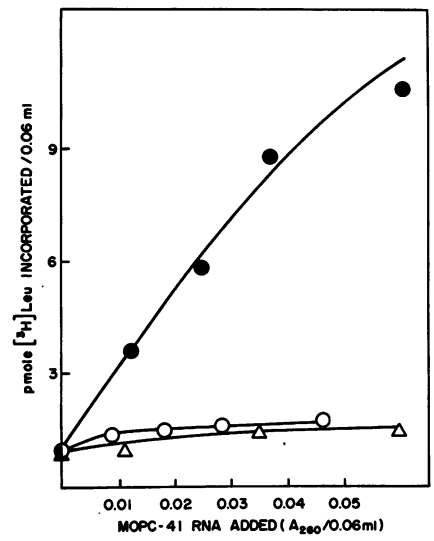

FIG. 2. Protein synthesis in response to MOPC-41 RNA fractions. Reaction mixtures were incubated for 60 min. The symbols represent the following: *Closed circles* (●——●), RNA retained by oligo(dT)-cellulose (Fig. 1, *Peak C*), *triangles* (Δ——Δ), RNA not retained by oligo(dT)-cellulose (Fig. 1, *Peak A*), and *open circles* (○——○), total MOPC-41 polysomal RNA.

6, 7; 0.5 M pyridine–0.25 M acetic acid, chambers 8, 9; 2.0 M pyridine–2.0 M acetic acid. 4-ml fractions were collected, dried, dissolved in 1 ml of water, and counted in a liquid scintillation counter in Aquasol.

RESULTS

Oligo(dT)–Cellulose Chromatography of MOPC-41, Membrane-Bound Polysomal RNA. Immunoglobulin synthesis occurs preferentially on polysomes associated with the membrane fraction of cell extracts (16, 22 and see Fig. 3). As a first step, we have isolated and used membrane-bound polyribosomes as the source of light-chain mRNA. Since various mammalian mRNAs contain extended sequences of adenylic acid (23–26), it seemed likely that this would be true of immunoglobulin mRNA as well. Therefore, we applied RNA derived from membrane-bound polysomes to an oligo(dT)–cellulose column (Fig. 1) previously used in the purification of poly(A)-rich rabbit globin mRNA (12). About 98% of the RNA was not retained by the column. This RNA that was not retained by oligo(dT)–cellulose (Peak A) is primarily ribosomal (12). When the column was eluted with buffer of low ionic strength, a condition that does not favor the association between complementary polynucleotides, two small RNA peaks (B and C) were eluted. We have tested the ability of each of these fractions to direct the synthesis of protein in the ascites cell-free system (Fig. 2). The crude polysomal RNA was virtually inactive in directing the incorporation of [^{3}H]leucine in the cell-free system at the concentrations used. This was also true for the RNA that was not retained by the oligo(dT)–cellulose column (Fig. 1, Peak A). In contrast, the RNA retained by the column and eluted with low ionic strength buffer (Peak C) was quite active, as little as 0.5 μg directing the incorporation of about 3 pmol of [^{3}H]leucine into Cl_3CCOOH-insoluble material (Fig. 2). The material eluted in Peak B was about 20% as active in directing protein synthesis as the material in Peak C (data not shown).

Characterization of the MOPC-41 mRNA-Directed Protein Products. The proteins synthesized in response to mRNA purified on oligo(dT)–cellulose have been separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and compared to an authentic MOPC-41 light-chain standard and to protein synthesized endogenously on MOPC-41 membrane-bound polysomes (Fig. 3). The membrane–bound polysomes

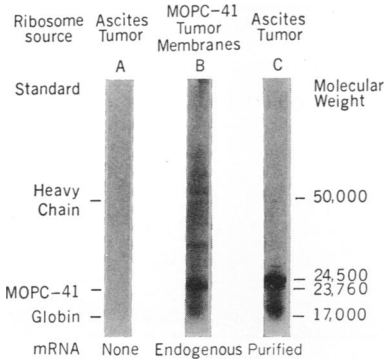

FIG. 3. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of proteins synthesized on membrane-bound myeloma tumor polysomes without additional mRNA and on ascites tumor ribosomes in response to oligo(dT)–cellulose-purified mRNA. Molecular weights were calculated from the indicated standards.

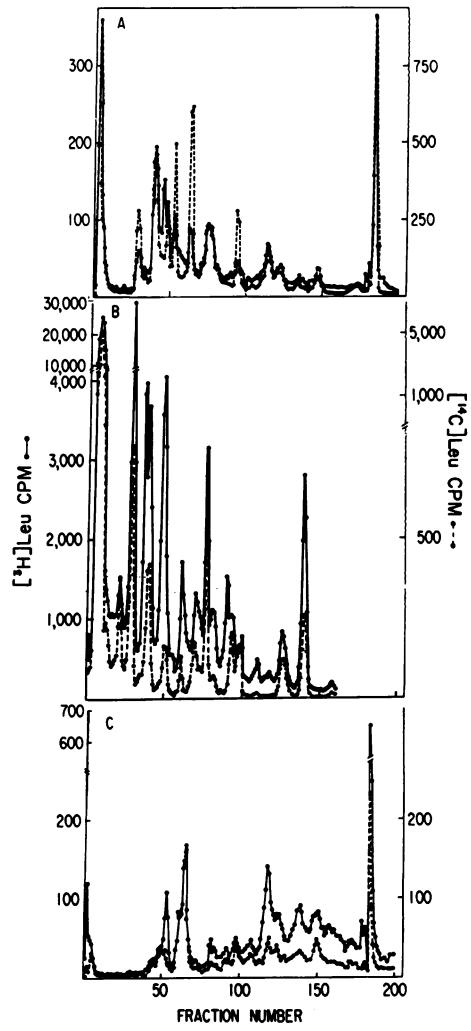

FIG. 4. Separation of tryptic peptides derived from protein synthesized in response to oligo(dT)–cellulose-purified mRNA. (*A*) Chromatography of total product on Dowex 1-X2; *closed circles* (●——●), [^{3}H]leucine-containing peptides derived from *in vitro* synthesized product; *open circles* (○——○), [^{14}C]leucine in peptides derived from MOPC-41 light-chain standard. (*B*) Chromatography of total product on Technicon-P. (*C*) Rechromatography of fractions 1–10 of Technicon-P column on Dowex 1-X2. The last fractions (175–280) were eluted with 40% CH_3COOH.

derived from MOPC-41 tumor direct the synthesis of several proteins; the major band comigrates with MOPC-41 light chain (molecular weight 23,760, calculated from ref. 27). Oligo(dT)–cellulose-purified MOPC-41 mRNA, from these same polysomes, directs the synthesis of several polypeptide chains in the ascites tumor cell-free system. One comigrates with the MOPC-41 light chain. The major product, however, is a protein having a molecular weight slightly greater than that of MOPC-41 light chain, and corresponds to a minor band synthesized endogenously on MOPC-41 polysomes. No bands appear in the absence of mRNA.

The tryptic peptides derived from the total product of such a reaction have been compared to those of an authentic MOPC-41 standard in two chromatographic systems. In an analysis by Dowex 1 chromatography (Fig. 4*A*), each standard MOPC-41 peak coeluted with a peak derived from the *in vitro* product. An additional peak (fractions 45–46) was derived

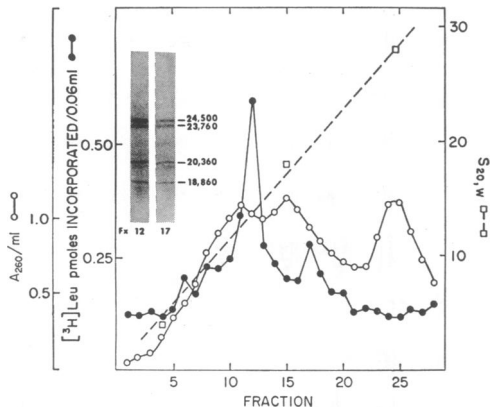

FIG. 5. Sucrose gradient centrifugation of oligo(dT)–cellulose-purified mRNA. *Open circles* (O——O) represent UV absorption. *Closed circles* (●——●) represent protein synthetic activity in the ascites tumor cell-free system. *Boxes* (□——□) represent $s_{20,w}$ values.

from the *in vitro* product that was absent in the *in vivo* product. When the product was analyzed by cation-exchange chromatography on Technicon P resin (Fig. 4*B*), each standard peak again coeluted with one derived from the *in vitro* product. There were two additional peaks (fractions 36–37 and 90–91) in the *in vitro* product that were absent in the *in vivo* product. The material that eluted with the application buffer (fractions 1–10) was further analyzed by Dowex 1 chromatography (Fig. 4*C*). The profiles of both *in vitro* and *in vivo* products corresponded.

Despite the fact that several discrete polypeptides were synthesized *in vitro*, almost all the peptides derived from the *in vitro* product correspond to authentic MOPC-41 peptides. The disparity in their relative radioactivities might be due to the presence of nascent or prematurely terminated polypeptide chains in reaction mixtures (28). Aminoethylation and tryptic digestion of MOPC-41 protein should result in the appearance of 11 leucine-containing peptides. The additional peaks derived from the authentic standard might be due to residual chymotryptic activity in the trypsin used in these studies.

Although the tryptic peptide analyses indicate that the products synthesized *in vitro* contain all the MOPC-41 amino-acid sequences, it was of interest to see if the product of the *in vitro* system would form a specific immunoprecipitate with anti-MOPC-41 antibody. As shown in Table 1, 34% (49,700 cpm) of the radioactivity incorporated into protein in response to the light-chain mRNA was recovered in the immunoprecipitate formed in the presence of carrier MOPC-41 protein, whereas only 10% (21,500 cpm) of the protein synthesized in the presence of rabbit globin mRNA was recovered in the immunoprecipitate. The amount of material in the immunoprecipitate might reflect only that percentage of the product present as complete light chain (see Fig. 3 and Fig. 5, *insert*).

Further Purification of Light-Chain mRNA by Sucrose Gradient Centrifugation. The mRNA purified by oligo(dT)–cellulose chromatography (Fig. 1, *Peak C*) represented about 2% of the RNA derived from the membrane-bound polysomes. This material was further purified by sucrose gradient centrifugation, and the resulting fractions were assayed for their ability to direct protein synthesis in the ascites tumor cell-free system. The absorbance and activity profiles are shown in Fig. 5. There was a prominent peak of mRNA activity that corresponded to an $s_{20,w}$ value of about 13, and a peak of lesser activity corresponding to an $s_{20,w}$ of about 19. Similar results have been obtained in several analyses. In addition, fractions sedimenting with $s_{20,w}$ values between 9 and 22 exhibited slight protein synthetic activity. The absorbance resolved into three peaks, two of which corresponded to ribosomal RNA. The third, at an $s_{20,w}$ of about 12, sedimented slightly more slowly than the major peak of mRNA activity. The protein products synthesized in response to both active fractions [($s_{20,w}$) 13 and 19] were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Fig. 5, *insert*). The products synthesized in response to both fractions were essentially identical. Four discrete polypeptides are seen. These migrate as proteins having molecular weights of 24,500, 23,760 (MOPC-41 light chain), 20,360, and 18,860.

DISCUSSION

An Approach to Purification of an Immunoglobulin mRNA. These studies indicate that the ascites cell-free system provides a sensitive assay for biologically active immunoglobulin light-chain mRNA. The system has an advantage over that described by Stavnezer and Huang (10), in that response to light-chain mRNA can be measured directly without recourse to lengthy immunoassays. It has the additional advantage of responding to less than 0.1% of the amount of mRNA used in the reticulocyte system (see ref. 10 and Fig. 5). In addition, the protocol described for the purification of the light-chain mRNA extends the usefulness of oligo(dT)–cellulose chromatography as an initial procedure for the purification of mRNAs in higher organisms (12).

Authenticity of the In Vitro Synthesized Product. An analysis of the products of the cell-free systems leads to an interesting paradox. The close correspondence of the tryptic peptides derived from the *in vitro* system to those derived from secreted MOPC-41 light chain indicates that a major portion of the *in vitro* product contains amino-acid sequences present in mature light chain (Fig. 4). The formation of a specific immunoprecipitate with anti-MOPC-41 antibody supports this conclusion. Nevertheless, gel electrophoresis (Fig. 5) indicates that four discrete polypeptides are synthesized in response to purified MOPC-41 mRNA. One of these comigrates with the secreted MOPC-41 light chain. Another band, corresponding to a molecular weight of 24,500, appears to be 6–8 amino acids

TABLE 1. *Immunoprecipitation of product synthesized in the presence of oligo(dT)–cellulose-purified mRNA*

mRNA Added	Total [^{3}H]-leucine incorporated (cpm/0.06 ml)	Total [^{3}H]-leucine precipitated with anti-MOPC-41 serum (cpm/0.06 ml)
None	7,000	1,300
MOPC-41	146,600	49,700
Rabbit globin	206,300	21,500

Where indicated, reaction mixtures contained 5 μg of oligo(dT)–cellulose-purified MOPC-41 or rabbit globin mRNA (12).

longer than the light chain. Two other bands, of molecular weight 20,360 and 18,860, appear to be 20–30 and 40–50 amino acids shorter, respectively. Additional tryptic peptide analyses suggest that these proteins contain extensive sequences identical to those of the MOPC-41 light chain, and that they do not arise as a result of out-of-phase translation of the MOPC-41 mRNA or in response to an extraneous mRNA. The presence of the smaller polypeptides is consistent with detailed studies of the *in vitro* translation of encephalomyocarditis virus mRNA, in which case the mRNA was initiated at a single initiation site, but translated incompletely, resulting in the production of several incomplete polypeptides (28).

The 24,500 dalton protein comigrates with a minor protein synthesized with endogenous mRNA on membrane-bound polysomes derived from the MOPC-41 tumor (Fig. 3*B*). There is a difference of only 6–8 amino acids between the length of this protein and MOPC-41 light chain. This might result from a processing step akin to those that occur in the N-terminal peptides of certain bacterial and mammalian proteins (29–31), or during activation of digestive enzymes and certain polypeptide hormones (for a review, see ref. 32). The larger protein might also result from the read-through of a termination signal. If this were the case, it would suggest the existence of a second termination signal, 6–8 codons from the first. The mechanism responsible for the production of the mature form of the light chain appears to operate more efficiently on membrane-bound polysomes derived from MOPC-41 than on ribosomes derived from ascites cells (Fig. 3).

Some Characteristics of the Light-Chain Message. The fact that the MOPC-41 light-chain mRNA anneals to oligo(dT)–cellulose suggests that it contains adenylic acid-rich sequences. While the chemical basis for the difference in elution of the two mRNA fractions from oligo(dT)–cellulose is not known, such a pattern suggests that there are two populations of mRNA, one containing more extensive adenylic acid-rich regions than the other (Fig. 1). A difference is also reflected in the biologic activity of the fractions in that the first eluted is the less active (Fig. 1, *Peak B*). Nevertheless, both fractions direct the synthesis of identical products (data not shown). The sucrose gradient step also indicates that, despite the fact that the most active mRNA fraction represents only 0.1% of the total membrane-bound polysomal RNA, the myeloma mRNA may not be pure. The activity peak does not correspond exactly to the absorbance peak, and it may be contaminated by RNA that is less active for protein synthesis.

The active form of the mRNA corresponds to a relatively uniform molecular species, suggesting that the light-chain mRNA is not *randomly* degraded from a much larger precursor. It further suggests that both variable and constant regions are encoded in a single mRNA molecule (33), and that assembly of the light chain does not occur via post-translational joining of these regions (see ref. 34). While it is difficult to estimate molecular weights of RNAs from sedimentation coefficients obtained under conditions in which the RNA has ordered structure, we can roughly estimate the molecular weight of the light-chain mRNA by a comparison with the molecular weight values determined for similarly analyzed RNAs (35). Such a comparison suggests that the light-chain mRNA is about 850 nucleotides long. This is about 200 nucleotides longer than would be necessary to encode more than one light-chain molecule. If these results can be taken as representative of the mechanism of antibody synthesis, they tentatively suggest that antibody protein subunits are synthesized from poly(A)-containing, monocistronic mRNAs that encode both variable and constant regions.

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