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Purification of 14S Messenger RNA of Immunoglobulin Light Chain That Codes for a Possible Light-Chain Precursor

(poly(dT) chromatography/mRNA molecular weight/mRNA translation/mouse myeloma)

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ABSTRACT **Polysomes released from microsomes of MOPC 41 mouse myeloma were used to prepare a poly(A)-containing fraction of RNA by chromatography on poly-(dT)-cellulose. From that fraction, a 14S RNA species was purified to a single peak by successive sucrose gradient centrifugations, followed by acrylamide gel electrophoresis. The RNA has an apparent molecular weight of 380,000 (1100 nucleotides), as estimated from the electrophoretic analyses. In a reticulocyte lysate this RNA directs the synthesis of a protein that migrates more slowly in sodium dodecylsulfate–acrylamide gels than does the light chain secreted by the same tumor. This difference in migration corresponds to a size difference appropriate for polypeptide chain about 20 amino acids longer than the light chain. The tryptic peptides of this protein correspond to those of the secreted light chain, except for the presence of two additional peptides from the product synthesized *in vitro* and for the absence of one light-chain peptide. The purified RNA is, therefore, the mRNA of the light chain, and it seems to code for a precursor protein slightly larger than the light chain. From the estimated size of the 14S mRNA, it appears that only 65% of the RNA is translated.**

Only two specific mRNAs have been purified to the point where they show a single peak by gel electrophoresis and code for a specific protein in cell-free extracts, those of hemoglobin (1) and silk fibroin (2). These mRNAs were prepared from highly specialized tissues, where they represent the predominant, and perhaps the single, mRNA species. In the case of cells that synthesize immunoglobulins, crude RNA fractions capable of directing the synthesis of immunoglobulin chains have been obtained from microsomes (3, 4), whole cytoplasm, and even nuclei (5). Fractionation procedures selecting for poly(A)-rich RNA, as used by Swan *et al.* (4) and Stevens and Williamson (5), have been especially useful in this respect. However, no mRNA purified to give a single peak on sucrose gradient centrifugation or on gel electrophoresis has yet been obtained and shown to direct exclusively the synthesis of immunoglobulin chains.

In this study, RNA was extracted from polysomes released from microsomes of a mouse myeloma, MOPC 41, that synthesizes immunoglobulin light chains (kappa type). When this polysomal RNA is chromatographed on poly(dT)–cellulose (6), it is possible to obtain a fraction that contains large poly(A) sequences, as discussed in detail elsewhere (27). This poly(A)-rich RNA, prepared under conditions that minimize contamination by ribosomal RNA, has been further fractionated, and a particular RNA species was purified (14S RNA). 14S RNA gives a single peak when analyzed by sucrose gradient centrifugation or by gel electrophoresis. In a reticulocyte lysate this RNA directs the synthesis of a protein that contains all the tryptic peptides of the MOPC 41 L-chain, with one exception, and that migrates slightly slower than does secreted MOPC 41 L-chain in dodecyl sulfate–acrylamide gels. The product of the 14S mRNA might, therefore, represent a precursor of the light chain.

As reported elsewhere (28), this purified L-chain mRNA can be used with the RNA-dependent DNA polymerase of avian myeloblastosis virus to direct the synthesis of complementary DNA.

MATERIALS AND METHODS

Preparation of Polysomes. Growth of myeloma tumor MOPC 41 (a generous gift of Dr. M. Potter), homogenization, and preparation of a postmitochondrial supernatant were as described (7). All glassware and buffers were treated with diethylpyrocarbonate (Baycovin, Bayer), and sterile precautions were used throughout. Microsomes were sedimented for 20 min at 30,000 rpm (Spinco 50 Ti rotor) through a 2-ml cushion of 0.8 M sucrose in Medium A-100 (50 mM Tris·HCl, pH 7.6–100 mM KCl–5 mM $MgCl_2$, ref. 8). After gentle resuspension of microsomes in Medium A-100 with a magnetic stirrer, 1 volume of liver postribosomal supernatant (8) from young rats and Nonidet P-40 (Shell) (final concentration 1.2%) were added. Released polysomes were collected by sedimentation at 50,000 rpm (Spinco 50 Ti rotor) for 3 hr through a 4-ml cushion of 2 M sucrose in Medium A-100, resuspended in the same medium, and stored at −75°. 100 A_{260} units of released polysomes were obtained from about 7 ml of tumor.

Extraction and Purification of RNA. Polysomes were diluted with H_2O to a concentration of 20 A_{260} per ml (KCl, final concentration 20 mM); Proteinase K (chromatographically pure, Merck) (200 μg/ml) and Na dodecyl sulfate (0.2%) were added, and the sample was incubated at 0° for 10 min. Then, 0.1 volume of 1 M Tris·HCl (pH 9) and 0.1 volume of 10% Na dodecyl sulfate were added, and RNA was extracted with H_2O-saturated phenol and precipitated as described (9). Polysomal RNA was applied to a column of poly(dT)–cellulose (6) in 0.5 M KCl at 30° and the column was washed with 50 mM KCl. The poly(A)-containing RNA fraction (about 2.5%) was then eluted with H_2O and precipitated with 0.2 M sodium acetate and 2.5 volumes of ethanol (27). This RNA was further fractionated on sucrose gradients [5–20% sucrose in 0.05 M sodium acetate (pH 5.5)] in a Spinco SW41 rotor at

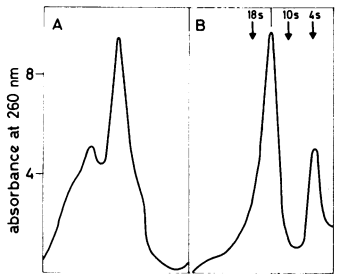

FIG. 1. Sedimentation profiles of RNA fractionated on sucrose gradients. (*A*) Poly(A)-rich RNA fraction obtained from myeloma polysomal RNA by poly(dT)-cellulose chromatography (see *Methods*) centrifuged for 13 hr. The position of the small peak (*left*) corresponds to 18S ribosomal RNA. (*B*) Major RNA peak from gradient *A*, precipitated with ethanol in the presence of 4S RNA and centrifuged for 12 hr. The positions of 18S and 10S RNA (*arrows*) were obtained from a parallel centrifugation of RNA fractions of rabbit reticulocyte polysomes.

40,000 rpm. RNA was also extracted from rabbit reticulocyte polysomes (10) as described above, and 28S, 18S, 10S, 5S, and 4S RNA, obtained from sucrose gradients (SW27 rotor), were prepared as markers. Electrophoresis in 3% acrylamide gels was as described (11). All RNA samples were treated with 75% dimethylsulfoxide (Merck) for 18 min at 37° (12) just before application to the gels. In some cases the peak myeloma RNA fraction (Fig. 2, *top*) was eluted, after slicing, by diffusion into 20 mM sodium acetate (pH 5.0)–0.5% Na dodecyl sulfate, for 24 hr at room temperature in the presence of carrier tRNA. The RNA was recovered by two successive ethanol precipitations, and the insoluble residue was discarded.

Translation in a Reticulocyte Lysate. Preparation of the lystate and incubation conditions were as described by Lingrel (10). The label was either [^{35}S]methionine (more than 100 Ci/mmol, a gift of R. Gesteland and B. Hirt), [^{14}C]leucine (342 Ci/mol) or [^{3}H]leucine (55 Ci/mmol), both from Amersham. 0.1 ml of incubation mixture contained 0.2 A_{260} unit of 14S RNA; incubation was for 40 min at 37°.

Analysis of the Cell-Free Product. The incubation mixture was treated for 20 min at 37° with 50 μg/ml of RNase (electrophoretically pure, Worthington) and analyzed either directly or after exposure to an immunoadsorbant, i.e., Sepharose coupled (13) to a rabbit antibody prepared against purified mouse kappa chain. The immunoadsorbant was stirred for 1 hr at 25° in 3 volumes of incubation mixture, then washed three times with Medium A-100, once with 0.5 M KCl, and once with 0.05 M Tris·HCl (pH 7.6). The material adsorbed by the Sepharose was released by incubation in the sample buffer used for the gel electrophoresis (14), 3% Na dodecyl sulfate–5% 2-mercaptoethanol–5% glycerol–0.05 M Tris·HCl (pH 6.8).

Acrylamide Gel Electrophoresis. All samples were incubated for 3 min at 90° in the sample buffer (14) just before electrophoresis, which was performed with the stacking gel procedure of Laemmli (14) in 12.5% acrylamide. After they were stained with Coomassie blue and destained, either the gels were sliced longitudinally, dried, and used for radioautography, or a 2-mm slice containing band A (Fig. 3) was removed and the protein was eluted in 0.05 M NH_4HCO_3–0.1% dodecyl sulfate–60 μg/ml bovine-serum albumin for 48 hr at 35°, with the pH maintained at about 7–8 (B. Hirt, personal communication). The protein was precipitated with 10% Cl_3CCOOH, dissolved in 0.05 M NH_4HCO_3, and washed several times with Cl_3CCOOH, then with alcohol–ether 2:1 and with ether.

Column Chromatography of Tryptic Peptides. Samples were reduced and alkylated, dialyzed, digested with trypsin (TPCK-treated, Worthington), and lyophilized as described (7). Chromatography on a 0.9 × 60 cm column of Dowex 1 × 2 was performed at 37° with a 9-chamber gradient of pyridine–acetate buffer (4). The flow rate was 20 ml/hr, and 3.5-ml fractions were collected, dried, suspended in 0.2 ml of 5 mM HCl, and counted in a Beckman LS-250 liquid scintillation counter, equipped with automatic quench correction. Corrections were made for spillover in double-label experiments, of counts into the other channel.

Preparation of Secreted, Labeled MOPC 41 L-Chains. Small tumor fragments were incubated in suspension for 3 hr (7) with either [^{35}S]methionine, [^{3}H]leucine, or [^{14}C]leucine. After centrifugation of the fragments and dialysis of the supernatant, acrylamide gel analysis of the culture fluid showed a single radioactive band ("*L*" in Fig. 3). Labeled L-chains were also purified from the dialyzed culture supernatant by DEAE-cellulose chromatography according to Potter (15).

RESULTS

Purification of 14S RNA

When RNA extracted from polysomes released from microsomal membranes is fractionated on poly(dT)–cellulose, a poly(A)-containing RNA fraction is obtained. When this fraction is sedimented through a sucrose gradient, a major peak of RNA is observed that is slightly lighter than 18S rRNA, and that represents 40–50% of the RNA analyzed (Fig. 1*A*). When this major peak is isolated and centrifuged on a second sucrose gradient, a single symmetrical RNA peak is obtained (Fig. 1*B*). It has an S value of about 14S, as compared with 18S, 10S, and 4S RNA from rabbit reticulocyte polysomes. The position of the 14S RNA peak coincides with

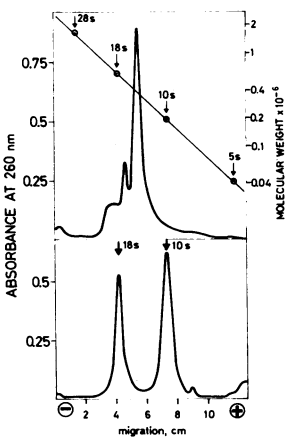

FIG. 2. Acrylamide gel electrophoresis of RNA fractions. *Top:* Electrophoretic pattern of the 14S RNA peak obtained after successive sucrose gradients (Fig. 1*B*). After electrophoresis (see *Methods*), the gels were scanned for absorbance at 260 nm. The position of migration of several RNA markers from rabbit reticulocyte polysomes are indicated in the *top* (⊙——⊙) and *bottom* (*arrows*) figures. Molecular weights of these RNA markers are as determined by others (16).

the position of a minor RNA species from reticulocyte polysomes (data not shown) that has also been observed by others (16, 17).

Analysis of this 14S RNA peak by acrylamide gel electrophoresis (Fig. 2) shows that it consists mainly of a sharp symmetrical peak. Since the distance of migration is directly proportional to the log of the molecular weight (18–20), it is possible to obtain an estimated molecular weight of 380,000 for the 14S RNA of myeloma by comparison with known RNA markers (Fig. 2). A smaller peak is also observed that is slightly heavier than 14S RNA, but is clearly not identical to 18S ribosomal RNA. This RNA peak has not been characterized.

Cell-free translation of 14S RNA into L-chain

The purified 14S RNA peak, obtained either from the second sucrose gradient (Fig. 1*B*) or from gel electrophoresis (Fig. 2), was tested for its ability to direct the synthesis of proteins *in vitro* in a reticulocyte lysate (14); [^{35}S]methionine was used to label the products. The radioactive proteins synthesized were analyzed by acrylamide gel electrophoresis, followed by radioautography. In the absence of added RNA, the lysate preferentially synthesizes hemoglobin and, in addition, discrete protein bands of high molecular weight (Fig. 3). The middle portion of the gel is free of radioactive proteins. In the presence of 14S RNA, a major additional polypeptide chain (Band *A* in Fig. 3) is synthesized. The added 14S RNA competes with the endogenous mRNA and hemoglobin synthesis is markedly depressed, as can best be seen with short exposure times of the radioautograph (Fig. 3-*1*). The 14S RNA obtained either from the sucrose gradient or from gel electrophoresis directs the synthesis of the same product (Fig. 3-*2*). When radioautographs are exposed for longer times, three minor protein bands can be seen (*B*, *C*, and *D* in Fig. 3) that are also synthesized exclusively in response to 14S RNA.

When the lysate incubated with 14S mRNA is exposed to Sepharose coupled to rabbit antibody against mouse kappa chains, the radioactive material eluted corresponds to the polypeptide chains specifically synthesized in response to 14S mRNA (Fig. 3-*3*). However, this result does not formally demonstrate that these bands possess the antigenicity of mouse kappa chain, since after exposure of the same lysate to an unrelated immunoadsorbant (Sepharose coupled to rabbit antibody specific for mouse lambda chain), a small amount of nonspecific binding of the same protein bands is also observed. Nevertheless, use of the immunoadsorbant removes most of the endogenous proteins of the lysate. This procedure allows a comparison of the 14S mRNA product with an internal L-chain marker without overloading the acrylamide gel (Fig. 3-*4*), (see below).

When the migration of band *A* is compared with that of a labeled L-chain marker ("*L*") on parallel gels, band *A* consistently migrates more slowly than does L-chain. However, electrophoretic mobility is influenced by the amount of protein loaded on a gel; therefore, a better comparison is obtained by the use of an internal marker. This comparison was done with either unlabeled L-chain marker (identified by staining) (Fig. 3-*4*, *left*) or labeled L-chain marker (Fig. 3-*4*, *right*), added to the cell-free product. This experiment shows unequivocally that band *A* has a slightly slower mobility than does L-chain. From this mobility, the molecular weight of band *A* can be estimated to be 26,000 (Fig. 3-*4*), which is

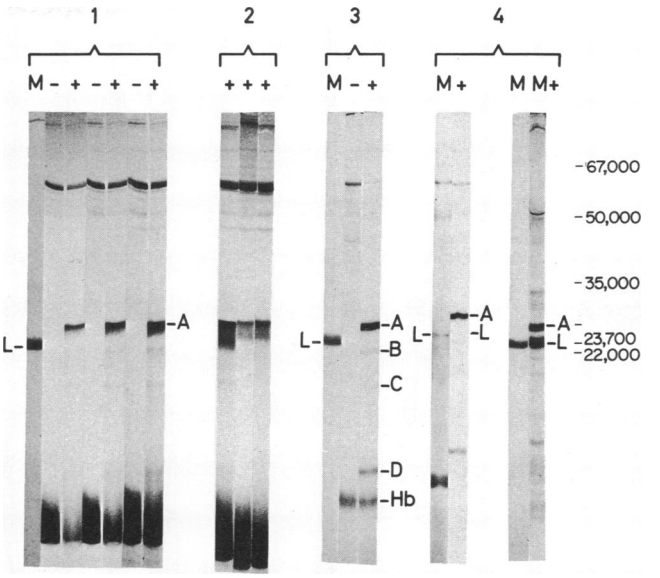

FIG. 3. Radioautographs of dodecyl sulfate-acrylamide gel electrophoreses comparing labeled marker L-chains ("*M*") [see *Methods*] with the products made in the reticulocyte lysate in the absence (−) or presence (+) of 14S poly(A)-rich RNA from myeloma. In all instances, [^{35}S]methionine was the labeled amino acid. (*1*). 30-μl Aliquot of the lysate incubated without (−) and with (+) 14S RNA obtained from sucrose gradients (Fig. 1*B*). Exposure time, from *left* to *right:* 12 hr, 1 day, and 2 days. (*2*). 30-μl Aliquot of the lysate incubated with 14S RNA obtained from the sucrose gradient peak (*left*), and from the peak of the gel electrophoresis (*right*, two different incubations). (*3*). Material from an incubated lysate retained by Sepharose-conjugated antibody prepared against kappa chains after repeated washing, released as described in *Methods* (*4*). Same as (*3*) except that internal MOPC 41 L-chain markers were run in the same gels *Left* gel pair: nonradioactive purified MOPC 41 L-chain (5 μg) was analyzed together with the labeled cell-free product, and the figure shows the results of staining (*left*) and of radioautography (*right*) of the same gel. *Right* gel pair: labeled marker L-chain analyzed either alone (*left*) or together with the labeled cell-free product (*right*). The proteins used to calibrate the molecular weight scale were bovine serum albumin (67,000), the heavy chain of IgG (50,000), pepsin (33,000), light chain of MOPC 41 (23,700, calculated from the aminoacid sequence) (21), and chymotrypsinogen (22,000).

2300 daltons, or about 20 amino acids, larger than MOPC 41 L-chain.

Tryptic peptides of ^{3}H-labeled L-chain marker were analyzed by chromatography on Dowex 1. Mixtures of ^{14}C- or ^{3}H-labeled band *A* and ^{3}H- or ^{14}C-labeled L-chain marker were similarly analyzed, with an excess of ^{3}H over ^{14}C counts added. The results showed an exact coincidence between the peptides of purified band *A* and of secreted L-chain (Fig. 4) with the following exceptions: the presence of two additional cell-free peptides not found in the L-chain peptides (around fractions 81 and 95), and the absence, in the cell-free peptides, of an L-chain peptide (around fraction 87). This result was observed with both combinations of ^{3}H and ^{14}C label, *in vitro* and *in vivo*.

DISCUSSION

The approach chosen for the purification of L-chain mRNA was based on the following considerations: (*i*) Membrane-

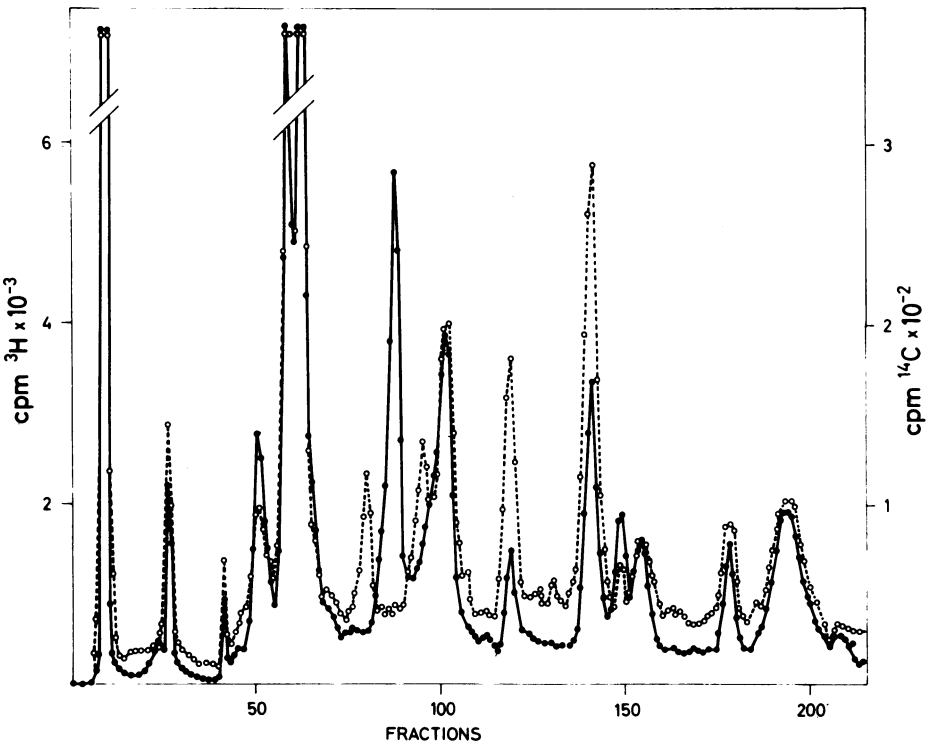

FIG. 4. Chromatography on Dowex 1 × 2 of the tryptic peptides obtained by digestion of a mixture of [^{3}H]leucine-labeled, secreted L-chain (92,000 cpm) and [^{14}C]leucine-labeled, purified band *A* from a cell-free incubation in the presence of 14S RNA (9000 cpm). ●——●: ^{3}H, L-chain. O- - -O: ^{14}C, band *A* from a cell-free incubation. The large peak around fraction 65 was found as a smaller single peak in other experiments involving the secreted L-chain alone or together with the cell-free product.

bound polysomes were selected for the extraction of RNA, because immunoglobulins are synthesized mainly on polysomes bound to the endoplasmic reticulum (22). The isolation of microsomes by sedimentation through a cushion of dilute sucrose reduces the contamination by free polysomes. (*ii*) Rather than extraction of RNA directly from microsomes, polysomes were first purified by releasing them from the microsomal membranes, since preliminary experiments had shown a high degree of RNA breakdown in RNA extracted directly from microsomes. The procedure chosen for the release and recovery of membrane-bound polysomes gives an optimal profile on sucrose gradients. (*iii*) Polysomes were incubated with Proteinake K at 0° before phenol extraction, since this procedure eliminated protein aggregation at the phenol interface, increasing the yield of RNA. It may also destroy any latent RNase present on polysomes. (*iv*) The use of poly(dT)–cellulose chromatography permits rapid purification of the small percentage of RNA that contains poly(A) sequences. The combination of all these steps results in an RNA fraction that shows no evidence of degradation and that contains predominantly 14S mRNA. Purification of this 14S RNA by successive sucrose gradients and gel electrophoresis is then relatively simple: when the procedure is used on a large scale (150 ml of tumor tissue), it yields mg of 14S RNA.

Purified 14S RNA is translated in a reticulocyte lysate, and the synthesis directed by 14S RNA takes place at the expense of the synthesis of endogenous proteins. The major translational product of 14S mRNA migrates slightly more slowly than do light chains in dodecyl sulfate gels; no protein with a mobility identical to L-chains is synthesized. However, from the correlation between the tryptic peptides of the product of 14S RNA and the peptides of the secreted L-chains, we can conclude that 14S RNA is the mRNA of the MOPC 41 L-chain. Swan *et al.* (4), using an RNA fraction from MOPC 41 microsomes prepared by poly(dT)–cellulose chromatography that showed a heterogenous pattern of sedimentation on sucrose gradients have described, in an ascites extract, the synthesis of several polypeptide chains whose peptides appear to correspond to those of L-chain. One of these chains presumably migrated to the same position as did L-chain on dodecyl sulfate gels, but the major product appeared to migrate slightly more slowly (estimated molecular weight, 24,500), although exact estimation is difficult without an internal marker in the same gel.

A difference in migration during dodecyl sulfate–gel electrophoresis does not necessarily reflect a difference in polypeptide length, since there is evidence that the addition of carbohydrate to immunoglobulin chains slows their migration in dodecyl sulfate–acrylamide gels (23). The present results cannot formally exclude the possibility that the reticulocyte lysate adds carbohydrate to the L-chain, or modifies the polypeptide structure in some way unrelated to its aminoacid composition. However, the observation that an L-chain peptide is missing in the cell-free product, and that two extra peptides are present, strongly suggests some difference in aminoacid composition, and is compatible with a greater length of the polypeptide chain. The unexpectedly large product of L-chain mRNA in the reticulocyte lysate might, therefore, correspond to an L-chain precursor, cleaved in myeloma cells but not in the reticulocyte extract. Biosynthesis of a longer precursor molecule occurs in the case of insulin (24) and of collagen, where it has been suggested that the additional amino acids are located at the amino-terminal end of the molecule (25). It would be of interest to investigate whe-

ther the additional peptide length is a common property of proteins synthesized on polysomes bound to the endoplasmic reticulum. Experiments in which 14S RNA was translated in *Xenopus laevis* oocytes show that the product synthesized under these conditions has the same size as L-chain. This result would be compatible with the existence of a cleavage enzyme in the oocyte that is absent from the reticulocyte lysate.

Minor bands of smaller molecular weight were observed when the product of 14S RNA was analyzed on gels. Smaller polypeptide chains were also observed when MOPC 41 myeloma RNA was translated in an ascites extract (4). These bands could result either from post-translational cleavage of the main product, or from premature termination of the synthesis directed by 14S mRNA.

From the gel electrophoretic analysis the molecular weight of L-chain mRNA was estimated to be 380,000 (about 1100 nucleotides). This estimation is limited by the possible effect of secondary structure of RNA on the rate of migration. Nevertheless, the value found for 14S RNA compares well with that of 360,000 found by others (16) for the "15S RNA" species of rabbit reticulocytes that coincides on sucrose gradient centrifugation with L-chain mRNA. If the primary translation product of myeloma 14S mRNA is about 235 amino acids long (Fig. 3), the portion of L-chain mRNA that is translated (700 nucleotides) represents about 65% of the entire 14S RNA. The remaining untranslated 400 nucleotides correspond roughly to the size (7S) of the RNase-resistant. poly(A)-rich fragment found in myeloma polysomal RNA (27). These two portions could account for the entire mRNA sequence. However, the existence of a short untranslated sequence, distinct from the poly(A)-rich fraction, cannot be ruled out.

The purified L-chain mRNA has been used as a template for the synthesis of complementary DNA with RNA-dependent DNA polymerase from avian myeloblastosis virus (28). Since the complementary DNA is made from purified L-chain mRNA, it will be useful for hybridization experiments involving light-chain-specific genes.

Since completion of this manuscript, Milstein *et al.* (26) have reported, in the case of another myeloma tumor (MOPC 21), that the cell-free product made either by myeloma polysomes released from microsomes, or by a reticulocyte lysate in the presence of myeloma RNA, is also of slightly slower electrophoretic mobility than secreted L-chains. As described here, this finding is compatible with the synthesis of a L-chain precursor that is subsequently cleaved. This additional observation renders more likely the possibility that the phenomenon observed is a general one, at least in the case of immunoglobulins.

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