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Purification of Biologically Active Globin Messenger RNA by Chromatography on Oligothymidylic acid-Cellulose

(poly(A)-rich mRNA/rabbit globin mRNA/ascites tumor cell-free system)

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ABSTRACT **A convenient technique for the partial purification of large quantities of functional, poly(adenylic acid)-rich mRNA is described. The method depends upon annealing poly(adenylic acid)-rich mRNA to oligothymidylic acid-cellulose columns and its elution with buffers of low ionic strength. Biologically active rabbit globin mRNA has been purified by this procedure and assayed for its ability to direct the synthesis of rabbit globin in a cell-free extract of ascites tumor. Inasmuch as various mammalian mRNAs appear to be rich in poly(adenylic acid) and can likely be translated in the ascites cell-free extract, this approach should prove generally useful as an initial step in the isolation of specific mRNAs.**

Various questions regarding the expression of genetic information in higher organisms depend upon the detection and isolation of gene-specific messenger RNA (mRNA). In contrast to bacterial systems, in which transducing phage simplify these procedures, isolation and detection of mRNA in animal cells has relied largely upon studies of the kinetics of radioactive labeling of polysomal RNAs and upon techniques that exploit differences in their molecular weights. While these techniques are useful, methods that take advantage of other unique properties of mRNA should prove of value as well.

Putative mRNAs isolated from animal cells differ from other RNA species in that they contain relatively long stretches of adenylic acid residues (1–4). The precise role of these poly(A)-rich regions in the metabolism of mRNA is not known, but it has been suggested that they are involved in the transport of mRNA from the cell nucleus to the cytoplasm, where protein synthesis occurs (4–5). Several workers have used the binding of poly(A)-rich RNA to oligo-(dT)-cellulose (6), to poly(U)-cellulose (7), or to nitrocellulose filters (4, 8) to detect poly(A) regions or putative mRNAs. Inasmuch as rabbit globin mRNA contains poly(A)-rich regions (1, 8, 9), it seemed likely that this feature, together with a highly-sensitive assay for the biological activity of the mRNA (10), would prove useful in the preparative purification of this and other biologically active mRNAs.

In the present work, we show that oligo(dT)-cellulose chromatography can be used conveniently to separate globin mRNA from the bulk of ribosomal RNA in crude polysomal extracts. Oligo(dT)-cellulose seems to have several unique advantages for this purpose. Purification of the globin message in this and subsequent steps is greatly facilitated by a sensitive assay for the *in vitro* synthesis of rabbit globin.

Abbreviation: SDS, sodium dodecyl sulfate.

MATERIALS AND METHODS

The sources of many of the reagents used in this study have been indicated (10). For the synthesis of oligo(dT)-cellulose, Whatman cellulose powder CC 41 was obtained from Reeve Angel; thymidine-5′-monophosphate, from Schwarz-Mann; and *N,N′*-dicyclohexylcarbodiimide, from Aldrich. [^{3}H]-Leucine (specific activity, 26 Ci/mmol); [^{14}C]leucine (specific activity, 263 Ci/mol) and [^{14}C]valine (specific activity, 219 Ci/mol), from New England Nuclear; [^{14}C]lysine (specific activity, 312 Ci/mol), from Amersham, [^{14}C]alanine (specific activity, 160 Ci/mol) from Schwarz BioResearch. Rabbit liver transfer RNA (tRNA) was obtained fom General Biochemicals. Trypsin ("chymotrypsin-free") was obtained from Worthington. Chromo-Beads cation exchange resin, type P, was obtained from Technicon.

Preparation of Oligo(dT)-Cellulose. Oligo(dT)-cellulose was prepared according to Gilham, using the *N,N′*-dicyclohexylcarbodiimide reaction for the polymerization of thymidine-5′-monophosphate on cellulose (11). One gram (dry weight) of oligo(dT)-cellulose prepared in this way retained and was saturated by about 160–200 μg of poly(A) under the conditions noted below.

Preparation of Rabbit Globin mRNA. Rabbit reticulocyte polysomes were isolated by described procedures (12). RNA was extracted from the polysomes by a modification of the procedure described by Lee, Mendecki, and Brawerman (4). The polysomes were suspended in 0.1 M Tris·HCl (pH 9.0)–0.1 M NaCl–1 mM EDTA at a concentration of 20 A_{260} units/ml and then were made 1% in sodium dodecyl sulfate (SDS). An equal volume of phenol–chloroform–isoamyl alcohol 50:50:1 was added, the mixture was shaken vigorously for 10 min at room temperature and chilled to 5°, and the phases were separated by centrifugation at 12,000 × *g* for 10 min. The aqueous phase was removed, extracted again as above, and made 2% in CH_3COOK (pH 5.5). Crude polysomal RNA was precipitated by the addition of two volumes of ethanol and was allowed to stand at −20° overnight. The RNA was collected by centrifugation at 12,000 × *g* at −20° for 20 min. The RNA pellet was washed twice with ethanol–0.2 M NaCl 2:1 and dissolved in H_2O or, if oligo(dT)-cellulose chromatography was to be used, in 0.01 M Tris·HCl (pH 7.5)–0.5 M KCl.

Oligo(dT)-Cellulose Chromatography. All operations were done at room temperature with glassware and reagents [except for oligo(dT)-cellulose] that had been autoclaved. 100 A_{260} units of crude rabbit reticulocyte polysomal RNA dissolved in application buffer containing 0.01 M Tris·HCl (pH 7.5)–0.5 M KCl was applied to a 2-ml (about 0.5 g, dry weight) oligo(dT)-cellulose column previously washed with application buffer. The nonabsorbed material was eluted by continued washing with the application buffer. The material retained by the column was eluted in two steps with buffers of reduced ionic strength (13). The first elution buffer contained 0.01 M Tris·HCl (pH 7.5)–0.1 M KCl; the second, 0.01 M Tris·HCl (pH 7.5). The material eluted in this way was immediately precipitated by the addition of CH_3COOK and two volumes of ethanol as noted above, or, if sufficiently concentrated, it was frozen directly and stored in liquid nitrogen. The procedure can be scaled up 20-fold to accommodate 2000 A_{260} units of polysomal RNA without difficulty, and the oligo(dT)-cellulose can be regenerated for repeated use by elution with 0.1 M KOH.

Cell-Free Protein Synthesizing Systems and Assays. A cell-free protein synthesizing system (S30) that was tRNA dependent was prepared from Krebs II ascites tumor cells (10). Globin mRNA was assayed in 0.06-ml reaction mixtures that contained 30 mM Tris·HCl (pH 7.5), 3.5 mM magnesium acetate, 100 mM KCl, 7 mM 2-mercaptoethanol, 1 mM ATP, 0.1 mM GTP, 0.6 mM CTP, 10 mM creatine phosphate, 8 μg creatine kinase, 40 μM (each) of 19 nonradioactive amino acids, 5 μM radioactive amino acid of indicated high specific activity ([^{3}H]leucine or [^{14}C]leucine, [^{14}C]valine, [^{14}C]lysine, and [^{14}C]alanine as indicated), 0.29 A_{260} units $tRNA_{rabbit}$, 15 μl of previously incubated extract of ascites tumor cells containing 14 mg/ml of protein, and mRNA, as indicated. Incubation was at 37° for 60 min. Reactions were stopped by the addition of 0.2 ml of 0.1 M KOH. Incubation was continued for 20 min, and 1 ml of ice-cold 10% Cl_3CCOOH was added. If the mixture was not to be used for analysis, it was cooled at 0° for 5 min, and the precipitate was collected over a 0.45-μm pore-size Millipore filter, washed three times with 3 ml (each) of 5% Cl_3CCOOH, dried, and counted in a liquid scintillation counter at an efficiency of 20–25% for ^{3}H and 50% for ^{14}C. All determinations were done in duplicate. Rabbit globin labeled with [^{14}C]leucine, used as a standard for tryptic peptide analysis, was synthesized in a rabbit reticulocyte lysate with endogenous rabbit globin mRNA (9).

ANALYTIC PROCEDURES

Sodium Dodecyl Sulfate (SDS)–2-Mercaptoethanol–Polyacrylamide Gel Electrophoresis. The products of reaction mixtures of ascites tumor cells containing [^{14}C]leucine, [^{14}C]-valine, [^{14}C]lysine, and [^{14}C]alanine were prepared and subjected to electrophoresis at 200 V for 5 hr in a slab containing a linear 7–28% gradient of polyacrylamide by procedures (14) virtually identical to those of Maizel (15). The dried slabs were exposed to x-ray film for 36 hr and developed.

Tryptic Peptide Analysis. The product of a reaction mixture directed by rabbit globin mRNA containing [^{3}H]leucine was made 0.1 M in ethylenediamine tetraacetic acid (EDTA), treated with 0.1 mg/ml of bovine pancreatic RNase, and incubated at 37° for 10 min. The product was then precipitated in 10% Cl_3COOH, and washed twice with a solution containing 5% Cl_3COOH, twice with acetone containing 0.035 M HCl and 3.5% (w/v) oxalic acid, and twice with acetone (9). The pellet (about 1 mg of protein) was suspended in 1 ml of 0.1 M NH_4HCO_3 (pH 8.0), 40 μg/ml of trypsin was added, and the mixture was incubated at 37° for 16 hr. Additional trypsin, 40 μg/ml, was added and the incubation was continued for 5 hr. The digested protein was lyophilized, dissolved in pyridine acetate (278 ml acetic acid, 16.1 ml pyridine, 705.9 ml H_2O; pH 3.1), and applied, together with similarly treated rabbit globin labeled with [^{14}C]leucine, to a 1 × 20-cm column containing Technicon type P cation exchange resin previously equilibrated in the same buffer. The product was eluted with a gradient consisting of 180 ml pyridine acetate (pH 3.1) and 180 ml pyridine acetate (139 ml acetic acid, 161 ml pyridine, 700 ml H_2O; pH 5.0) (16). Samples of about 1.8 ml were collected and ^{14}C and ^{3}H radioactivity was determined by scintillation counting.

Sucrose Gradient Sedimentation. RNA samples were layered on a 5–20% sucrose gradient containing 0.01 M Tris·HCl (pH 7.5)–0.1 KCl–0.001 M EDTA in an SW 27 rotor carrying the 1044 adapter bucket and centrifuged at 27,000 rpm for 17 hr. About 40 fractions were collected, and their A_{260} was determined. The ability of 0.005-ml aliquots to direct protein synthesis in the ascites tumor cell-free system was determined as noted above.

Polyacrylamide Gel Electrophoresis of RNA. Electrophoresis in a 4% polyacrylamide–0.5% agar slab gel was done at 75 V for 240 min (17). A 3-μg sample was applied to the gel and detected by the sensitive "stains all" technique (17).

RESULTS

Oligo(dT)-Cellulose Chromatography of Recticulocyte Polysomal RNA. As shown in Fig. 1, over 90% of the total polysomal RNA applied to the oligo(dT)-cellulose column was eluted with the application front and was not retained by the column (*peak A*). Subsequent studies, using more extensively washed columns, indicated that the amount of RNA retained was frequently less than 3% of the total RNA applied. When the KCl concentration of the elution buffer was reduced from

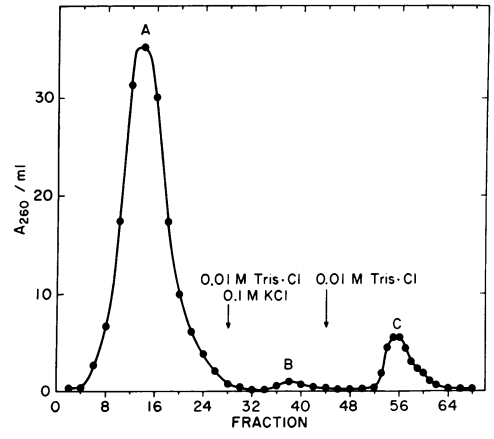

FIG. 1. Oligo(dt)-cellulose chromatography of reticulocyte polysomal RNA. *Peak A*, material that is not retained by the column; *peak B*, material eluted with the indicated buffer of intermediate ionic strength; *peak C*, material eluted with the indicated buffer of low ionic strength.

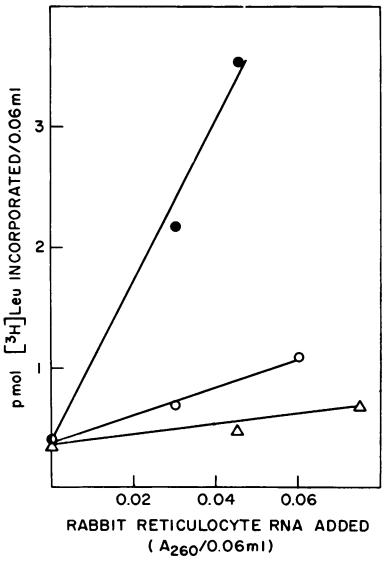

FIG. 2. Protein synthesis directed by reticulocyte RNA in the ascites tumor cell-free system. (●——●), RNA purified on oligo(dT)-cellulose (*peak C*, Fig. 1); (○——○), crude reticulocyte polysomal RNA; (△——△), reticulocyte RNA not retained by oligo(dT)-cellulose (*peak A*, Fig. 1).

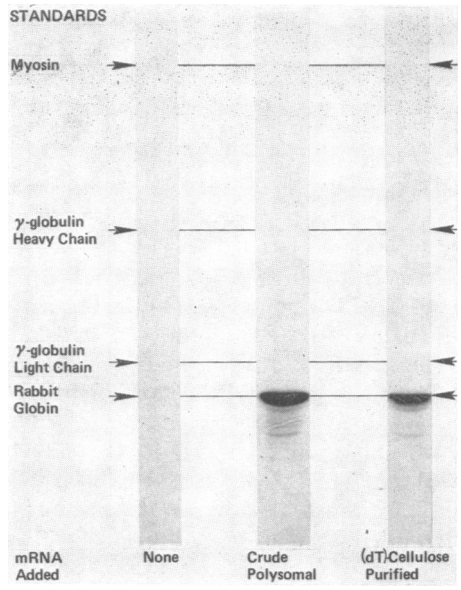

FIG. 3. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of products synthesized in the ascites tumor cell-free system. The RNA used to make each product is indicated. Comigration of unlabeled proteins is indicated at the margin of the figure.

0.5 to 0.1 M, a small amount of UV absorbing material was eluted (*peak B*), but when the ionic strength of the elution buffer was further reduced by omission of KCl, an additional and larger peak appeared (*peak C*). The two larger peaks were concentrated, and their ability to direct cell-free protein synthesis in the ascites tumor cell-free system was determined.

mRNA Activity of the Oligo(dT)-Cellulose Fractions and Characterization of Their Products. As indicated in Fig. 2, small amounts of crude reticulocyte RNA stimulated incorporation of [^{3}H]leucine into protein in the ascites tumor cell-free system. The material that was not originally retained by oligo(dT)-cellulose (*peak A*) was relatively inactive in stimulating the incorporation of [^{3}H]leucine into protein. In contrast, that material retained by oligo(dT)-cellulose and eluted at lowest ionic strength (*peak C*) was most active as a mRNA in the cell-free system. The very limited amount of material eluted at the intermediate ionic strength (*peak B*) was not tested for this purpose.

The protein synthesized in response to the two active RNA fractions has been characterized by SDS–polyacrylamide gel electrophoresis and by analysis of their tryptic peptides. The major product synthesized in response to crude reticulocyte polysomal RNA and RNA purified on oligo(dT)-cellulose (*peak C*) was a polypeptide of about 17,000 daltons that comigrated with rabbit globin on SDS–polyacrylamide electrophoresis (Fig. 3). In addition, small amounts of peptides lighter than rabbit globin appear in the presence of both polysomal RNA and RNA purified on oligo(dT)-cellulose. These constitute about 4% of the total counts on each gel. No labeled product was formed in the absence of mRNA (Fig. 3).

The product synthesized under the direction of the RNA purified on oligo(dT)-cellulose was treated with trypsin, and its peptides labeled with [^{3}H]leucine were compared by cation exchange chromatography to peptides labeled with [^{14}C]-leucine derived from globin formed endogenously in a rabbit reticulocyte lysate (Fig. 4). The elution profiles were coincident with respect to 22 peaks. All the reticulocyte-derived peaks have a counterpart in the profile of the ascites tumor cell-free product. Three peaks (fractions 41, 81, and 84) derived from the ascites tumor cell-free product, however, did not appear in the reticulocyte profile. The origin of these is not clear, but they may be related to unprocessed initiation peptides. Theoretically 17 tryptic peptides containing leucine can be derived from the α and β subunits of rabbit globin. Chromatography of trypsin-treated globin derived from the rabbit reticulocyte lysate yielded 23 peaks, including 4–6 minor ones. This degree of variation between theoretically expected product and result is consistent with prolonged action of trypsin, presumably contaminated with small amounts of chymotryptic activity, on relatively small amounts of substrate.

Characterization of the Purified Globin mRNA. The RNA eluted from the oligo(dT)-cellulose column at lowest ionic strength (*peak C*) was analyzed by sucrose gradient centrifugation (Fig. 5). This resolved the material into four UV-absorbing peaks with $s_{w,20}$ values corresponding to about 9, 12, 18, and 28. These gradient fractions were assayed for their ability to direct incorporation of [^{3}H]leucine into protein in the ascites tumor cell-free system. The major activity resided in the 9S fraction, but a small shoulder and an additional peak of mRNA activity appear under, but not precisely corresponding to, the 12S and 18S regions as well. The two upper fractions of the 9S peak were pooled, concentrated, and, together with other reticulocyte RNA fractions, subjected to polyacrylamide gel electrophoresis (Fig. 6). Reticulocyte polysomal RNA, before purification, (Fig. 6, *gel 2*) consisted largely of ribosomal RNA. 9S RNA does not appear as a stained band in this material, probably due to the fact that globin mRNA constitutes but a small proportion of the total

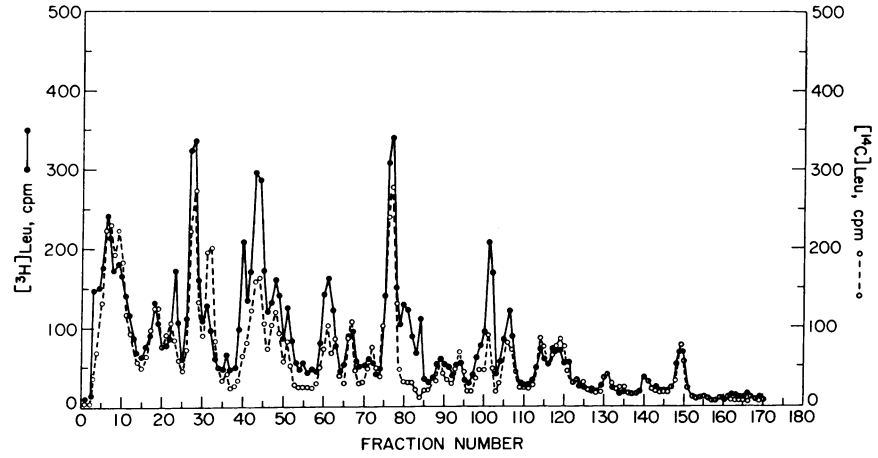

FIG. 4. Cation-exchange chromatography of protein derived from tryptic peptides synthesized *in vitro*. (●——●), [^{3}H]leucine in product synthesized in response to RNA purified on oligo(dT)-cellulose (*peak C*, Fig. 1) in the ascites tumor cell-free system; (○——○), [^{14}C]-leucine in product synthesized endogenously on rabbit reticulocyte polysomes.

RNA. In contrast, the RNA retained by the column and eluted at the lowest ionic strength (*peak C*) contained a major component corresponding to 9S RNA (Fig. 6, *gel 4*). RNA that was not retained by the oligo(dT)-cellulose column (Fig. 6, *gel 3*) consisted of 18 and 28S, the latter of which barely entered the 4% polyacrylamide gel. Two fractions of the absorbed RNA that were further fractionated by sucrose gradient centrifugation (Fig. 5) are shown in Fig. 6, *gels 1 and 5*. The 9S material (*gel 5*) migrates as a single homogeneous component and can be compared to the 18S material (*gel 1*). The 9S material was used as template in other studies directed toward synthesis of DNA complementary to rabbit globin mRNA (18).

DISCUSSION

Two major difficulties have complicated the isolation of gene-specific mRNA in higher organisms. The first of these has been the lack of a sufficiently sensitive, functional assay for a specific mRNA. The second has been the difficulty involved in freeing the small fraction of RNA that constitutes a gene-specific message from the bulk of intact and partially degraded ribosomal RNA. To overcome the first difficulty, we have used a cell-free protein synthesizing system derived from ascites tumor cells (10). Under the conditions we describe, the system is both a sensitive and convenient tool for detecting as little as 0.25 pmol of purified mRNA (Fig. 5) and exhibits a negligible amount of endogenous amino acid incorporation (Fig. 3). Furthermore, the system appears to translate both viral and host mRNAs, yielding authentic translation products (10, 14, 19–23), (Figs. 3 and 4).

The problem of the isolation of a small amount of specific mRNA from the bulk of cellular RNA is less easily overcome. Still, the observations that several putative mRNAs are

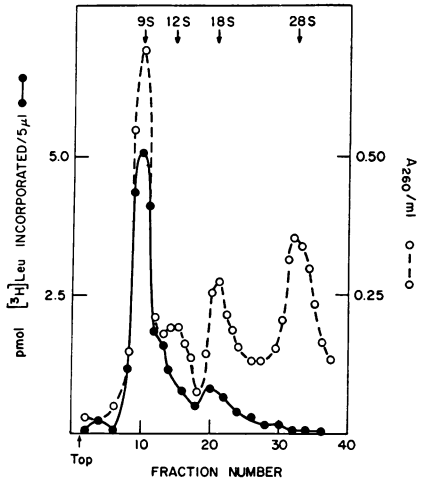

FIG. 5. Protein synthetic activity of RNA purified on oligo-(dT)-cellulose after sucrose gradient centrifugation. (●——●), [^{3}H]leucine incorporated in response to a 0.005-ml aliquot of each fraction in the ascites tumor cell-free system; (○——○), A_{260}/ml.

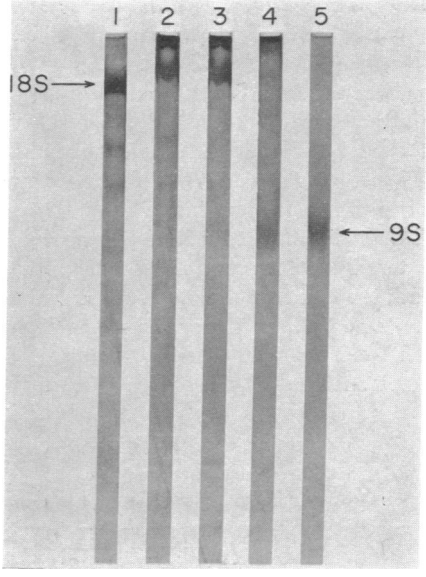

FIG. 6. Polyacrylamide (4%) gel electrophoresis of reticulocyte RNA fractions. *1*, 18S RNA (*fraction 21–22*, Fig. 5); *2*, total reticulocyte polysomal RNA; *3*, RNA not retained by globin mRNA purified on oligo(dT)-cellulose (*peak C*, Fig. 1); *5*, 9S globin mRNA (*fractions 10, 11*, Fig. 5).

rich in poly(A) sequences (1–4, 9) and that this property can be used for detection of such mRNAs quite naturally suggested its usefulness for preparative purposes as well (4, 7, 8). In this regard, oligo(dT)-cellulose column chromatography has certain advantages. The oligodeoxynucleotide is chemically stable and can be used repeatedly after treatment with alkali. These columns have a relatively high capacity and can be used to isolate several milligrams of poly(A)-mRNA from several hundred milligrams of crude polysomal RNA. With suitable precautions to avoid nuclease contamination, the procedure can be done at room temperature. In addition, the product isolated under these conditions retains its functional activity and is conveniently applied to small sucrose gradients for a further, and quite different, purification step. While it is not clear that all mammalian mRNAs contain poly(A)-regions, it is reasonable to expect that this procedure will prove generally useful in the isolation of various mammalian mRNAs. Our preliminary studies have already indicated its usefulness in the preparation of avian and human globin mRNA. Further refinements of the chromatographic procedure involving temperature and ionic strength gradient elution should prove useful as well.

During these studies, several interesting observations have emerged. Sucrose gradient centrifugation of the oligo(dT)-purified globin mRNA revealed a heavy (16–17S) peak of RNA with messenger activity (Fig. 5). In preliminary experiments, we find that protein synthesized with this mRNA is identical to that synthesized in the presence of 9S RNA. While it is possible that this material represents an aggregated form of 9S RNA, it is also possible that it represents a higher molecular weight form of the globin message. Maroun, Driscoll and Nardone (24) recently demonstrated a rapidly labeled, 17S RNA species that accumulates during avian reticulocyte starvation. Their experiments suggest that this RNA is not an aggregated form of 9S RNA and that, on relief of starvation, this species acts as a precursor of 9S RNA. Our evidence for the biologic activity of a similar RNA species in the rabbit suggests that the rabbit globin mRNA may exist in a high molecular weight form that is relevant to the metabolism of mRNA.

In addition, we have found that less than 5% of the product synthesized in the presence of purified globin mRNA consists of discrete polypeptides of molecular weight less than that of rabbit globin (Fig. 3). While it is possible that these proteins are unrelated to globin, it seems more likely that they represent discrete degradation products of the globin proteins or prematurely terminated nascent globin chains. This view is consistent with the fact that we have not seen minor proteins of molecular weight *greater* than that of globin when using purified mRNA of mammalian or avian source. In addition, Gesteland (personal communication) frequently observes discrete, premature termination products during the translation of phage mRNA in bacterial systems. Such premature termination might arise as a result of discrete cleavages in the mRNA during the protein synthetic reaction, or from arrests at specific codons in the globin mRNA. In any case, the very limited extent to which these occur and the discrete nature of the major product formed, suggest that the tRNA-dependent ascites tumor system in conjunction with purified globin mRNA should be useful for testing potential tRNA suppressors of mammalian termination codons.

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