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Synthesis of α and β Chains of Rabbit Hemoglobin in a Cell-Free Extract from Krebs II Ascites Cells

(CM-cellulose/tryptic peptides/Sephadex/10S RNA)

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ABSTRACT **An RNA sedimenting at 10 S, with a molecular weight of 2.3 × 10^5, was isolated from rabbit reticylocyte polyribosomes. When this RNA is added to a cell-free extract from Krebs II ascites cells, both α and β chains of rabbit hemoglobin are synthesized; β chains are made in about 50% excess over α chains.**

Isolation and characterization of the messenger RNA for α and β chains of rabbit hemoglobin has been reported by several groups (1–7). The informational content of this RNA has been assayed by adding it to a eukaryotic cell-free extract that was already synthesizing a homologous hemoglobin (3, 7) or myosin (5), or to a bacterial cell-free extract (4). The protein made in these extracts was analyzed by separation of the polypeptide chains into those whose synthesis was directed by endogenous messenger RNA and those whose synthesis was the result of added RNA. We report here the synthesis of both α and β chains of rabbit hemoglobin when 10S messenger RNA from rabbit reticulocytes is added to a cell-free extract from Krebs II ascites cells. This cell-free extract can be incubated to eliminate the synthesis of endogenous protein, while it retains the ability to respond to added viral RNA (8, 9). Our results extend the use of previously incubated extracts of Krebs II ascites cells to characterization of proteins encoded by isolated mammalian messenger RNAs.

METHODS

The 10S messenger RNA was prepared from rabbit reticulocytes by the method of Labrie (2). Polyribosomes were dissociated in EDTA, and the ribonucleoprotein particle that contained 10S messenger RNA was separated from ribosomal subunits on a sucrose density gradient (2). The RNA was then precipitated by adjusting the appropriate fractions to 0.1 M NaCl and 0.5% sodium dodecyl sulfate (SDS), followed by the addition of 3 volumes of ethanol. After 2 hr at −20°C, the precipitate was collected by centrifugation, dissolved in 0.1 M NaCl–0.5% SDS–1 mM EDTA–0.01 M Tris [pH 7.4 (SDS buffer)], layered over a 15–30% sucrose density gradient made in the same buffer, and centrifuged for 21 hr at 22°C and 27,000 rpm in the Beckman SW 27 rotor. The gradient was monitored for A_{260nm}; fractions containing RNA sedimenting at 10 S were pooled and precipitated with ethanol. The precipitated RNA was dissolved in SDS buffer and extracted with phenol–chloroform, as described by Penman (10). After precipitation with ethanol, the RNA was washed at least three times with ethanol at −20°C to remove SDS, dissolved in water at a concentration of 1 mg/ml, and stored at −70°C. 40 μg of purified 10S RNA was prepared from 10 mg of polyribosomes.

Maintenance of Krebs II ascites cells in mice has been described (8). A cell-free extract was prepared from these cells according to a method communicated to us by M. B. Matthews, and used by him and others (8, 9). Mice bearing tumors were killed 7–10 days after inoculation, and the ascites cells were removed from the peritoneal cavity into 0.146 M NaCl–35 mM Tris (pH 7.5). They were collected by centrifugation at 800 rpm (120 × *g*) for 2 min at 0°C in an IEC-PR6 centrifuge, and washed five times with the same buffer. All subsequent procedures were performed at 0°C unless otherwise noted. The pelleted cells were resuspended in 2.5 volumes of 15 mM KCl–1.5 mM Mg (acetate)$_2$–6 mM 2-mercaptoethanol–10 mM Tris (pH 7.5), allowed to swell for 5 min, and homogenized with 10–30 strokes of a glass Dounce homogenizer. After the addition of one-tenth volume of 1.2 M KCl–50 mM Mg (acetate)$_2$–60 mM 2-mercaptoethanol–0.20 M Tris [pH 7.5 (10 × medium K)], the homogenate was centrifuged 15 min at 30,000 × *g*. It was then incubated at 37°C for 40 min in 1 mM ATP, 0.2 mM GTP, 0.05 mM (each) of 20 amino acids, 2 mg/ml of creatine phosphate, and 20 International Units/ml of creatine phosphokinase (all supplied by Calbiochem) to reduce endogenous protein synthesis. This incubated extract was again centrifuged for 15 min at 30,000 × *g* and the supernatant was filtered through a column of Sephadex G-25 (Pharmacia) equilibrated with medium K. The fractions containing the highest concentration of excluded material were pooled, divided into small aliquots, and frozen at −70°C. This cell-free extract remained active for several weeks.

The 10S RNA was assayed in the following reaction mixture: 1 mM ATP, 0.2 mM GTP, 6 mM creatine phosphate, 50 IU/ml of creatine phosphokinase, 0.05 mM (each) of 20 amino acids (except the one used as a radioactive label), 0.1 M KCl, 5 mM Mg (acetate)$_2$, 20 mM Tris (pH 7.5), 6 mM 2-mercaptoethanol, 0.6 ml of cell-free extract/ml, and either 25 μCi/ml of [^{3}H]leucine (55 Ci/mmol) or 25 μCi/ml of [^{3}H]-tyrosine (50 Ci/mmol); both supplied by New England Nuclear. The reaction mixtures were incubated at 37°C for 40 min.

For the experiment described in Table 1, the reactions were stopped by the addition of 0.5 ml of 0.1 N NaOH and further incubated at 37°C for 10 min; 0.5 ml of 10% trichloroacetic acid was added and the samples were collected on fiberglass filters (Whatman GF/A, 25 mm diameter) and counted in a

toluene-based scintillation fluid in a Beckman Scintillation counter.

To examine the [^{3}H]leucine-labeled products by Sephadex G-75 chromatography, we first removed ribosomes from the 0.5-ml reaction mixture by centrifugation at 165,000 × *g* for 2 hr. The supernatant was treated with 100 μg of pancreatic RNase (Sigma Biochemicals) at 37°C for 30 min, and carrier globin was added. Total globin was prepared by acid–acetone precipitation, dissolved in 6 M urea–0.1 M acetic acid–2 mM 2-mercaptoethanol, and layered on a 1.5 × 50 cm column of Sephadex G-75 in the same buffer (11). Fractions of 0.35 ml were collected, and 10 ml of a scintillation fluid [composed of 5.3 g of butyl-PBD (Ciba pharmaceuticals), 600 ml of toluene, and 400 ml of ethylene glycol monomethyl ether] was added to alternate fractions. The samples were counted in a Beckman scintillation counter. The positions at which rabbit globin and blue dextran chromatographed were determined in a separate run through the same column.

Material eluting from the Sephadex column at the position of rabbit globin was pooled, dialyzed against 0.2 M formic acid–0.02 M pyridine–1 mM 2-mercaptoethanol, mixed with rabbit globin uniformly labeled with [^{14}C]leucine (a generous gift of Mr. M. Jacobs-Lorena), applied to a 0.9 × 23 cm column of carboxymethyl (CM) cellulose (CM-32, Whatman) and eluted as in ref. 12, except that the volumes used in the pyridine–formic-acid gradient were doubled. Fractions of 3 ml were collected; 2 ml of each fraction was added to 15 ml of Aquasol (New England Nuclear) and counted in a scintillation counter. All of the ^{3}H applied to the column was eluted.

Tyrosine-containing tryptic peptides were analyzed by paper ionophoresis. A 0.5-ml reaction mixture containing 10 μg of 10S RNA was incubated for 40 min at 37°C. Labeled protein was digested with trypsin and the peptides were isolated as described (12). [^{14}C]Tyrosine-labeled rabbit globin (50,000 cpm) was added immediately before digestion with trypsin. One-dimensional paper ionophoresis was performed at pH 6.5 in a Varsol-cooled tank for 2.5 hr at 4 kV, as described by Ingram (13). The paper was dried and cut into 1-cm strips. Each strip was treated with 1 ml 0.1 N NaOH for 15 min at 65°C in a vial, 10 ml of Aquasol was added, and the samples were allowed to stand for 24 hr before they were counted in a scintillation counter.

RESULTS

The 10S RNA was analyzed for purity on a 3.5% polyacrylamide gel (Fig. 1). It migrated as a single peak. The migration rate of 10S RNA was compared to that of marker RNAs on 2.6% polyacrylamide gels to determine the molecular weight by the method of Loening (14). A value of 2.3 × 10^5 was obtained, compared with values for the molecular weight of the messenger RNA for rabbit hemoglobin of 1.93 × 10^5 obtained by Labrie (2) and 2.2 × 10^5 obtained by Gaskill and Kabat (6).

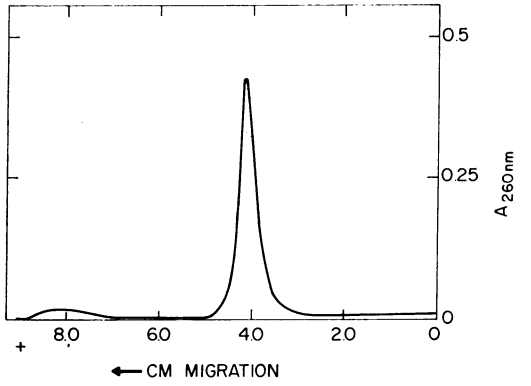

FIG. 1. Electrophoresis of 5 μg of purified rabbit reticulocyte 10S RNA on a 9-cm, 3.5% polyacrylamide gel for 4 hr at 10 V/cm, as described by Weinberg *et al.* (23).

The data in Table 1 show that rabbit 10S RNA can stimulate 10-fold the incorporation of tyrosine into protein by a cell-free extract from Krebs II ascites cells. When the reaction mixture contained 1 μg of 10S RNA (5 μmol), 0.3 μmol of globin chains were synthesized. RNA from mouse encephalomyocarditis virus also stimulates the incorporation of amino acids into protein in this cell-free extract (8, 9).

The products of the cell-free extract stimulated by 10S RNA were characterized in several ways. Fig. 2 shows that much of the [^{3}H]leucine-labeled material eluted from Sephadex G-75 at the same position as a marker of rabbit globin. The small amount of radioactively-labeled material that chromatographed with blue dextran was also present in the products isolated from a cell-free extract incubated without added RNA. The ^{3}H found in the last fractions of the included volume of the Sephadex column (Fig. 2) presumably represents free [^{3}H]leucine contaminating the isolated products. Addi-

TABLE 1. *Effect of added 10S RNA on [^{3}H]tyrosine incorporation by the cell-free extract*

RNA added	cpm [^{3}H]tyrosine incorporated
1 μg of rabbit 10S RNA	16,600
3 μg of rabbit 10S RNA	35,750
None	3,573

Reactions were performed in a reaction volume of 50 μl, containing about 4 μmol of ribosomes. 20,000 cpm of [^{3}H]tyrosine are equivalent to about 1 pmol of tyrosine.

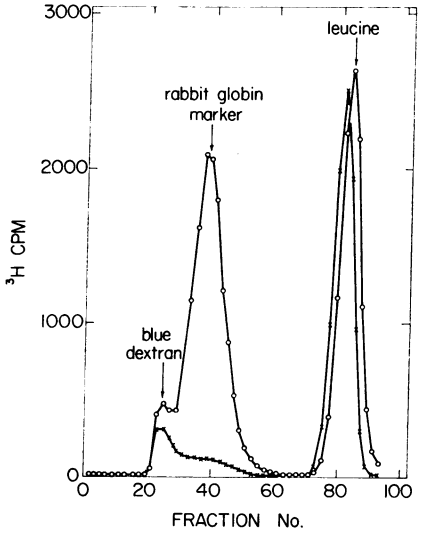

FIG. 2. Sephadex G-75 chromatography of the products of the cell-free extract. O—O, 10 μg of 10S RNA added. ×—×, no added RNA.

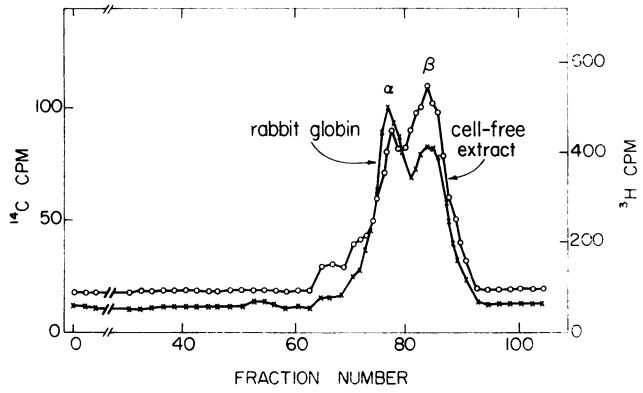

FIG. 3. CM-cellulose chromatography of the product of a cell-free extract with added 10S RNA. The material in fractions 32–50 of the Sephadex G-75 column illustrated in Fig. 2 was concentrated and chromatographed. O—O, [^{3}H]leucine in the product of the cell-free extract. ×—×, [^{14}C]leucine in an internal standard of rabbit globin.

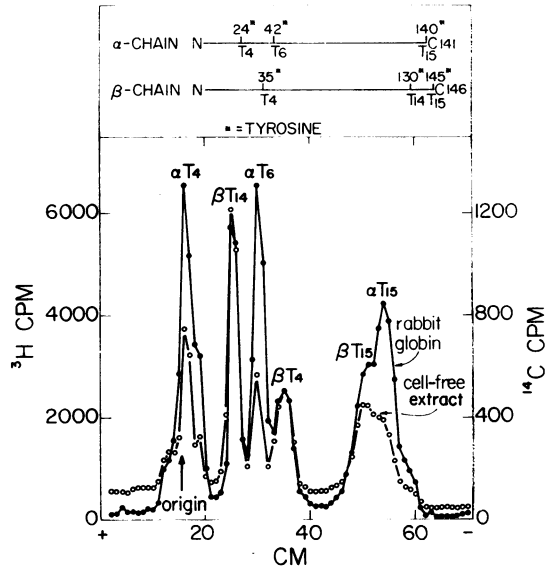

FIG. 4. Ionophoresis of the tryptic peptides from the product of the cell-free extract with added 10S RNA. The extract was incubated with 10 μg of added 10S RNA, and tyrosine-containing peptides were examined by paper ionophoresis at pH 6.5. The 10S RNA used in this experiment was prepared as in ref 3, and was the generous gift of Dr. G. Temple. O—O, [^{3}H]tyrosine in the product of the cell-free extract. ●—●, [^{14}C]tyrosine in an internal standard of rabbit globin.

tion of 10S RNA to the cell-free extract therefore resulted in the synthesis of a new product, which chromatographed as a single peak on Sephadex G-75 with a marker of rabbit globin.

We further analyzed the [^{3}H]leucine-labeled products of the cell-free extract stimulated with 10S RNA by chromatography of some of the material eluted from Sephadex G-75 on CM-cellulose in the presence of ^{14}C-labeled rabbit globin. The CM-cellulose column separates the α and β chains of rabbit globin (15). Fig. 3 shows that most of the product eluted coincidentally with α and β chains of rabbit globin. The ratio of β/α chains in the product is about 1.5:1, as compared with 1:1 in authentic globin.

As additional confirmation that both α and β chains are synthesized when 10S RNA is added to the cell-free extract we analyzed the tryptic peptides from the [^{3}H]tyrosine-labeled product. α and β chains each have three tyrosine-containing tryptic peptides [αT_4, αT_6, αT_{15} and βT_4, βT_{14}, βT_{15} in the nomenclature of vonEhrenstein (16)]; these six peptides are well separated by paper ionophoresis at pH 6.5 in one dimension (^{14}C cpm in Fig. 4). When the [^{3}H]tyrosine-labeled product from a cell-free extract stimulated with 10S RNA was added to [^{14}C]tyrosine-labeled rabbit globin, digested with trypsin, and electrophoresed, all six peptides contained ^{3}H (Fig. 4). If no RNA was added to the cell-free extract and a similar analysis was made no ^{3}H was present in any of these six peptides. The ^{3}H/^{14}C ratio is higher in the β peptides than in the α peptides, again suggesting that more β than α chains are produced when 10S RNA is added to this cell-free extract.

DISCUSSION

These experiments clearly demonstrate that a cell-free extract from Krebs II ascites cells can synthesize rabbit globin when 10S RNA from rabbit is added. The product of the cell-free extract stimulated with 10S RNA elutes with rabbit globin from a Sephadex G-75 column, elutes with the α and β chains of authentic rabbit globin from CM-cellulose, and has six tyrosine-containing peptides that coelectrophorese with those of authentic rabbit globin. Heywood (5) found that translation of chicken-globin messenger RNA by chick-muscle ribosomes was greatly enhanced by the addition of reticulocyte initiation factors, and suggested that highly differentiated tissues may effect translational control of protein synthesis by a requirement for tissue-specific initiation factors (5, 17, 18). Our experiments, however, show no absolute requirement for added initiation factors. (It should be noted that we did not wash the ribosomes with buffers that contained a high concentration of salt to remove any endogenous initiation factors that might have been present.) Stavnezer and Huang (19) recently demonstrated that RNA from a mouse myeloma can direct the synthesis of (mouse) immunoglobulin light-chains in a cell-free extract from rabbit reticulocytes; their extract also did not require the addition of initiation factors. Further study of the translation of specific messenger RNA molecules by cell-free extracts from various tissues will help to elucidate the role of tissue-specific factors in protein synthesis.

Rabbit reticulocytes synthesize essentially equal amounts of the α and β chains of hemoglobin (20–22). The experiments shown in Figs. 3 and 4 suggest that on addition of 10S RNA to the cell-free extract, more β than α chains are synthesized. This differential synthesis might be the result of more efficient recovery of messenger RNA coding for β chains than that coding for α chains when we prepare 10S RNA, or it could be a characteristic of the cell-free extract.

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