IDENTIFICATION OF GLOBIN mRNA IN 10s RNA OF RABBIT RETICULOCYTES

by Kazazian, Haig H., Jr., Moore, Patricia A., and Snyder, Pamela G. Department of Pediatrics, Johns Hopkins University School of Medicine, Baltimore, Maryland

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Electrophoresis on 6% polyacrylamide gels splits 10s RNA of detergent-treated polysomes from rabbit reticulocytes into two major bands. After these two RNAs are isolated separately, the first 10s RNA[1] directs the synthesis of both α and β chains in the Krebs II ascites cell-free system. In contrast, the second 10s RNA is inactive in directing globin synthesis. This result is further documented by separation of the two 10s RNAs by oligo dT-cellulose chromatography and by isolation of globin mRNA after EDTA-treatment of reticulocyte polysomes. Therefore, globin mRNA containing both α- and β-chain synthetic capacity moves as a single RNA species on electrophoresis in polyacrylamide gels.

10s RNA of the reticulocytes of various species contains the mRNA for the α- and β-globin chains (1-4). We and others have suggested that the two 10s RNA bands seen after electrophoresis on 6% polyacrylamide gels of SDS-treated polysomal RNA from reticulocytes are the α and β mRNAs (5,6). We now show that one 10s RNA band from rabbit reticulocytes contains the mRNA activity for both α- and β-globin chains. Recently Lanyon et al have reached a similar conclusion for globin mRNA from mouse reticulocytes (7).

METHODS

Isolation of polyribosomal RNA from rabbit reticulocytes, gradient separation of the RNA, and polyacrylamide gel electrophoresis were carried out by methods previously described (8-10). RNA in polyacrylamide gels was stained with Stains-all (11). The Krebs II ascites cell-free system was used with ^{3}H-L-leucine as the radioactive amino acid (12,13). Oligo dT-cellulose was supplied by Collaborative Research, Inc. and RNA was applied to oligo dT-cellulose

1. The 10s RNA with greater electrophoretic mobility toward the anode is designated "first 10s RNA." The slower 10s RNA is "second 10s RNA."

columns in 0.5 M KCl, .01 M Tris (pH 7.5), and polyadenylic-acid-rich RNA was eluted in .01 M Tris (pH 7.5) (14,15).

RNA was eluted from 6% polyacrylamide gels by the following method (16). The RNA solution was subjected to electrophoresis on four to six gels 1.5 cm in length, and after an experimentally-determined time, a dialysis sac filled with sterile buffer (40 mM Tris-acetate, pH 7.8; 20 mM sodium acetate; 2 mM EDTA) was fitted over the gel end. Electrophoresis was then continued until the desired RNA had moved off the gel and into the dialysis sac. The dialysis sac was removed, washed with buffer, and the RNA recovered by ethanol precipitation after the solution was made 0.1 M in sodium acetate (pH 5.0). The two 10s RNAs were collected separately in the following way. After a 140 minute electrophoresis, the first 10s RNA was collected in a dialysis sac during a subsequent 30 minute electrophoresis. Electrophoresis without sac for 10 minutes was again followed by a 30 minute electrophoresis with dialysis sac to collect the second 10s RNA. By re-electrophoresis, we estimate that 10-30% of the 10s RNA applied to the gels is recovered by this technique.

The product made in the mRNA-directed Krebs II ascites system was analyzed for synthesis of specific α- and β-chain tryptic peptides. Each 60 μl cell-free mixture containing ^{3}H-L-leucine was incubated first for 1 hour at 37° C and then, after the addition of 0.4 ml of 0.1 N KOH, for a subsequent 20 minutes. To each mixture 4 mgm of uniformly-labeled ^{14}C-L-leucine globin (8-11,000 cpm) obtained from an incubation of rabbit reticulocytes (17) was added. Protein was precipitated by acid-acetone, digested with trypsin, and various α- and β-chain peptides were separated by paper electrophoresis and chromatography (18). The peptides were identified by ninhydrin staining, eluted into 0.1 N HCl, and assayed for radioactivity. The product of an mRNA-directed assay was compared with that of an assay lacking exogenous mRNA by relating the ^{14}C dpm observed

than that observed with ascorbate-TPD. Thus, one must ask why substrates which use the same portion of the respiratory chain would differ in their ability to support active transport.

METHODS AND MATERIALS

Membrane Preparations - The growth conditions and the preparation of the ETP from M. phlei (ATCC 354) have been described (8). The ETP were suspended in water.

Protein Estimation - Protein was estimated by a modification of the Biuret method (9).

Proline Uptake - The assay system for proline uptake by membrane preparations was similar to that used previously (1). The level of transport refers to the steady state level of transport, i.e., the amount of proline accumulated 15 min following substrate addition, and the rate of transport refers to the initial rate of proline transport, measured within the first minute.

Rate of Oxidation - Oxygen consumption of ETP was measured at 30° with a Yellow Springs Instrument Model 53 Oxygen Monitor with ascorbate-TPD, ascorbate-phenazene methosulfate (PMS), or generated NADH as substrate. Both oxygen consumption and rate and/or level of proline transport were examined as a function of pH from pH 7.5 to pH 9.0, using Tris-HCl buffers. The rates of auto-oxidation (in the absence of ETP) for both the ascorbate-TPD and ascorbate-PMS systems were significant and all rates were corrected for auto-oxidation at each pH value.

Cytochrome Reduction - The rate of cytochrome reduction was followed with an Aminco DW-2 double beam spectrophotometer. The wavelength pairs used were 551-540 nm for cytochrome c and 598-623 nm for cytochromes $a + a_3$ (7).

RESULTS AND DISCUSSION

The rate of oxygen consumption as a function of pH with generated NADH as electron donor was maximal at pH 8.0 (Fig. 1). Under the same conditions, the steady state level of proline transport with generated NADH was found to parallel the rate of oxygen consumption (Fig. 1).

Table 1

RNA Source	^{3}H-L-leucine incorporation over blank (cpm)	Relative Synthesis		
		β chain	α chain	β/α
1. 5-18s region of sucrose gradient	18,000	13.6	5.6	2.4
2. Gel eluted 10s RNA:				
A) First 10s RNA	3,200	4.7	2.7	1.75
B) Second 10s RNA	-1,200	-0.2	-0.1	--
C) Both 10s RNAs	9,600	8.1	3.8	2.10
3. RNA from oligo dT-cellulose				
A) Poly A-poor (5 of 100 μl)	1,300	0.4	<0.3	--
B) Poly A-rich (5 of 30 μl)	13,200	21.0	9.2	2.2
4. EDTA-isolated mRNA	27,000	20.0	10.0	2.0

Incorporation of ^{3}H-L-leucine in assays lacking mRNA was 3-6,000 cpm without exogenous tRNA and 7-11,000 cpm with exogenous tRNA. Purified mRNA samples were completely dependent on tRNA added to the cell-free system. 10s RNA, 0.2-1.0 μg, was added to 60 μl mixtures containing in part 15 μl ascites cell S-30 and 3.7 mM magnesium acetate. In each assay cited, 11,000 cpm ^{14}C-L-leucine globin was added. Chain synthesis was calculated from the average synthesis of 3-4 purified tryptic peptides of each chain, e.g. βT1, βT2, βT9. Leucine content in a peptide was corrected for by dividing the RNA-directed ^{3}H dpm minus ^{3}H dpm of the blank peptide by the ^{14}C dpm of the blank peptide, e.g. βT1 sample of 2A equaled $\frac{406-11}{78}$ or 5.0. With the exception of peptides from assays 2B and 3A, ^{3}H-L-leucine incorporation was found in all α and β peptides studied.

second 10s RNA was eluted free of the first 10s RNA, and in both cases it did not contain globin mRNA activity when tested in the Krebs II ascites system. When the first 10s RNA eluted from the gels was assayed at the same time as a sample of both 10s RNAs eluted together (Fig. 2, A), the ratio of β- to α- chain

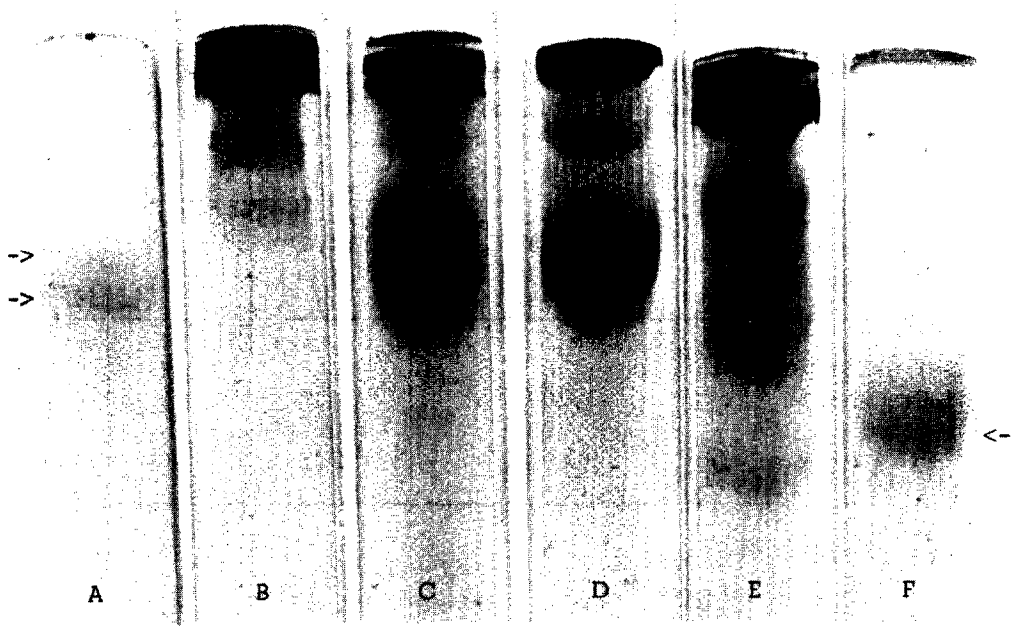

Figure 2: Electrophoresis on 6% polyacrylamide gels of the following samples. A. 10s RNA region eluted from gels; B. RNA of EDTA-isolated 40s subunit; C. 5-18s RNA of SDS-treated polysomes; D. RNA of EDTA-isolated 14s RNP; and E and F. RNA of 5-18s RNA eluted from oligo dT-cellulose in 0.5 M KCl (E) and in KCl-free buffer (F). Both E and F were subjected to electrophoresis for 4 hrs instead of the 2 hrs used for the other samples. After a 2-hr electrophoresis, the RNA in F moved with the mobility of the first 10s RNA of A, C, and D. Occasionally the presence of 1) a double second 10s RNA (see C) and 2) a 9s RNA having a faster mobility than the first 10s RNA (see C, D, and E) were noted.

synthesis directed by these samples was similar (Table 1). In other experiments, the first 10s RNA directed the synthesis of globin chains with a β/α ratio which suggested that 1.5 times more α chains than β chains were made.

B. Polyadenylic-acid-rich RNA from Reticulocytes

Gradient purified 5-18s RNA from SDS-treated polysomes was subjected to oligo dT-cellulose chromatography at 25° C. In two experiments the second 10s RNA eluted with rRNA and tRNA in 0.5 M KCl. When KCl was removed from the buffer, only the first 10s RNA eluted from the column (Fig. 2, F). This RNA presumably contains polyadenylic acid characteristic of globin mRNA (19). At least 95% of the globin mRNA capacity for α- and β-chain synthesis was recov-

ered with this preparation. The rRNA, tRNA fraction which contained the second 10s RNA had <5% of the total globin mRNA activity (Table 1).

C. mRNA from EDTA-treated Polyribosomes

When rabbit reticulocyte polyribosomes are treated with 30 mM EDTA, a 14s mRNP particle can be separated from the ribosomal subunits (1). When the RNA of this particle was obtained by SDS-phenol extraction, the 10s RNA region consisted of a single band with the mobility of the first 10s RNA (Fig. 2, D). This preparation of 10s RNA from EDTA-treated polyribosomes directed the synthesis of both α and β chains in the cell-free system (Table 1). In contrast to observations from mouse reticulocytes (7), the second 10s RNA was not found associated with the RNA of the 40s subunit (Fig. 2, B).

DISCUSSION

By use of gel elution and oligo dT-cellulose chromatography, we have isolated RNA which 1) migrates as a single band on electrophoresis in 6% polyacrylamide gels, and 2) directs the synthesis of both α and β chains in ratios similar to those obtained with crude polysomal RNA or gradient-separated 10s RNA. Moreover, the EDTA-released mRNP yields an active RNA preparation which contains a single 10s RNA band of the same electrophoretic mobility as the pure mRNA preparations.

The origin and function of the second 10s RNA from SDS-treated rabbit polysomal RNA is unknown. Although we find that the RNA of 40s subunits made by EDTA treatment of polyribosomes contains 12 RNA, the second 10s RNA was not found in these preparations (Fig. 2, B). A previous experiment suggested that the second 10s RNA was β mRNA and the first 10s RNA was α mRNA. The experiments presented here suggest a more acceptable explanation which follows. When L-O-methylthreonine is used to partially separate α- and β-chain poly-

somes of rabbit reticulocytes (17,20), the large β polysomes with 8-10 ribosomes per mRNA contain more second 10s RNA than first. In contrast, the small α polysomes with 2-3 ribosomes per mRNA contain an excess of the first 10s RNA (6). If the second 10s RNA is derived from ribosomes and the first 10s RNA is globin mRNA, β polysomes having a greater ratio of rRNA to mRNA than α polysomes would contain the observed excess of second 10s RNA. Conversely, in control cells the large polysomes contain only 6-7 ribosomes and the small polysomes have about 4 ribosomes. Thus, in control cells the 10s RNA pattern isolated from large polysomes is little different from that obtained from small polysomes.

The predicted 2-3% size difference in the α and β mRNAs is not sufficient to separate these molecules in 6% polyacrylamide gel electrophoresis, even when electrophoresis is carried out for times up to 4 hours (Fig. 2, F). This lack of separation of α and β mRNAs and the diffuse banding of globin mRNA on polyacrylamide gel electrophoresis may be related to variable lengths of polyadenylic acid attached to the 3' OH end of the messengers.

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