SEPARATION OF α- and β-GLOBIN MESSENGER RNAs BY FORMAMIDE GEL ELECTROPHORESIS

by Kazazian, Haig H., Jr., Snyder, Pamela G., and Cheng, Tu-chen, Johns Hopkins University School of Medicine, Baltimore, Maryland.

Received June 24,1974

Electrophoresis on 7.5% polyacrylamide gels in 98% formamide splits the 10s RNA of EDTA-treated polysomes from rabbit reticulocytes into two bands of similar intensity. Fractions containing first 10s RNA[1], both 10s RNAs, and nearly-pure second 10s RNA were obtained and tested in a cell-free system derived from ascites tumor cells. These RNA preparations directed the synthesis of product composed of 85-100% α chains, 55-70% β chains, and 75-87% β chains, respectively. The relative amounts of the two 10s RNAs in certain fractions were determined by formamide electrophoresis and correlated well with the relative synthesis of β and α chains directed by these fractions in the cell-free system. Therefore, the α and β globin mRNAs are separated by this procedure. Moreover, electrophoresis of globin mRNA further purified by oligo-dT cellulose chromatography indicates that the concentration of α mRNA exceeds that of β mRNA on rabbit reticulocytes polysomes.

The 10s fraction of reticulocyte RNA contains the globin messenger RNAs (1-4). A means to separate reproducibly the mRNAs for the α- and β-globin chains would facilitate the quantitation of these mRNAs in various species and in certain hemoglobinopathies of man. The ability to separate the globin mRNAs would also assist in their sequence analysis and in the production of cDNAs specific for one or another of the globin genes.

To date, separation of the α- and β-globin mRNAs by electrophoresis in aqueous polyacrylamide gels has been unsuccessful (5,6). However, Gould and Hamlyn have reported the division of 10s RNA of rabbit reticulocytes into two bands by electrophoresis in buffered formamide and

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

have suggested that these bands represent the α- and β-globin mRNAs (7). Lingrel has reported that supernatant globin mRNA which directs the synthesis of predominantly α chains contains only the 10s RNA of faster mobility on formamide electrophoresis (8). Now we have isolated highly-enriched fractions of one or the other 10s RNA after formamide electrophoresis. Studies of the translational specificity of each RNA fraction lead us to conclude that the α- and β-globin mRNA molecules are physically separated by this procedure.

METHODS

Isolation of polyribosomal RNA from rabbit reticulocytes by EDTA treatment of polysomes, gradient separation of the RNA, and oligo-dT cellulose chromatography were carried out by methods described previously (9-13). Isolation of supernatant globin mRNA was begun by acid precipitation as described (14), followed by purification of mRNA by ultracentrifugation through a sucrose gradient, and oligo-dT cellulose chromatography. The Krebs II ascites cell-free system was used to translate globin mRNAs with ^{3}H-L-leucine as the radioactive amino acid (15,16).

Formamide electrophoresis in polyacrylamide gels was carried out with minor modifications of described methods (17). Gels containing 7.5% acrylamide, 1.13% bisacrylamide, 0.02 M NaCl (made by dilution of 5 M NaCl), 98% formamide, were run for 2.5 - 4.5 h at 3.5 ma/gel in 0.02 M NaCl, 98% formamide, and stained for RNA with Stains-all (18). The formamide for gel use (Fisher certified) was deionized with Dowex AG50W-X8, 20-50 mesh-hydrogen form. The electrophoresis buffer was made with reagent grade formamide (Aldrich Chemical Co.) treated with activated charcoal in addition to Dowex AG50W-X8, and was subjected to preelectrophoresis

for 2 h with a blank gel prior to use. The buffer was reused up to four times, and gels were run one to two days after their preparation.

RNA was eluted from formamide gels by the following procedure. The RNA was located by scanning the unstained gel at 270 nanometers. Gel portions containing the desired RNA fractions were sliced, each gel slice was placed in the constricted region of a Pasteur pipette, and the RNA was removed by electrophoresis into a dialysis sac for 1.5 h under aqueous conditions (40 mM Tris-acetate, pH 7.8; 20 mM sodium-acetate; 2mM EDTA) (6). The eluted RNA was then precipitated with ethanol and tested in the ascites cell-free system. The product of the mRNA-directed cell-free assay was analyzed for synthesis of specific α- and β-chain tryptic peptides as described (6,19). Certain eluted samples were also subjected to electrophoresis on formamide gels.

RESULTS

Under the conditions described in the "Methods," formamide electrophoresis reproducibly splits the 10s RNA from EDTA-treated polysomes of reticulocytes into two well-separated bands of nearly equal staining intensity (Fig. 1). However, when NaCl was added to the formamide as the solid salt instead of in a concentrated solution, or the buffer was not subjected to preelectrophoresis with a blank gel, no separation of the 10s RNA bands was seen.

Assignment of Globin mRNA Activity to the 10s RNA Bands

The following RNA fractions were located and eluted from gel slices: 1) The bottom half of the first 10s RNA, 2) the top half of that RNA along with the bottom half of the second 10s RNA, and 3) the top half of the second RNA. The curvature of the RNA within the gels following electro-

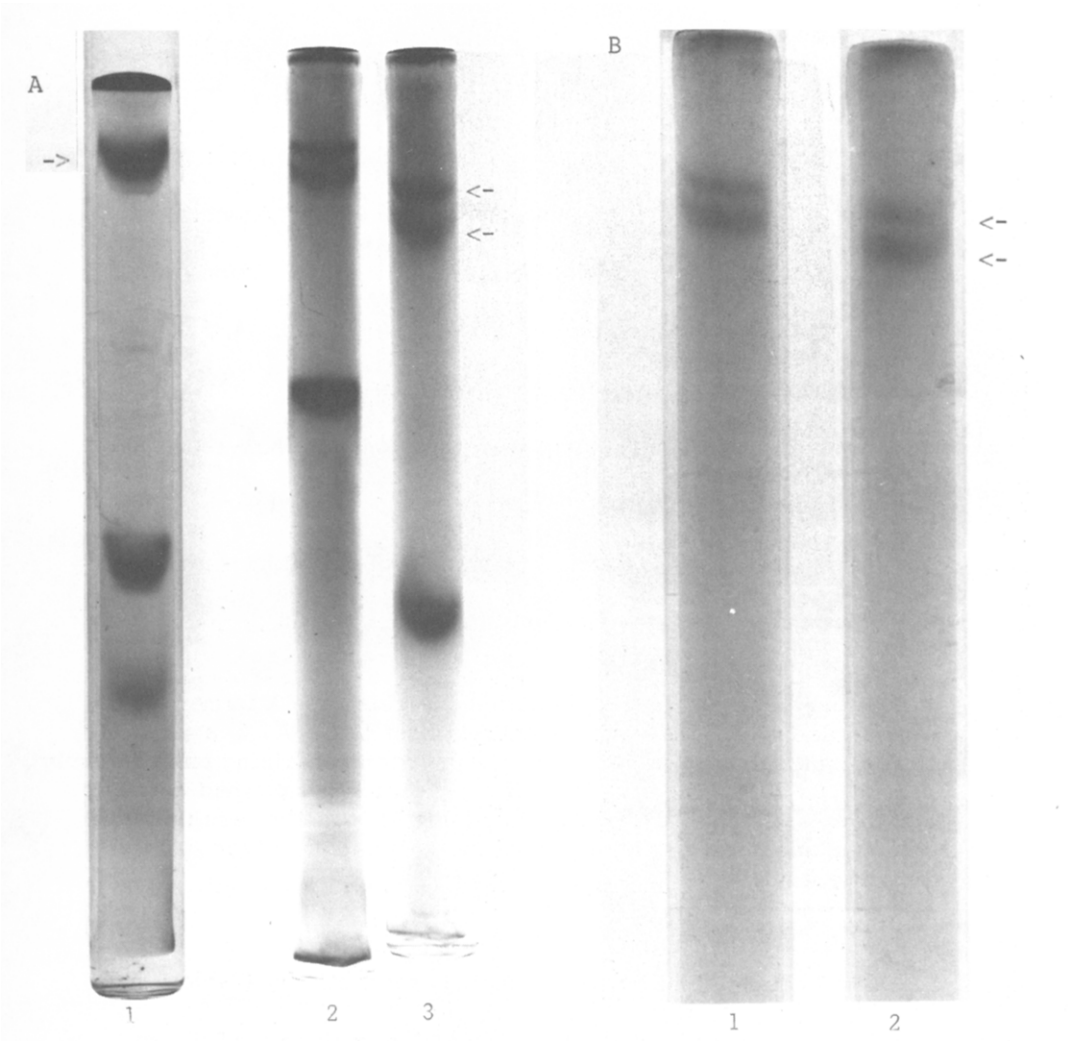
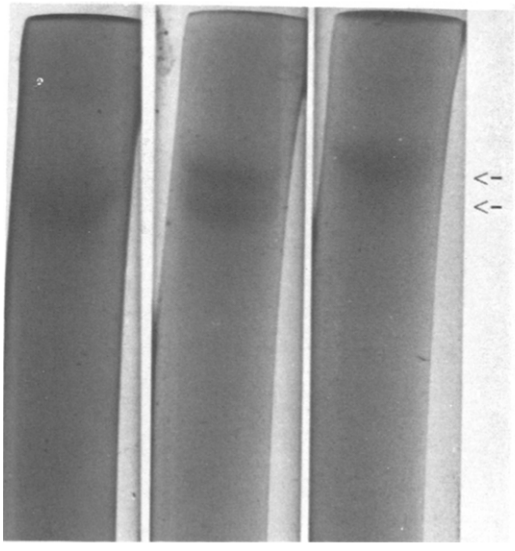


Figure 1:

A. Electrophoresis in polyacrylamide gels of EDTA-treated polysomal RNA of rabbit reticulocytes: 1) 6% polyacrylamide gel run under aqueous conditions (22). 2) and 3) 7.5% gels made with and run in 98% formamide for 3.5 and 4.5 h. Note single 10s RNA (arrow) in 1) and two 10s RNAs (arrows) in 2) and 3).

B. Formamide electrophoresis in 7.5% polyacrylamide gels for 3.5 and 4 h of globin mRNA purified by oligo-dT cellulose chromatography from rabbit polysomal RNA. The gels were stained and scanned at 610 nanometers. Relative amounts of the two 10s RNAs were estimated from the area under the curve for each.

phoresis made it difficult to obtain samples highly enriched in the second 10s RNA. In various experiments, RNA fractions enriched for the first 10s RNA, both RNAs, and the second 10s RNA directed the synthesis of 83-100%



1 2 3

Figure 2: Reelectrophoresis on 7.5% polyacrylamide gels in 98% formamide of eluted 10s RNAs. The samples are: 1) first 10s RNA, 2) a mixture of first and second 10s RNA, and 3) a fraction containing predominantly second 10s RNA. The 10s RNAs of a preelution sample had the same electrophoretic mobility as the eluted 10s RNAs. The results of the cell-free assays with aliquots of these samples are given in Table I, experiment #3.

α chains, 55-70% β chains, and 75-87% β chains, respectively (Table I and footnote to Table). One sample of the first 10s RNA contained α mRNA but completely lacked β mRNA by translation analysis, while the greatest enrichment of β mRNA in a preparation of the second 10s RNA was 84-87% (Table I).

Three RNA samples isolated from different gel regions were subjected to reelectrophoresis in formamide gels in addition to translation analysis (Fig. 2 and Table I, Exp. #3). In these samples the concentration ratio of the second 10s RNA to first 10s RNA on reelectrophoresis was estimated by densitometry of the stained gels at 610 nanometers to be 71:29, 48:52, and 5:95, respectively. Under the direction of these RNA samples in the cell-

Table I

Experiment	Fraction	Relative Synthesis		
		β-chain	α-chain	β/α*
#1	1st 10s RNA	0.12	0.96	0.12
	Both 10s RNAs	5.90	3.23	1.8
	2nd 10s RNA	4.45	0.68	6.6
#2	1st 10s RNA	3.1	9.0	0.34
	Both 10s RNAs	4.4	1.7	2.6
	2nd 10s RNA	7.8	2.0	3.9
#3	1st 10s RNA	0.12	1.50	0.08
	Both 10s RNAs	1.38	1.25	1.1
	2nd 10s RNA	1.75	0.57	3.1
#4	1st 10s RNA	0	0.67	0
	2nd 10s RNA	0.38	0.08	4.8

Incorporation of ^{3}H-L-leucine in assays lacking mRNA was 3-4,000 cpm, and 5-10,000 cpm were incorporated when purified mRNA samples (< 1μg) were added to the cell-free system. In all assays shown the mRNA concentration was below saturation levels. To each assay tube after incubation 5,000 cpm of ^{14}C-L-leucine globin was added as described (6). Chain synthesis was calculated from the average synthesis of 3-5 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. in experiment #4, αT10 equaled $\frac{236-51}{340}=0.55$ and αT9 equaled $\frac{183-63}{168}=0.71$. ^{3}H-L-leucine incorporation was observed in all α and β peptides studied except the β-chain peptides of experiment #4 (1st 10s RNA). No trace of ^{3}H-L-leucine incorporation was observed in any of four β peptides studied in this experiment.

*When EDTA-treated polysomal RNA is added to the cell-free system, the β/α synthetic ratio is about 1.4. The β/α synthetic ratio of a 50:50 mixture of globin mRNAs after formamide gel electrophoresis is unknown, but a β/α ratio of 1.2-1.4 was assumed for the chain percentages presented in "Results."

free system, the ratio of β-chain synthesis to α-chain synthesis was 3.1 for the first sample, 1.1 for the second, and 0.08 for the third. Thus, the relative concentrations of the two 10s RNAs correlated well with the

relative synthesis of β and α chains in the cell-free system further indicating that the first 10s RNA is α mRNA and the second 10s RNA is nearly-pure β mRNA.

Quantitation of Relative α and β mRNA Amounts

Globin mRNA of reticulocyte post-ribosomal supernate is reported to be greatly enriched for α mRNA (14,20) and in our laboratory has been 75-85% α mRNA by translation analysis. When supernatant globin mRNA prepared from rabbit reticulocytes through purification by oligo-dT cellulose chromatography was subjected to formamide electrophoresis, the amount of first 10s RNA greatly exceeded that of second 10s RNA (figure not shown). However, the second 10s RNA has always been present in these samples.

The staining intensity of the first and second 10s RNAs is nearly equal in EDTA-treated polysomal RNA which has not undergone oligo-dT cellulose chromatography. However, after removal of small amounts of non-mRNA in the 10s fraction of EDTA-treated polysomal RNA by oligo-dT cellulose chromatography, formamide electrophoresis demonstrates that the first 10s RNA exceeds the second 10s RNA in concentration by about 50% (Fig. 1B), as estimated by scans of the stained gels at 610 nanometers.

DISCUSSION

Our data demonstrate the following: 1) Since RNA fractions have been obtained which are exclusively first 10s RNA by electrophoretic analysis and 95-100% α mRNA by translation analysis, the first 10s RNA contains α mRNA free of β mRNA. 2) The second 10s RNA is nearly pure in β mRNA by translation analysis. However, the curvature of the RNA bands after electrophoresis makes it likely that small amounts of the first 10s RNA (α mRNA) have been included in the gel slices aimed at obtaining pure second

10s RNA. 3) The contamination of active β mRNA by inactive α mRNA and vice versa, while not excluded, is unlikely. Thus, after oligo-dT cellulose chromatography the two 10s RNA bands are in all likelihood the nearly pure α and β mRNAs and the relative quantities of these mRNAs can be estimated after chromatography by gel electrophoresis. In confirmation of the indirect estimates of globin mRNA quantities on polysomes of rabbit reticulocytes (21), on direct analysis we find that the amount of α mRNA exceeds that of β mRNA by about 50%.

If the separation of mRNA observed by formamide electrophoresis occurs strictly on a molecular weight basis, β mRNA must be roughly 10% larger than α mRNA (7). Since under slightly modified conditions of formamide electrophoresis we have failed to separate the 10s RNAs, the separation of the α and β globin mRNAs obtained by this procedure may not be wholly due to a molecular weight difference in the two molecules. In any event, this procedure provides the opportunity to prepare nearly pure α and β globin mRNAs from any species for sequence analysis and for the production of complementary DNA specific for individual globin genes.

ACKNOWLEDGMENT

This work was supported in part by NIH research grant AM 13983.

REFERENCES

1. Chantrenne, H., Burny, A., and Marbaix, G., Prog. Nucleic Acid Res. Mol. Biol. 7, 173 (1967).
2. Lockard, R.E., and Lingrel, J.B., Biochem. Biophys. Res. Commun. 37, 204 (1969).
3. Mathews, M.B., Osborn, M., and Lingrel, J.B., Nature 233, 206 (1971).
4. Nienhuis, A.W., Laycock, D.G., and Anderson, W.F., Nature New Biol. 231, 205 (1971).
5. Lanyon, G., Paul, J., and Williamson, R., Eur. J. Biochem. 31, 38 (1972).
6. Kazazian, H.H., Jr., Moore, P.A., and Snyder, P.G., Biochem. Biophys. Res. Commun. 51, 564 (1973).

7. Gould, H.J., and Hamlyn, P.H., FEBS Letters 30, 301 (1973).
8. Lingrel, J.B., Morrison, M., Gorski, J., and Merkel, C.G., Ann. N.Y. Acad. Sci. (in press).
9. Evans, M.J., and Lingrel, J.B., Biochemistry 8, 3000 (1969).
10. Labrie, F., Nature 221, 1217 (1969).
11. Kazazian, H.H., Jr., Nature New Biol. 238, 166 (1972).
12. Gilham, P.T., J. Amer. Chem. Soc. 86, 4982 (1964).
13. Aviv, H., and Leder, P., Proc. Nat. Acad. Sci. 69, 1408 (1972).
14. Gianni, A.M., Giglioni, B., Ottolenghi, S., Comi, P., and Guidotti, G., Nature New Biol. 240, 183 (1972).
15. Mathews, M.B., and Korner, A., Eur. J. Biochem. 17, 328 (1970).
16. Aviv, H., Boime, I., and Leder, P., Proc. Nat. Acad. Sci. 68, 2363 (1971)
17. Staynov, D.Z., Pinder, J.C., and Gratzer, W.B., Nature New Biol. 235, 108 (1972).
18. Dahlberg, A.E., Dingman, C.W., and Peacock, A.C., J. Mol. Biol. 41, 139 (1969).
19. Kazazian, H.H., Jr., and Itano, H.A., J. Biol. Chem. 243, 2048 (1968).
20. Jacobs-Lorena, M., and Baglioni, C., Proc. Nat. Acad. Sci. 69, 1425 (1972).
21. Lodish, H.F., J. Biol. Chem. 246, 7131 (1971).
22. Loening, U.E., Biochem. J. 102, 251 (1967).