THE SYNTHESIS OF MOUSE HEMOGLOBIN β-CHAINS IN A RABBIT RETICULOCYTE CELL-FREE SYSTEM PROGRAMMED WITH MOUSE RETICULOCYTE 9S RNA

Raymond E. Lockard and Jerry B. Lingrel

Department of Biological Chemistry, University of Cincinnati
College of Medicine, Cincinnati, Ohio 45219

Received August 6, 1969

SUMMARY. The 9S RNA fraction of mouse reticulocyte polysomes has been purified and shown to direct the synthesis of mouse hemoglobin β-chains. This has been accomplished by adding mouse 9S RNA to a rabbit reticulocyte cell-free system and incubating in the presence of labeled leucine. After the incubation, carrier mouse hemoglobin was added, globin prepared and mouse β-chains isolated by column chromatography. Labeling of the mouse β-chains in the rabbit cell-free system was strictly dependent upon addition of mouse 9S RNA, demonstrating that the fraction contains the mRNA for mouse hemoglobin β-chains. This is the first definitive demonstration of protein synthesis in a mammalian cell-free system under the direction of a mRNA isolated from a different mammalian species.

When reticulocyte polysomes incubated with sodium dodecyl sulfate to dissociate protein and RNA are centrifuged in a sucrose gradient, a small fraction of RNA migrating between transfer RNA and ribosomal RNA is observed (1,2). Although many properties of this RNA are consistent with it being the hemoglobin mRNA (1-5), unequivocal evidence can only be obtained by showing that this RNA directs the synthesis of hemoglobin. This can best be accomplished by using a cross-system; i.e., adding the RNA to a cell-free protein synthesizing system which does not synthesize the protein in question. The 9S RNA was isolated from mouse reticulocytes and the cell-free system was prepared from rabbit reticulocytes. This communication describes experiments which demonstrate that the 9S RNA isolated from mouse polysomes is capable of directing the synthesis of mouse hemoglobin β-chains in a rabbit reticulocyte cell-free system.

METHODS

Mouse and rabbit reticulocytes were obtained as previously described

(2,4) and ribosomes were isolated by the procedure of Evans and Lingrel (2). Dissociation of ribosomes by sodium dodecyl sulfate and sucrose density gradient analysis of RNA fractions were carried out as previously reported (2).

The conditions of the cell-free incubation were similar to those of Adamson et al. (6). The incubation mixture contained, in a final volume of 1.0 ml, 0.40 ml of lysate and 0.10 ml of energy mixture containing the following components (the final concentration of each in the cell-free system is given): 100 mM ammonium acetate, 10 mM Tris·HCl, pH 7.4, 2 mM magnesium acetate, 5 mM mercaptoethanol, 1 mM ATP, 0.2 mM GTP, 15 mM creatine phosphate, 60 μg creatine phosphokinase, and one-tenth the concentration of amino acids minus leucine described by Lingrel (7). RNA and labeled and non-labeled leucine were added as indicated in the figures, and the reaction mixture was made up to 1 ml with 0.05 M Tris·HCl, pH 7.4. L-Leucine-^{14}C was obtained from New England Nuclear Corporation and L-leucine-4,5-^{3}H from Schwarz BioResearch. Globin was prepared according to the procedure of Rabinovitz and Fisher (8).

Mouse β-chains were separated from mouse α- and rabbit α- and β-chains as outlined by Grossbard et al. (9) on a 1.65 cm x 15 cm Whatman CM52 carboxymethyl cellulose column. The column was eluted with a 680 ml gradient from 0.01-0.02 M $Na_2HPO_4 \cdot 7H_2O$ at pH 6.8, containing 8 M urea and 0.05 M mercaptoethanol. Ten-milliliter fractions were collected.

After the addition of 5 mg of carrier serum albumin, the protein in each fraction was precipitated with 10% final concentration of trichloroacetic acid. The precipitated protein was collected by centrifugation, washed twice with 5% trichloroacetic acid and then dissolved in 1 ml of 0.1 N NaOH. A 0.5 ml aliquot of this solution was added to 15 ml of liquid scintillation counting solution (10) containing 0.9 g of Cab-O-Sil. ^{14}C and ^{3}H were determined in a Packard Tricarb liquid scintillation spectrometer.

RESULTS

The mouse 9S RNA was isolated from reticulocyte ribosomes by sucrose

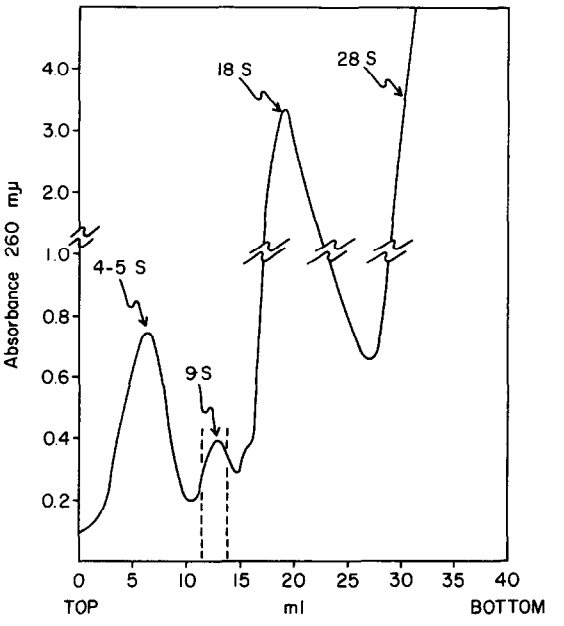

Figure 1. Preparation of Mouse Reticulocyte 9S RNA.

Eight milligrams of polysomes in 1 ml of 5 mM Tris·HCl buffer, pH 7.4, was treated with 0.05 ml of 10% sodium dodecyl sulfate for 5 min at 37° and layered on a 5-20% exponential sucrose gradient. Centrifugation was performed at 1.8° in a Spinco SW27 rotor for 26 hours at 27,000 rpm. Fractions designated by the dashed lines were collected from several gradients, pooled and further purified.

---

density gradient centrifugation. Figure 1 shows the results of a gradient analysis of sodium dodecyl sulfate treated mouse ribosomes. The 9S RNA appears as a sharp peak between the transfer and small ribosomal RNA. The peak fractions were pooled, concentrated, treated with a final concentration of 0.25% sodium dodecyl sulfate and recentrifuged on a 15-30% sucrose gradient (Fig. 2). The recentrifugation shows a major peak with a small amount of contaminating material migrating in the tRNA region. The 9S RNA does not appear to have been degraded during the concentration and recentrifugation; it migrates in the same position as the original RNA liberated from polysomes as shown by the total RNA pattern superimposed on this figure. Fractions enclosed by the dotted lines were used in the cell-free system studies.

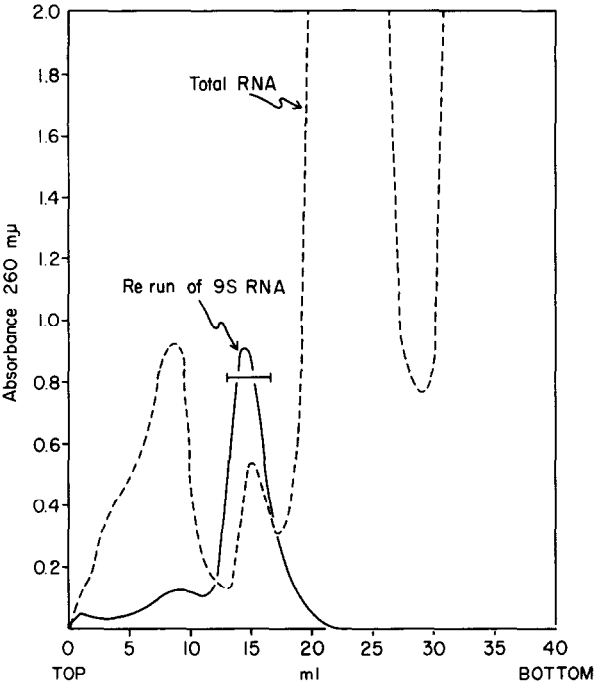

Figure 2. Purification of Mouse 9S RNA.

The pooled fractions of 9S RNA obtained from gradients similar to those shown in Fig. 1 were concentrated by centrifugation at 105,000 x g for 20 hr. The RNA in the bottom ml of the tubes was treated with 0.025 ml of 10% sodium dodecyl sulfate for 1 min at 25° and rerun for 46 hr in a 15-30% exponential sucrose gradient (solid line). The profile of 4 mg of total RNA run under identical conditions is superimposed on this figure and is indicated by a dotted line. The fractions represented by the bar were pooled and then concentrated by centrifugation for 20 hr at 105,000 x g. To the RNA located in the bottom ml of the tube was added 10 volumes of 0.05 M Tris·HCl, pH 7.4, to dilute the sucrose. The 9S RNA was again concentrated by centrifugation and used for cell-free analyses.

Separation of Mouse β-Chains from Mouse α- and Rabbit α- and β-Chains.

The synthesis of mouse β-chains in the rabbit reticulocyte cell-free system can be determined by separating the mouse β-chains from the mouse α- and rabbit α- and β-chains according to the method of Grossbard *et al.*(9)

Figure 3 shows the separation of a mixture of L-leucine-^{14}C labeled rabbit globin and L-leucine-^{3}H labeled mouse globin. The mouse β-chain is well resolved from the mouse α- and rabbit α- and β-chains. Although the rabbit globin elutes as two peaks, it is not known which of these are the α-chains and which are the β-chains.

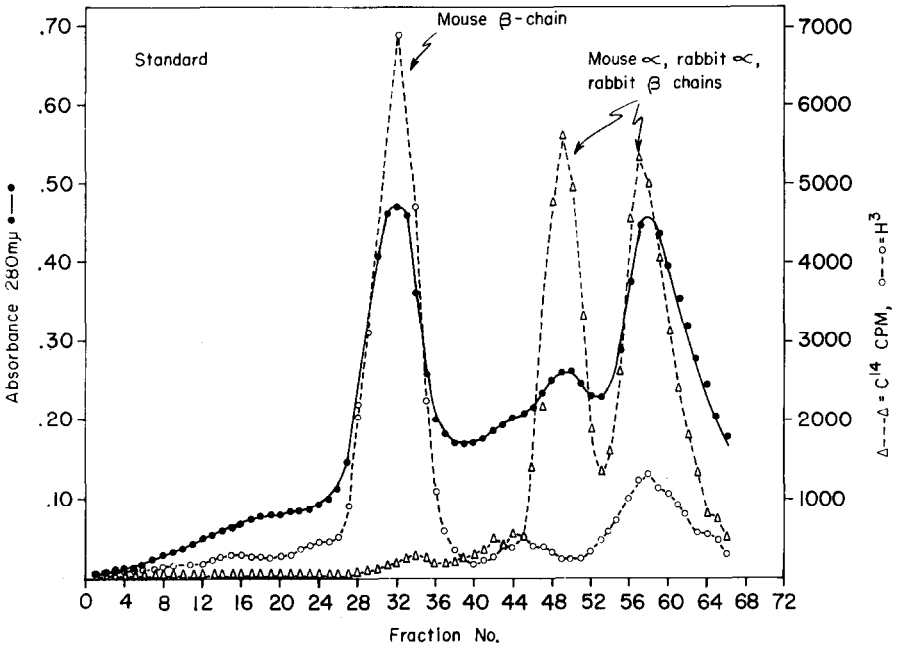

Figure 3. Separation of Mouse β-Chains from Mouse α-, and Rabbit α- and β-Chains.

Two 1.0 ml cell-free incubation mixtures were used as described in Methods. One mixture contained rabbit reticulocyte lysate with 8 μc L-leucine-^{14}C (278 μc/μmole). The other mixture contained mouse reticulocyte lysate plus 80 μc L-leucine-^{3}H (5910 μc/μmole). Both mixtures were brought up to 1.0 ml with 0.05 M Tris·HCl, pH 7.4, buffer and incubated at 28° for 60 min. At the end of the incubation the two cell-free systems were pooled, 100 mg of unlabeled mouse hemoglobin was added, and the mixture dialyzed against distilled water for 24 hr at 3°. Globin was prepared and chromatographed on a carboxymethyl cellulose column as described in Methods. ^{3}H labeled mouse globin (-o--o-), ^{14}C labeled rabbit globin (-Δ-Δ-), 280 mμ absorbance (-•-•-•-).

Synthesis of Mouse β-Chains by Mouse 9S RNA.

In order to synthesize protein from an exogenous mRNA, the cell-free preparation must be capable of initiating new chains. Unfractionated preparations from rabbit reticulocytes have this capability (6,11,12) and were therefore used in these studies. Treatment to lower endogenous mRNA was avoided in order to minimize possible loss of initiation properties of the system. Thus, the added mRNA may have to compete with that already present.

To detect the synthesis of mouse hemoglobin, two tubes, each containing

the rabbit cell-free system were used. Leucine-^{3}H and mouse 9S RNA were added to one tube; leucine-^{14}C and no mouse 9S RNA were added to the other. After incubation, the two cell-free systems were combined and mixed with unlabeled carrier mouse hemoglobin. The globin prepared from this mixture was then chromatographed on a carboxymethyl cellulose column to separate mouse β-chains from mouse α-chains and rabbit α- and β-chains. The ^{14}C elution profile provides a control representing the synthesis of rabbit hemoglobin in the rabbit cell-free system, while the tritium elution profile is a composite of endogenous hemoglobin synthesis and the synthesis of any new protein made under the direction of mouse 9S RNA.

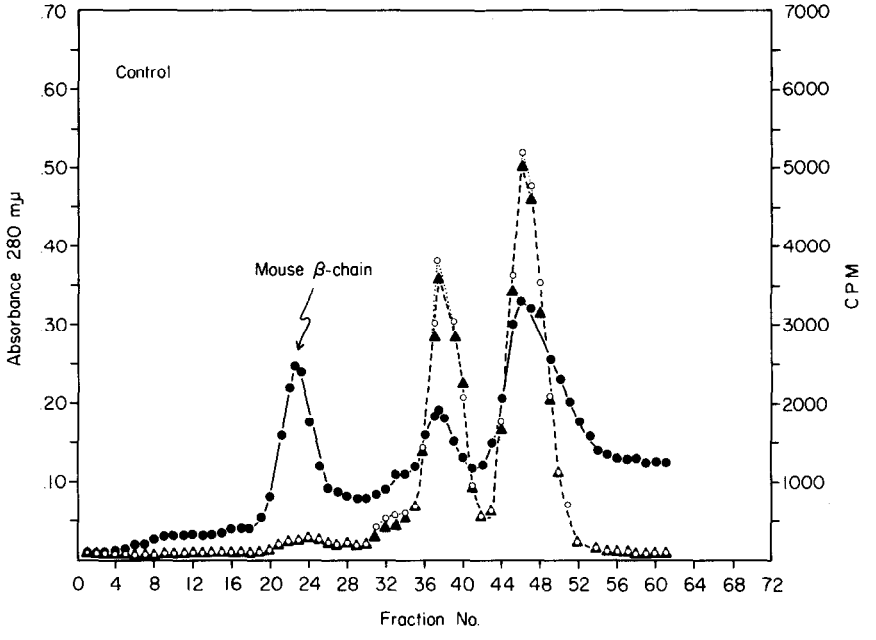

Figure 4. Comparison of Leucine-^{14}C and Leucine-^{3}H in Labeling Globin in the Cell-Free System.

Two 1.0 ml cell-free systems were used. To the first, L-leucine-^{14}C (278 μc/ mole) was added at a concentration of 3.1×10^{-5} M with 6.9×10^{-5} M non-radioactive leucine giving a final specific activity of 85 μc/μmole. To the second cell-free system L-leucine-^{3}H (2000 μc/μ mole) was added at a concentration of 2×10^{-5} M with 8×10^{-5} M non-radioactive leucine resulting in a specific activity of 400 μc/μmole. The two cell-free systems were then incubated at 28° C for 60 min. At the end of the incubation they were cooled to 0°, pooled, and 90 mg of mouse carrier hemoglobin was added. The mixture was then dialyzed against 2 liters of water over a 24-hr period after which globin was prepared, and chromatographed on a carboxymethyl cellulose column. Absorbance at 280 mμ (-●-●-●-), leucine-^{14}C cpm (-▲-▲-), leucine-^{3}H (-o-o-o-).

As a control the globins prepared from two rabbit cell-free systems, one with leucine-^{14}C and the other with leucine-^{3}H, but with neither containing mouse 9S RNA, were combined and chromatographed. The results are shown in Figure 4. The specific activities of the leucine-^{14}C and leucine-^{3}H were adjusted with non-radioactive leucine so that the final specific activities of these isotopes would give a $^{3}H/^{14}C$ ratio of one throughout the elution profile. This study indicates that no differences exist between leucine-^{14}C and leucine-^{3}H.

The column chromatographic analysis of a mixture of two rabbit cell-free systems, one containing leucine-^{3}H and mouse 9S RNA, and the other leucine ^{14}C but without the added RNA, is shown in Figure 5. A substantial amount of tritium relative to ^{14}C elutes in the mouse β-chain region suggesting that 9S RNA directed the synthesis of mouse β-chains. If mouse 9S RNA is added at the end of incubation, no labeling of β-chains occurs. When mouse 4S, 5S, 18S, and 28S RNA's were added to the cell-free system only control

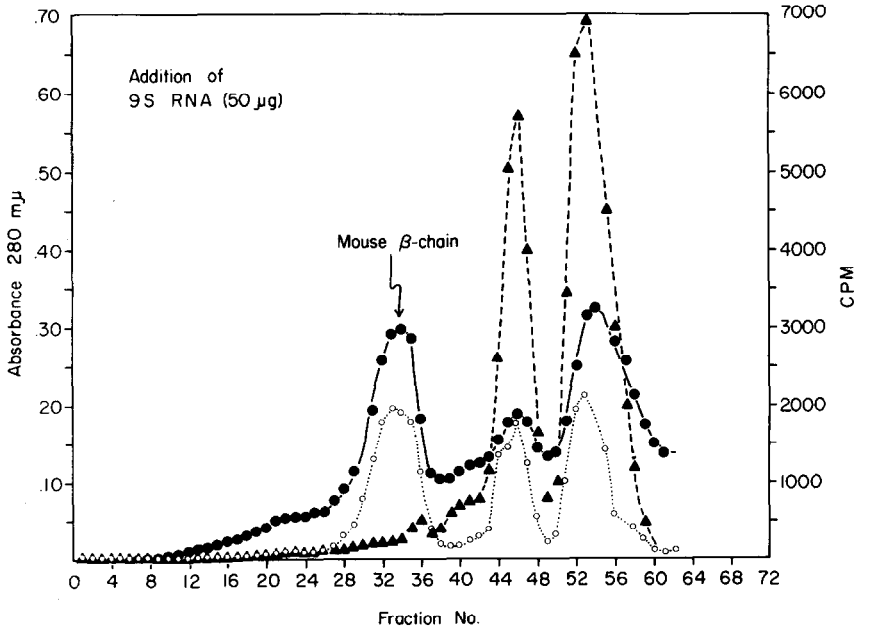

Figure 5. The Synthesis of Mouse β-Chains in the Rabbit Cell-Free System.

Identical to Figure 4 except 50 g of mouse 9S RNA (see Fig. 2) was added to the leucine-^{3}H containing tube. Absorbance at 280 mµ (-●-●-●-), leucine-^{14}C cpm (-▲-▲-), leucine-^{3}H (-o-o-o-).

values of ^{3}H appeared in the mouse β-chain region, thus suggesting that only 9S RNA can specifically direct the synthesis of mouse β-chains (13).

DISCUSSION

These studies suggest that the 9S RNA fraction isolated from mouse ribosomes is capable of directing the synthesis of mouse hemoglobin β-chains. Mouse α-chains may also be synthesized under the direction of the added 9S RNA, but since these chains are not resolved by this column no conclusions can be drawn regarding their synthesis.

It is of interest to note that the syntheses of rabbit chains are depressed upon addition of mouse 9S RNA suggesting that the added RNA may be competing with endogenous mRNA for template activity.

While these experiments were in progress a report appeared showing that an RNA fraction isolated from rabbit reticulocytes could direct the synthesis of globin when added to an E. coli cell-free system (14). The translation required the addition of N-acetylvalyl tRNA. A reticulocyte RNA fraction isolated from different rabbits has also been added to a rabbit reticulocyte cell-free system to determine the basis of amino acid sequence ambiguities in hemoglobin (15).

ACKNOWLEDGEMENTS

This investigation was supported by U. S. Public Health Service research grant GM-10999, National Science Foundation grant GB-5041, and the Denver M. Eckert Memorial grant for Cancer Research from the American Cancer Society, E-479.

J.B.L. is a Career Development Awardee (1-Kr-GM-8673) of the National Institutes of Health, U.S.P.H.S.

REFERENCES

1. Marbaix, G. and Burny, A., Biochem. Biophys. Res. Commun. 16, 522 (1964).
2. Evans, M J. and Lingrel, J. B., Biochemistry 8, 3000 (1969).
3. Burny, A. and Marbaix, G., Biochem. Biophys. Acta 103, 409 (1965).

4. Evans, M. J. and Lingrel, J. B , Biochemistry 8, 829 (1969).
5. Chantrenne, H., Burny, A., and Marbaix, G., Progress Nucleic Acid Research and Molecular Biology 7, 173 (1967).
6. Adamson, S. D., Herbert, E., and Godchaux, W., III., Arch. Biochem. Biophys. 125, 671 (1968).
7. Lingrel, J. B. and Borsook, H., Biochemistry 2, 309 (1963).
8. Rabinovitz, M. and Fisher, J. M., Biochim. Biophys. Acta 91, 313 (1964).
9. Grossbard, L., Banks, J., and Marks, P. A., Arch. Biochem. Biophys. 125, 580 (1968).
10. Lingrel, J. B., Biochem. Biophys. Acta 142, 75 (1967).
11. Lamfrom, H. and Knopf, P. M., J Mol. Biol. 9, 558 (1964).
12. Zucker, W. V. and Schulman, H. M , Proc. Natl. Acad. Sci. U.S. 59, 582 (1968).
13. Lockard, R. E. and Lingrel, J. B., unpublished experiments (1969).
14. Laycock, D. G and Hunt, J. A., Nature 221, 1118 (1969).
15. Schapira, G., Dreyfus, J. C., and Maleknia, N., Biochem. Biophys. Res. Commun., 32, 558 (1968).