Defect in Messenger RNA for Human Hemoglobin Synthesis in Beta Thalassemia

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ABSTRACT Functional messenger RNA for human hemoglobin synthesis was prepared from reticulocyte lysates of patients with homozygous beta thalassemia and sickle cell anemia. The messenger RNA stimulated the synthesis of human globin chains by a cell-free system derived from Krebs mouse ascites cells. In the presence of beta thalassemia messenger RNA, the system synthesized much less beta chain than alpha chain whereas in the presence of sickle cell anemia messenger RNA, nearly equal amounts of alpha and beta chains were synthesized. The beta/alpha synthetic ratios obtained in the cell-free system were similar to those obtained by incubating intact beta thalassemia and sickle cell anemia reticulocytes in the presence of radioactive leucine. The experiments provide direct evidence of a defect in messenger RNA for beta chains as a cause for the decreased synthesis of beta chains observed in beta thalassemia.

INTRODUCTION

Beta thalassemia is an inherited disorder of hemoglobin synthesis characterized by decreased synthesis of structurally normal beta chains of adult human hemoglobin (1–7). It is currently believed that the basic defect in beta thalassemia is decreased synthesis of structurally normal messenger RNA (mRNA)[1] for beta chains, or synthesis of a structurally and/or functionally abnormal mRNA for beta chains (8, 9). There is much experimental evidence which indirectly supports this hypothesis: thalassemic ribosomes synthesize polyphenylalanine normally when primed by polyuridylic acid in a cell-free system (10); the rate of beta chain translation and chain termination in thalassemic reticulocytes is normal as determined by pulse labeling and analysis of newly synthesized globin chains by peptide fingerprinting (Dintzis plots) (11, 12); in a fractionated rabbit reticulocyte cell-free system, thalassemic ribosomes respond normally to added initiation factors (0.5 M KCl ribosome wash) but continue to synthesize less beta chain than alpha chain (13); and in the same type of cell-free system, thalassemic ribosomes translate added rabbit globin mRNA and synthesize rabbit globin chains in the same beta/alpha ratio as do nonthalassemic human ribosomes (14). Supporting the concept that the beta chain mRNA attached to ribosomes is functionally normal but deficient in amount, is the fact that the distribution of nascent beta and alpha chains across light and heavy polysomes is normal in beta thalassemic reticulocytes (15).

To provide direct evidence of a defect in availability of normal mRNA for beta chains in beta thalassemia, we have adopted the use of a mRNA-dependent cell-free system in which the only added reticulocyte component is mRNA. With this system, we have demonstrated that the mRNA of beta thalassemia reticulocytes stimulates the synthesis of much less beta than alpha chain whereas mRNA from nonthalassemic reticulocytes stimulates synthesis of nearly equal amounts of alpha and beta chains.

METHODS

Whole reticulocyte RNA was prepared from membrane-free reticulocyte lysates by the phenol-cresol extraction procedure of Parish and Kirby (16) as modified by Loening (17), except that 4-aminosalicylic acid was omitted. The RNA was allowed to precipitate overnight at −20°C after addition of ½ volume 20% sodium acetate, pH 5.4, and 2 volumes of 95% ethanol, resuspended in 2% sodium acetate, pH 5.4, and 10 mM $MgCl_2$, and dialysed for 5 hr against buffer A: 0.04 M Tris-acetate, pH 7.2, 0.02 M sodium acetate, and 2 mM EDTA. After a second ethanol precipitation, the RNA was resuspended in buffer A, and 5 or 10% so-

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[1] *Abbreviations used in this paper:* mRNA, messenger RNA; SDS, sodium dodecyl sulfate.

dium dodecyl sulfate (SDS) was added to a final concentration of 0.5%. The RNA was then fractionated by centrifugation at 100,000 *g* for 15 hr at 4°C through 5–20% linear sucrose density gradients which contained buffer A and 0.5% SDS. SDS was removed from the sucrose gradient fractions by addition of 4 M KCl to a final concentration of 0.2 M (18) and removal of the potassium dodecyl sulfate precipitate was accomplished by centrifugation. The RNA was then precipitated from the clear supernatant solution by addition of $\frac{1}{10}$ volume 20% sodium acetate, pH 5.4, and 2 volumes of 95% ethanol. After centrifugation, the RNA was dried under a stream of nitrogen, resuspended in 0.1 mM $MgCl_2$, and frozen at −20°C until used.

Whole lysate RNA was used in preference to RNA released from polysomes by EDTA or SDS because we have observed that ribosome-free, thalassemic as well as non-

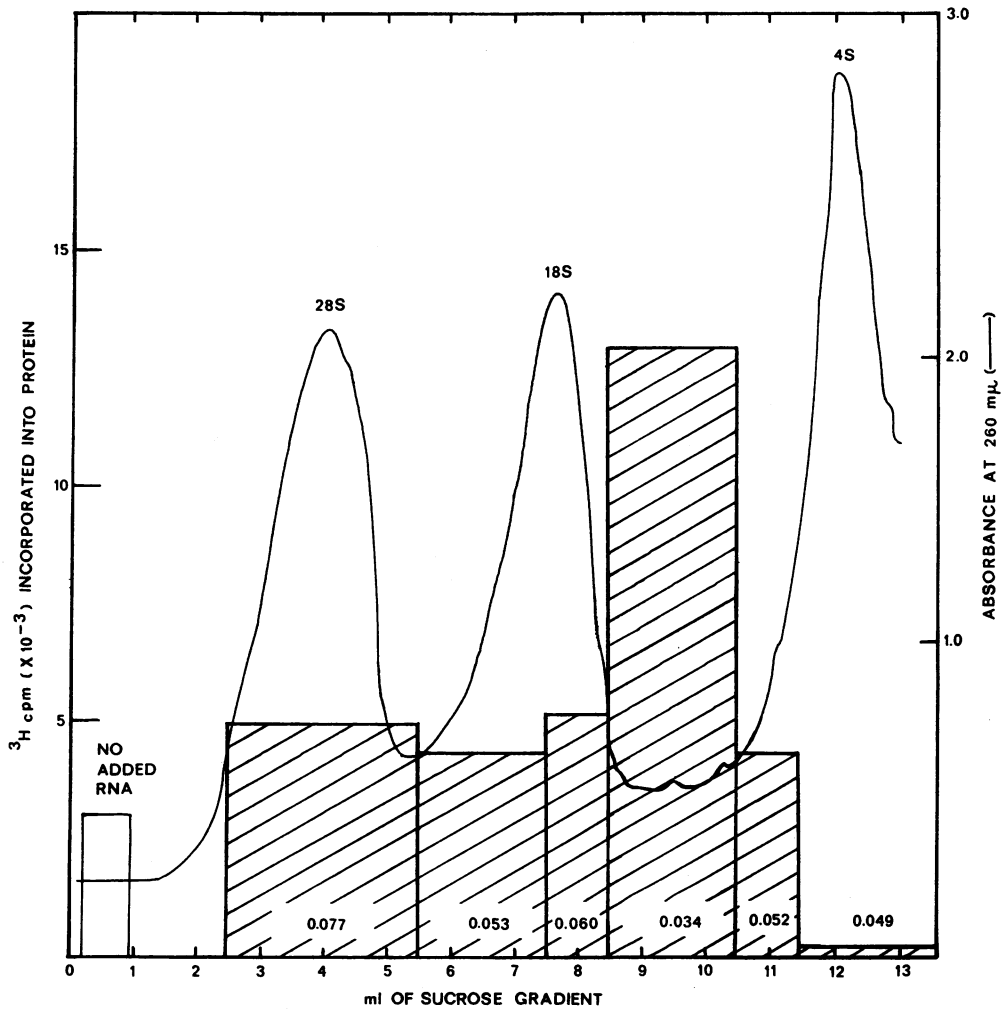

FIGURE 1 Sucrose gradient fractionation of beta thalassemia reticulocyte RNA. RNA was prepared from membrane-free lysate of beta thalassemic reticulocytes by phenol-cresol extraction (16, 17). 1.2 mg (30 A_{260} U) of RNA were fractionated by centrifugation through a 5–20% linear sucrose gradient containing 0.04 M Tris-acetate pH 7.2, 0.02 M sodium acetate, 2 mM EDTA, and 0.5% SDS (30,000 rpm for 15 hr at 4°C in a SW 40 rotor). 1 ml fractions were collected using an Isco (Instrumentation Specialties Co., Lincoln, Nebr.) model 182 gradient fractionator (3 ml/min), while the optical density at A_{260} (solid line) was continuously recorded by means of a Gilford 2400 spectrophotometer (Gilford Instrument Labs, Inc., Oberlin, Ohio.) (5 mm path length, model 203 flow through cuvette, chart speed 0.5 min/inch). The SDS was removed from the fractions by precipitation with KCl, and the RNA subsequently ethanol precipitated.

The crosshatched bars illustrate the capacity of 1.4–3 μg of RNA from each fraction (0.034–0.077 A_{260} U, as indicated in each bar) to stimulate incorporation of leucine-^{3}H into protein by a 50 μl reaction mixture of a Krebs mouse ascites tumor cell lysate system (see text).

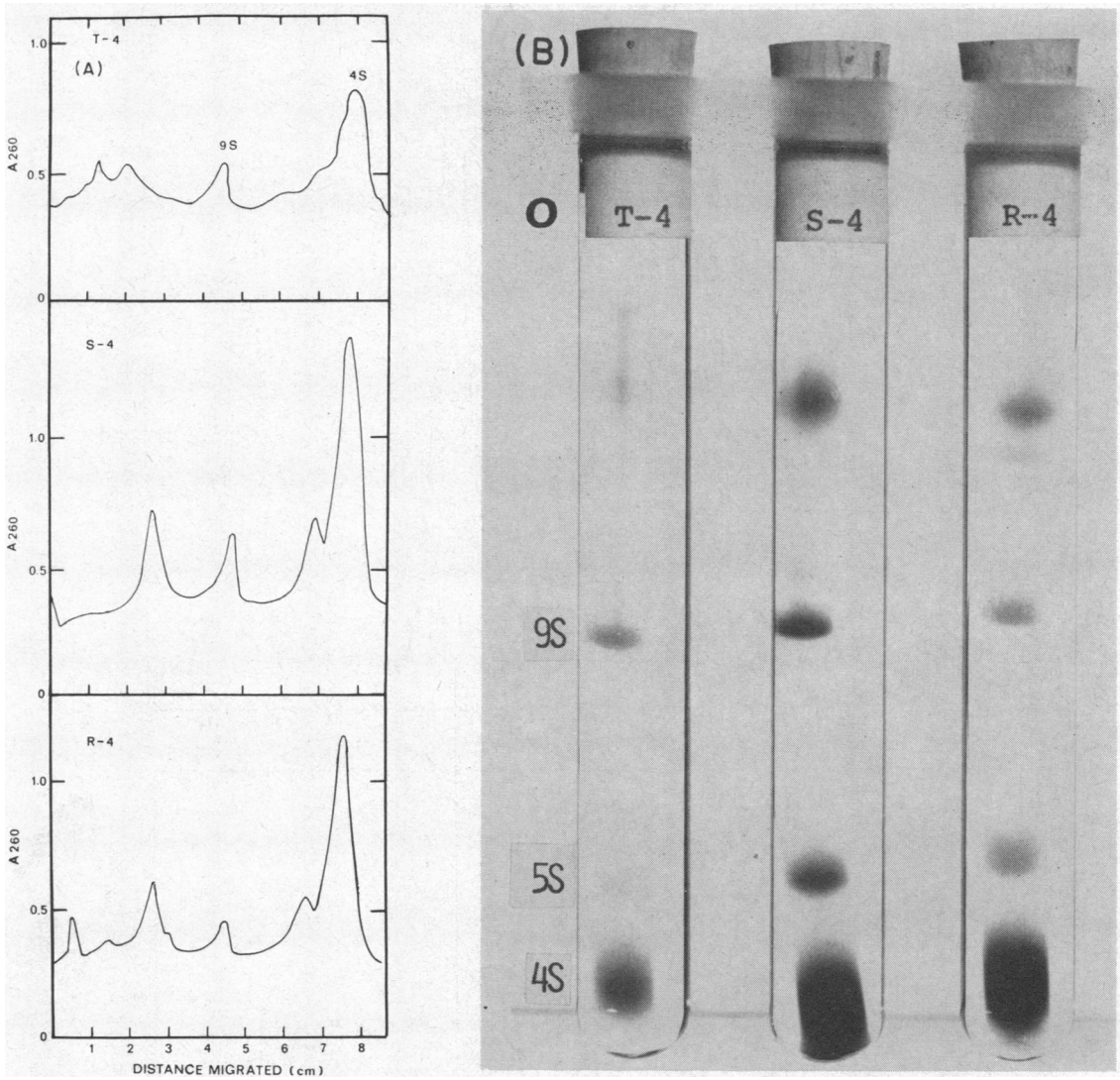

FIGURE 2 Acrylamide gel electrophoresis of mRNA fractions. The RNA of fraction 8.5–10.5 ml of Fig. 1 was analyzed by electrophoresis in 9 × 0.5 cm cylindrical gels of 5% acrylamide (5 ma/gel for 2 hr at 4°C in buffer A: 0.04 M tris-acetate, pH 7.2, 0.02 M sodium acetate, 2 mM EDTA) (22). (A) Densitometer scan of unstained gels at A_{260} using a Gilford 2400 spectrophotometer equipped with a model 2410 linear transport system (scan speed 2 cm/min, chart speed 1 min/inch, scanning slit aperture 0.1 × 2.36 mm). T-4, thalassemic mRNA 0.514 A_{260} U. S-4, sickle cell anemia mRNA 0.630 A_{260} U. R-4, similarly prepared rabbit reticulocyte mRNA 0.488 A_{260} U. (B) Photo of gels stained with methylene blue (23). O indicates the origin.

thalassemic, supernatants from human reticulocyte lysates, spun by centrifugation at 250,000 *g* for 3 hr, contain approximately 50% of the whole lysate 9S RNA (the presumed mRNA for hemoglobin: [19–21]). The amount of 9S RNA present in the human ribosome-free supernatants is much greater (as a per cent of the total soluble RNA) than in rabbit reticulocyte lysates. Human supernatant 9S RNA cannot be accounted for as being derived from unsedimented 40S ribosomal subunits since very little or no 18S ribosomal RNA is present in RNA preparations from the supernatants.[2] The human mRNA used in these experiments was obtained from the following sources: (*a*) sickle cell anemia mRNA: Pooled whole lysate RNA from 250 ml of peripheral blood from five different patients with sickle cell anemia having reticulocyte counts of 15–25% and hematocrits of 20–26% provided 8.2 mg of RNA. This was fractionated on three sucrose gradients after which the 9S fractions of each gradient were pooled. (*b*) beta thalassemia mRNA: Pooled whole lysate RNA from 300

[2] Forget, B. Unpublished results.

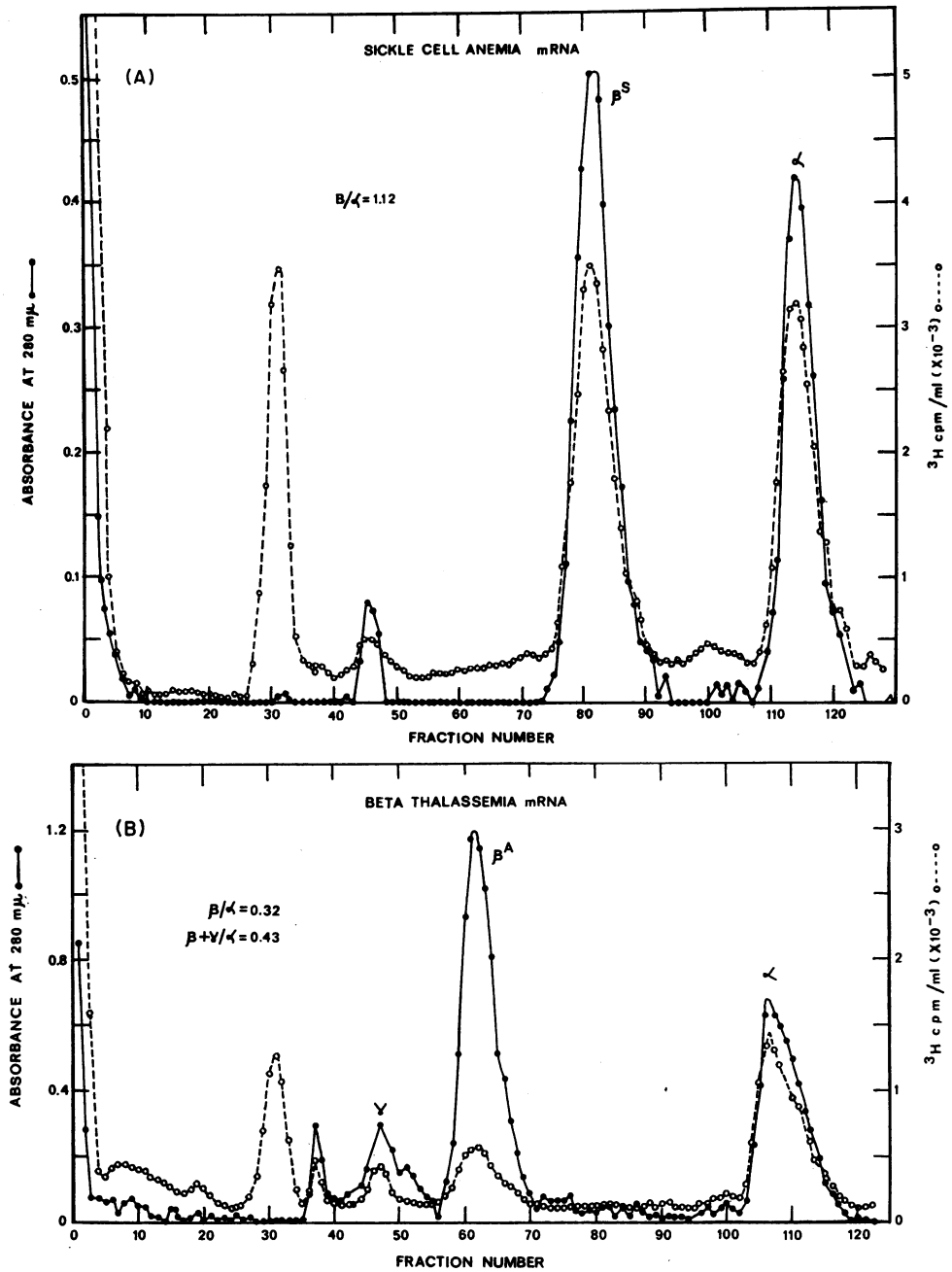

FIGURE 3 Carboxymethyl cellulose column chromatograms of radioactive protein synthesized in the presence of human reticulocyte mRNA. 350 μl Krebs ascites tumor cell lysate reaction mixtures (see text) were incubated for 40 min at 37°C in the presence of human reticulocyte mRNA. At the end of the incubation, the reaction mixture was chilled to 0°C and non-radioactive human hemoglobin was added in the form of membrane-free lysate. Globin was prepared by acid acetone precipitation, dialyzed against starting buffer for 3 hr, and applied to a 15 × 0.5 cm column of carboxymethyl cellulose (Whatman CM 52) eluted with a phosphate gradient in 8 M urea (27) (flow rate 25 ml/hr; fraction size 3.2 ml). The globin chains are identified by appropriate symbols above the peaks. The radioactive nonglobin peaks were observed when the system was incubated without added human reticulocyte messenger RNA but no radioactivity cochromatographed with human globin chains unless mRNA was added. (A) Reaction mixture incubated with 0.473 A_{260} U of sickle cell anemia mRNA, 50 mg of sickle cell hemoglobin added at the end of the incubation. (B) Reaction mixture incubated with 0.514 A_{260} U of beta thalassemia mRNA, 50 mg of nonthalassemic hemoglobin A and 50 mg of beta thalassemic hemoglobin added at the end of the incubation. (●——●, A_{280}, ○——○, leucine-^{3}H cpm/ml).

ml of peripheral blood from two unrelated patients with typical homozygous beta thalassemia, having reticulocyte counts of 6–10%, 20,000–80,000 nucleated red cells/mm^3 and hematocrits of 24–28% provided 3.6 mg of RNA. This was also fractionated on three sucrose gradients after which the 9S fractions of each gradient were pooled.

Acrylamide gel electrophoresis of RNA was carried out as described by Loening (*22*), using purified reagents obtained from Bio-Rad Laboratories, Richmond, Calif. The main gel contained 5% acrylamide and the spacer gel 3.0% acrylamide. Electrophoresis was carried out in buffer A at 5 ma/gel for 2 hr at 4°C. The unstained gels were scanned at A_{280} using a Gilford 2400 spectrophotometer (Gilford Instrument Labs, Inc., Oberlin, Ohio.) equipped with a linear transport device. They were then stained with methylene blue (*23*).

The cell-free system utilized was the Krebs mouse ascites tumor cell lysate system (*24, 25*). Such a preparation was kindly supplied to us by Dr. David Housman, Dr. Gary Temple, and Dr. Harvey Lodish of the Department of Biology, Massachusetts Institute of Technology, Cambridge, Mass. This system has very low endogenous activity and has been shown to synthesize rabbit and mouse globin chains when primed by rabbit or mouse reticulocyte mRNA (*25, 29*). The reaction mixtures contained per milliliter, 0.6 ml of ascites tumor cell lysate, 0.2 ml H_2O containing the mRNA to be tested, and 0.2 ml of a master mix containing the following components to yield the indicated final concentrations: *N*-2-hydroxyethylpiperazine-N′-2-ethane sulfonic acid (HEPES) buffer, pH 7.0 (26.6 mM), ATP (1.33 mM), guanosine 5′-triphosphate (0.26 mM), 19 nonradioactive amino acids minus leucine (0.067 mM each), magnesium acetate (2.33 mM), creatine phosphate (8.6 mM), creatine phosphokinase (60 U/ml), and tritiated leucine, specific activity 38.8 Ci/mmole, (75 μCi/ml). The mixture was incubated at 37°C for 40 min. Incorporation of radioactivity into protein was assayed by adding 50 μl of the reaction to 0.5 ml of 0.1 M KOH, incubating at 37°C for 15 min, then precipitating the protein by addition of 10 volumes of 10% trichloroacetic acid (TCA) at 0°C. The precipitate was collected on nitrocellulose filters, dried, and counted in a liquid scintillation counter with an efficiency of 22% for tritium.

For analysis of the end products synthesized by the cell-free system, 50–100 mg of nonradioactive hemoglobin (in the form of membrane-free lysate) was added to the reaction mixture at the end of the incubation, and globin prepared by acid-acetone precipitation. The globin was then fractionated by carboxymethyl cellulose column eluted by a phosphate gradient in 8M urea (*26, 27*). 1 ml portions of each sample were counted in Aquasol (New England Nuclear Corp., Boston, Mass).

RESULTS

The pattern obtained after sucrose gradient fractionation of beta thalassemia reticulocyte RNA is shown in Fig. 1. Similar profiles were obtained with whole reticulocyte RNA from patients with sickle cell anemia and from phenylhydrazine-treated rabbits. Fig. 2 shows the results of analytical acrylamide gel electrophoresis of the pooled mRNA fractions, the region of the sucrose gradient between 18S and 4S RNA (fractions 8.5–10.5 ml of Fig. 1). The mRNA contains a prominent discrete 9S RNA component, but also contains some 4S RNA, 5S RNA, and variable amounts of larger RNA components (12–15S) which are thought to result from degradation of ribosomal RNA (*19*).

The effect of addition of RNA from various fractions of the sucrose gradient on incorporation of radioactivity into protein in the Krebs ascites tumor cell system is shown by the crosshatched bars in Fig. 1. These are superimposed over the optical density profile of the RNA fractionation. The only significant stimulation was provided by the RNA fraction between the 18S and 4S RNA region of the gradient. The overall stimulation per μg of mRNA from nonthalassemic human reticulocyte RNA and rabbit reticulocyte mRNA was approximately double that obtained from a similar amount of beta thalassemia mRNA (data not shown).

The radioactive products produced by the cell-free systems are shown in Fig. 3A and B. In the presence of mRNA, derived from sickle cell reticulocytes, the radioactive proteins synthesized cochromatographed with the nonradioactive carrier sickle beta (β^s) chain and alpha chain (Fig. 3A). Nearly equal amounts of β^s and alpha chains were synthesized; the precise beta/alpha ratio obtained by summing the radioactivity obtained in each peak was 1.12, a value very similar to that obtained by incubating intact sickle cell anemia reticulocytes in the presence of radioactive leucine (*5, 6*).[3]

In the presence of beta thalassemia mRNA, the radioactive proteins synthesized cochromatographed with the nonradioactive carrier gamma, normal beta (β^A), and alpha chains (Fig. 3B). However, the amount of radioactive beta chain synthesized was much less than the amount of radioactive alpha chain, beta/alpha = 0.32; the synthesis of gamma chains did not compensate for this imbalance since gamma and beta/alpha was only 0.4. These results are similar to those obtained when intact beta thalassemia major reticulocytes are incubated in the presence of radioactive leucine (*2–7, 28*).

DISCUSSION

In a Krebs mouse ascites cell-free system where the only added human reticulocyte component is phenol extracted and SDS-sucrose density gradient purified reticulocyte mRNA, we have demonstrated that mRNA from patients with beta thalassemia major stimulates the synthesis of unequal amounts of beta and alpha globin chains (as in intact thalassemic reticulocytes), whereas mRNA from nonthalassemic reticulocytes (sickle cell anemia) stimulates the synthesis of nearly equal amounts of beta and alpha chains (beta/alpha = 1.12). These results provide direct evidence that the basic defect in

[3] Kan, Y. W. Personal Communication.

beta thalassemia resides in a deficient and/or functionally abnormal mRNA for beta globin chains.

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REFERENCES

1. Weatherall, D. J. 1965. The Thalassemia Syndromes. Blackwell Scientific Publications Ltd., Oxford.
2. Heywood, J. D., M. Karon, and S. Weissman. 1964. Amino acids: incorporation into α and β chains of hemoglobin by normal and thalassemic reticulocytes. *Science (Washington).* **146:** 530.
3. Heywood, D., M. Karon, and S. Weissman. 1965. Asymmetrical incorporation of amino acids in the alpha and beta chains of hemoglobin synthesized by thalassemic reticulocytes. *J. Lab. Clin. Med.* **66:** 476.
4. Weatherall, D. J., J. B. Clegg, M. A. Naughton. 1965. Globin synthesis in thalassemia: an in vitro study. *Nature (London).* **208:** 1061.
5. Bank, A., and P. A. Marks. 1966. Excess α chain synthesis relative to β chain synthesis in thalassemia major and minor. *Nature (London).* **212:** 1198.
6. Weissman, S. M., I. Jeffries, and M. Karon. 1967. The synthesis of alpha, beta and delta peptide chains by reticulocytes from subjects with thalassemia or hemoglobin Lepore. *J. Lab. Clin. Med.* **69:** 183.
7. Bank, A., A. S. Braverman, J. V. O'Donnell, and P. A. Marks. 1968. Absolute rates of globin synthesis in thalassemia. *Blood J. Hematol.* **31:** 226.
8. Itano, H. A. 1965. Abnormal Haemoglobins in Africa. Blackwell Scientific Publications Ltd., Oxford. 3.
9. Ingram, V. M. 1964. A molecular model for thalassemia. *Ann. N. Y. Acad. Sci.* **119:** 485.
10. Bank, A., and P. A. Marks. 1966. Protein synthesis in a cell-free human reticulocyte system: ribosome function in thalassemia. *J. Clin. Invest.* **45:** 330.
11. Clegg, J. B., D. J. Weatherall, S. Na-Nakorn, and P. Wasi. 1968. Hemoglobin synthesis in beta thalassemia. *Nature (London).* **220:** 664.
12. Rieder, R. F. 1971. Translation of beta chain mRNA in beta thalassemia. *J. Clin. Invest.* **50:** 77a. (Abstr.)
13. Gilbert, J. M., A. G. Thornton, A. Nienhuis, and W. F. Anderson. 1970. Cell-free hemoglobin synthesis in beta thalassemia. *Proc. Nat. Acad. Sci U. S. A.* **67:** 1854.
14. Nienhuis, A. W., D. G. Laycock, and W. F. Anderson. 1971. Translation of rabbit hemoglobin messenger RNA by thalassemic and non-thalassemic ribosomes. *Nature New Biology.* **231:** 205.
15. Nathan, D. G., H. Lodish, Y. W. Kan, and D. Housman. 1971. Beta thalassemia and translation of globin RNA. *Proc. Nat. Acad. Sci. U. S. A. messenger* **68:** 2514.
16. Parish, J. H., and K. S. Kirby. 1966. Reagents which reduce interactions between ribosomal RNA and rapidly labeled RNA from rat liver. *Biochim. Biophys. Acta.* **129:** 554.
17. Loening, U. E. 1969. The determination of the molecular weight of ribonucleic acid by polyacrylamide-gel electrophoresis. *Biochem. J.* **113:** 131.
18. Heywood, S. M., and M. Nwagwu. 1969. Partial characterization of presumptive myosin messenger ribonucleic acid. *Biochemistry.* **8:** 3839.
19. Labrie, F. 1969. Isolation of an RNA with the properties of hemoglobin messenger. *Nature (London).* **221:** 1217.
20. Chantrenne, H., A. Burny, and G. Marbaix. 1967. The search for the messenger RNA of hemoglobin. *Prog. Nucl. Acid Res. Mol. Biol.* **7:** 173.
21. Lockard, R. F., and J. B. Lingrel. 1969. The synthesis of mouse hemoglobin beta chains in a rabbit reticulocyte cell-free system programmed with mouse reticulocyte 9S RNA. *Biochem. Biophys. Res. Commun.* **37:** 204.
22. Loening, U. E. 1967. The fractionation of high molecular weight ribonucleic acid by polyacrylamide gel electrophoresis. *Biochem. J.* **102:** 251.
23. Peacock, A. C., and C. W. Dingman. 1967. Resolution of multiple ribonucleic acid species by polyacrylamide gel electrophoresis. *Biochemistry.* **6:** 1818.
24. Mathews, M. B., and A. Korner. 1970. Mammalian cell-free protein synthesis directed by viral ribonucleic acid. *Eur. J. Biochem.* **17:** 328.
25. Housman, D., R. Pemberton, and R. Taber. 1971. Synthesis of alpha and beta chains of rabbit hemoglobin in a cell-free system from Krebs II ascites cells. *Proc. Nat. Acad. Sci. U. S. A.* In press.
26. Clegg, J. B., M. A. Naughton, and D. J. Weatherall. 1965. An improved method for the characterization of human hemoglobin mutants: identification of $\alpha_2\beta_2$ 95 glu, hemoglobin N (Baltimore). *Nature (London).* **207:** 945.
27. Kan, Y. W., E. Schwartz, and D. G. Nathan. 1968. Globin chain synthesis in the alpha thalassemia syndromes. *J. Clin. Invest.* **47:** 2515.
28. Kan, Y. W., and D. G. Nathan. 1970. Mild thalassemia: the result of interactions of alpha and beta thalassemia genes. *J. Clin. Invest.* **49:** 635.
29. Mathews, M. B., M. Osborn, and J. B. Lingrel. 1971. *Nature New Biology.* **233:** 206.