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Isolation and Translation of Hemoglobin Messenger RNA from Thalassemia, Sickle Cell Anemia, and Normal Human Reticulocytes

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ABSTRACT Human hemoglobin messenger RNA was isolated by sucrose gradient centrifugation from reticulocytes of patients having various hemolytic anemias. Using a messenger RNA-dependent cell-free system derived entirely from rabbit reticulocytes, the human hemoglobin messenger RNA has been translated and the products analyzed by carboxymethylcellulose column chromatography. Normal messenger RNA directs synthesis of normal human α- and β-globin chains in nearly equal amounts. Sickle cell anemia messenger RNA directs the synthesis of normal α- and sickle β-chains. β-thalassemia messenger RNA directs the synthesis of normal α- and β-chains, but the amount of β-globin synthesized is markedly reduced. Thus the inability of the thalassemia reticulocyte to produce β-globin is clearly attributable to the β-globin messenger RNA.

INTRODUCTION

Depressed synthesis of the β-globin chain of hemoglobin in homozygous β-thalassemia has been well established (1). Bank and Marks first proposed a defect in the messenger RNA (mRNA)[1]-ribosome complex in β-thalassemia (2). Our previous studies have shown depressed synthesis specifically of β-globin by the mRNA-ribosome complex from thalassemia cells (3), but normal translation of rabbit α- and β-globin mRNA's by thalassemia ribosomes in a cell-free system (4). In the experiments to be described here we have isolated human hemoglobin mRNA and developed a mRNA-dependent cell-free system from rabbit reticulocytes. This experimental procedure was utilized in order to investigate whether or not mRNA is directly responsible for the depressed β-globin synthesis by thalassemia erythroid cells.

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

METHODS

Cell-free protein synthesis system. Preparation of DEAE-treated ribosomal wash fraction (containing the initiation factors), supernatant enzyme fraction, and transfer RNA (tRNA) from phenylhydrazine-induced rabbit reticulocytes was as previously described (5, 6). Assays were performed in a 0.1 ml reaction mixture and contained: Tris-HCl, (pH 7.5), 20 mM; $MgCl_2$, 3.75 mM; KCl, 90 mM; dithiothreitol, 1 mM; ATP, 1.5 mM; GTP, 0.3 mM; phosphoenolpyruvate, 2.25 mM; pyruvate kinase, 0.05 IU; amino acids other than leucine, 10 μM; and leucine-^{14}C (331 mCi/mmole), 5 μM; 300 μg of supernatant enzyme fraction, 200 μg of DEAE-treated ribosomal wash fraction; 0.16 OD_{260} U of rabbit reticulocyte tRNA; 0.03–0.09 OD_{260} U of mRNA; and 0.15 OD_{260} U (rate-limiting) of ribosomes. The reaction was linear for 50 min. Determination of hot trichloroacetic acid precipitable counts was as previously described (5, 6).

Preparation of mRNA-dependent rabbit reticulocyte ribosomes. Polysomes were prepared by centrifugation from the lysate fraction (5) and were diluted to 30 OD_{260} U/ml in 20 mM Tris-HCl, pH 7.5. Pancreatic ribonuclease A (0.1 μg/ml) was added and the mixture was incubated for 20 min at 37°C. Selective digestion of mRNA occurs under these conditions (7). The solution was layered over 25% sucrose containing KTM buffer (KCl, 500 mM; Tris-HCl, pH 7.5, 50 mM; $MgCl_2$, 2 mM) and centrifuged at 386,000 × g for 90 min at 2°C. The ribosome pellet was suspended in KTM buffer, puromycin was added to 2.5 mM, and the mixture was incubated for 20 min at 37°C. Centrifugation was performed as described above. This pellet was suspended in 0.25 M sucrose, 0.1 mM EDTA, and 1 mM dithiothreitol, and stored in small portions in liquid nitrogen. The endogenous activity of ribosomes prepared in this manner is approximately 0.2% of the activity of standard salt-washed reticulocyte ribosomes (5).

Preparation of human hemoglobin mRNA. Heparinized blood was obtained from two siblings with homozygous β-thalassemia (N. L. and J. L.), two patients with sickle cell anemia (J. W. and H. Y.), and a patient with autoimmune hemolytic anemia (D. H.). Human reticulocyte polysomes were prepared as previously described (3). Polysomes (50 OD_{260} U/ml) were incubated with 0.5% sodium dodecyl sulfate (SDS) for 5 min at 37°C and the 8-16S RNA fraction isolated by sucrose gradient centri-

fugation (8) (Fig. 1A). This fraction was made 0.3 M in KCl and 2 volumes of ethanol were added. After 8 hr at −20°C the precipitated RNA was collected by centrifugation, dissolved in 5 mM Tris-HCl, pH 7.5, and again incubated in 0.5% SDS for 5 min at 37°C. Sucrose gradient centrifugation was again performed, the 9S peak isolated (Fig. 1B), dialyzed, lyophilyzed, dissolved in water, and stored in liquid nitrogen.

Product analysis by carboxymethylcellulose chromatography. Six identical 0.1 ml reaction mixtures saturated in the mRNA to be tested and containing leucine-^{3}H (17,000 mCi/mmole) were incubated. Addition of leucine-^{14}C uniformly labeled globin obtained from intact thalassemia reticulocytes, chromatography in phosphate-urea buffer, and double-label scintillation counting of the radioactive globin products were as previously described (3, 4).

RESULTS

Protein synthesis under the standard incubation conditions is dependent on added 9S mRNA (Fig. 2). When several different human and rabbit 9S mRNA fractions were tested, a range of 0.03–0.09 OD_{260} U of RNA was required to saturate the standard 0.1 ml reaction mixture. Maximal protein synthesis ranged from 3 to 10 pmoles of leucine-^{14}C incorporated into protein in a 40 min incubation. There is negligible leucine-^{14}C incorporation into protein in the absence of ribosomes. In the absence of added mRNA, product analysis demonstrates the synthesis of only rabbit α- and β-globin chains in small amounts.

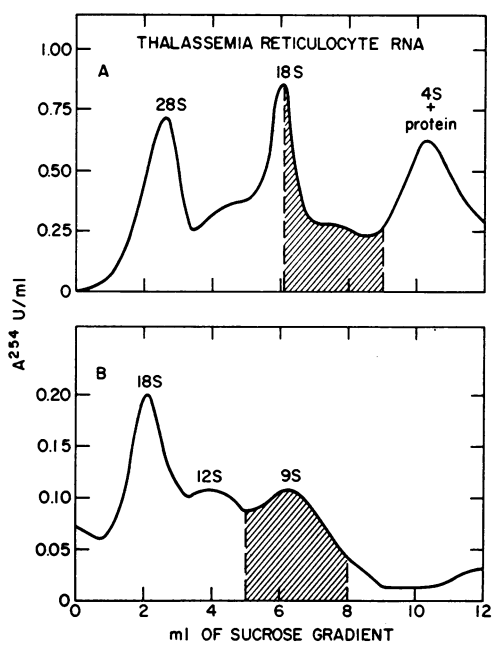

FIGURE 1 Fractionation of thalassemia reticulocyte RNA. A. Polysomes treated with SDS (see Methods) were layered on six identical 12.5 ml exponential 5–22% sucrose gradients and centrifuged at 41,000 rpm for 10 hr at 2°C in an SW-41 rotor. The indicated fractions containing 8–16S RNA from each of the six gradients were pooled. B. The concentrated 8–16S fraction was layered on three identical exponential 5–18% sucrose gradients and centrifuged at 41,000 rpm for 15 hr at 2°C. The 9S peak from each gradient was pooled (see Methods) and utilized for subsequent studies.

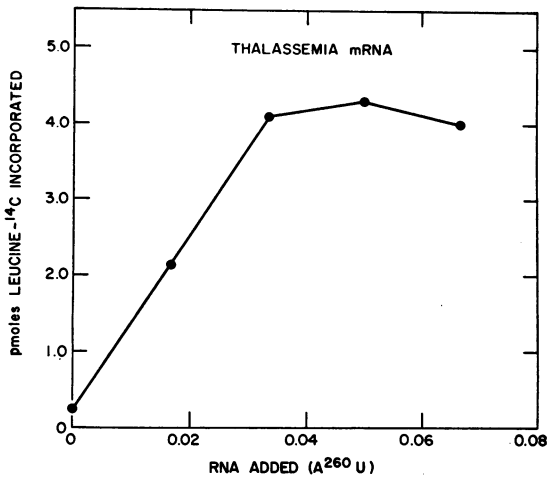

FIGURE 2 Concentration curve for the translation of thalassemia mRNA in the rabbit mRNA-dependent cell-free system. The RNA added was the 9S fraction shown in Fig. 1B. (0.02 OD_{260} U of RNA is approximately equal to 1 μg.)

Fig. 3A illustrates the translation of thalassemia mRNA on mRNA dependent rabbit ribosomes. An imbalance in the ratio of α- to β-globin chains is produced similar to that seen in the intact thalassemia reticulocyte. Fig. 3B illustrates the translation of sickle cell anemia mRNA; β^{S}- and α^{A}-globin chains are produced. In separate experiments it has been demonstrated that the β^{S}-globin produced in the mRNA-dependent cell-free system cochromatographs with β^{S}-globin made in the intact reticulocyte both on column chromatography and by tryptic digestion and fingerprint analysis. mRNA isolated from autoimmune hemolytic anemia reticulocytes direct synthesis of β^{A}- and α^{A}-globin (data not shown). When mRNA other than that isolated from thalassemia cells is translated in the cell-free system, 10–40% more β- than α-globin is produced similar to the result obtained in our previous studies using rabbit hemoglobin mRNA (4).

DISCUSSION

We have described the isolation of a specific human messenger RNA and its use in the elucidation of the mechanism of a human disease. Our data indicate that the depressed synthesis of β-globin in homozygous β-thalassemia is due to a mutation affecting the amount or sequence of the mRNA. Whether this is true for all the varied thalassemia syndromes remains to be determined.

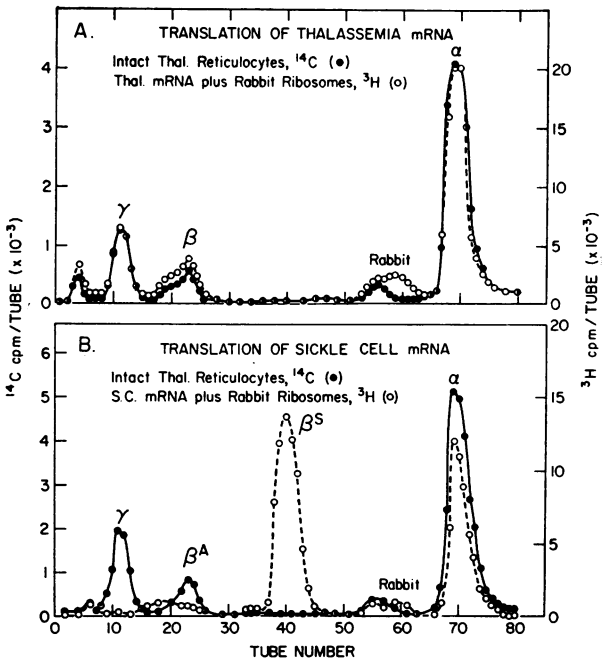

FIGURE 3 Chromatography of the products from the translation of human hemoglobin mRNA on rabbit reticulocyte ribosomes (see Methods). The human globin peaks are labelled: α, β, and γ. The rabbit peak, labeled "Rabbit," includes both the rabbit α- and β-globin chains. A. Translation of thalassemia mRNA. Six 0.1 ml reaction mixtures, each containing leucine-^{3}H and 0.053 OD_{260} U of the thalassemia 9S fraction shown in Fig. 1B, were pooled. After the incubation, leucine-^{14}C uniformly labeled globin isolated from intact thalassemia reticulocytes which had been incubated with leucine-^{14}C, was added. B. Translation of sickle cell anemia mRNA. Six 0.1 ml reaction mixtures, each containing leucine-^{3}H and 0.042 OD_{260} U of sickle cell anemia 9S fraction, were pooled. After the incubation, leucine-^{14}C uniformly labeled globin isolated from intact thalassemia reticulocytes was added just as in Fig. 3A above.

Translation of a given mRNA depends on the attachment of ribosomes to the mRNA (initiation), addition of amino acids sequentially to the growing peptide chain (elongation), and release of the completed protein from the ribosome (termination). Thus decreased synthesis of the β-globin chain in homozygous β-thalassemia could be due to a mutation in the β-mRNA slowing initiation, elongation, or termination, or could be due to a reduction in the amount of β-globin mRNA.

Elongation and release of β-globin chains have been reported to be normal in the intact β-thalassemia cell (9, 10). In the cell-free system we have described, mRNA is added independently and therefore each globin chain produced must be newly initiated. We have not yet found a defect in the initiation step involving the thalassemia β-mRNA. The most likely explanation for the molecular basis of β-thalassemia, therefore, would appear to be that the thalassemia β-globin mRNA is produced in the erythroid cell in reduced amount by virtue of a mutation affecting transcription from the β-chain structural gene.

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