

Globin Messenger RNA in Hemoglobin H Disease

By Edward J. Benz, Jr., Paul S. Swerdlow, and Bernard G. Forget

Functional messenger RNA (mRNA) for human globin synthesis was isolated from reticulocytes of each of two patients with hemoglobin H disease. The RNA was tested for its capacity to direct globin synthesis in a messenger RNA-dependent cell-free system derived from Krebs Type II mouse ascites tumor cells. In each case, hemoglobin H disease mRNA directed the synthesis of a great excess of β -globin chains relative to α -globin chains of hemoglobin A. The β/α synthetic ratios obtained in the cell-free system at saturating concentrations of mRNA were >22 and >15 , respectively, for the two hemoglobin H disease mRNA preparations, whereas

the β/α synthetic ratios obtained by incubation of intact reticulocytes from these same patients were 2.6 and 2.8, respectively. The β/α synthetic ratio obtained in the cell-free system did not vary when lower concentrations of hemoglobin H disease mRNA were used. A marked decrease in the amount of functional α -globin-chain mRNA relative to β -chain mRNA is therefore associated with the decreased α -chain synthesis observed in hemoglobin H disease. This decrease in α -chain-specific mRNA activity is greater than expected from the β/α synthetic ratio of intact reticulocytes in hemoglobin H disease.

HEMOGLOBIN H DISEASE is a form of α -thalassemia which results from the interaction of two α -thalassemia genes of different severity: the readily detectable α -thalassemia₁ gene and the barely recognizable α -thalassemia₂ (or silent carrier) gene.¹ The human α -chain locus is probably duplicated in which case the α -thalassemia₂ trait would result from involvement of only one out of four α -chain genes; the α -thalassemia₁ trait, from involvement of two α -chain genes (*in cis*); and Hb H disease, from involvement of three out of the four α -chain genes.¹

Peripheral blood reticulocytes from patients with hemoglobin H disease in-

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Abbreviations used: mRNA, messenger RNA; SDS, sodium dodecyl sulfate

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cubated in the presence of ^{14}C -leucine, synthesize approximately 2–3 times more β -globin chain than α -globin chain of Hb A.^{2,3} These excess β chains combine to form β_4 tetramers which constitute hemoglobin H, the precipitation of which is important in the pathophysiology of the hemolytic anemia observed in this disorder.^{4–6}

In homozygous β -thalassemia, the opposite occurs: There is decreased β -chain relative to α -chain synthesis, and α -chain precipitates accumulate in the cells. The molecular pathology of β -thalassemia has been studied in great detail, and a deficiency of functional β -chain mRNA has been identified in β -thalassemia reticulocytes: We,⁷ as well as Nienhuis and Anderson,⁸ have demonstrated that mRNA isolated from β -thalassemia reticulocytes will direct the synthesis, in a heterologous cell-free system, of much less β chain than α chain. We now report the isolation of functional globin mRNA from two patients with hemoglobin H disease and the capacity of this mRNA to direct globin chain synthesis in a heterologous cell-free system capable of translating added exogenous mRNA. In this system, at rate-limiting concentrations of mRNA, non-thalassemic human globin mRNA directs the synthesis of nearly equal amounts of α and β chains of Hb A, while β -thalassemia mRNA directs the synthesis of much less β than α chain. In contrast, mRNA from the patients with Hb H disease directs the synthesis of a great excess of β chains of Hb A, compared to little or no detectable α -chain synthesis. These results indicate that the deficient α -chain synthesis which occurs in hemoglobin H disease reticulocytes is due to the presence in these cells of a decreased amount of functional mRNA for α -chain synthesis. Similar observations have been made in one other case of hemoglobin H disease.⁹

MATERIALS AND METHODS

Patients

The two patients are unrelated, young adult males of Mediterranean origin with hemoglobin H disease documented by hematologic and family studies. Patient one (J.M.) is virtually asymptomatic and has not had a splenectomy. He has a Hb of 9.5–10.5 g/100 ml; MCV 61 cu μ ; MCH 19 pg, and MCHC 29%, with a reticulocyte count of 5–10%. Patient 2 (A.R.) has had a more symptomatic course and has had a splenectomy for moderate hemolytic anemia. His Hb is 9–10 g/100 ml; MCV 67 cu μ ; MCH 19 pg, and MCHC 28.5%, with 4–10% reticulocytes. Both patients, in addition to 15% Hb H, have low normal levels of Hb A₂ and F on hemoglobin electrophoresis, and no other abnormal hemoglobin. Family study reveals, in each case, one parent with classic α -thalassemia one trait, whereas the other parent is hematologically normal.

Preparation of Human Globin mRNA

The mRNA was prepared as previously described⁷ by phenol-cresol extraction of membrane-free reticulocyte lysates, and fractionated by sucrose density-gradient centrifugation in the presence of 0.5% SDS. The fractions containing RNA sedimenting between 4S and 18S RNA were pooled, made 0.3 M with respect to NaCl and the RNA allowed to precipitate overnight at -20°C after addition of 3 volumes of 95% ethanol. The RNA was then pelleted, resuspended in 0.2 M KCl, and the residual potassium-SDS precipitate was removed by centrifugation. After a second ethanol precipitation, the RNA was dissolved in H_2O at a concentration of 1–2 mg/ml and stored at -20°C .

Rabbit reticulocyte tRNA was prepared in a similar manner, from membrane-free reticulocyte lysates obtained from phenylhydrazine-treated rabbits. The 4S RNA region of the sucrose gradient fractionation was then recovered as described above and shown to be free of 10S globin mRNA by acrylamide gel electrophoresis.

Rabbit Reticulocyte Ribosomal Salt Wash Fraction

Rabbit reticulocyte polyribosomes were treated with KCl (final concentration 0.5 M) as previously described.^{10,11} The crude KCl wash fraction was then twice treated with DEAE cellulose to remove mRNA and tRNA,¹¹ and concentrated by precipitation with 68% ammonium sulfate.¹² The precipitate was resuspended at one-tenth of its original volume in 25 mM Tris-HCl, pH 7.0, 200 mM KCl, 1 mM DTT, 0.1 mM EDTA, and dialysed for 12 hr against the same buffer (A.W. Nienhuis, personal communication). After centrifugation at 15,000 g for 15 min, the supernatant solution was stored in small volumes at -80° until used. Rabbit ribosomal salt wash fraction has been shown to increase the efficiency of protein synthesis by the Krebs Type II mouse ascites tumor cell cell-free system.^{13,14}

Cell-Free Protein Synthesizing System

Krebs Type II mouse ascites tumor cell lysates were prepared and preincubated to reduce endogenous protein synthesizing activity, according to the technique of Mathews and Korner.¹⁵

Reaction mixtures containing 225 μ l were used for subsequent analysis of the products of protein synthesis by carboxymethyl-cellulose column chromatography. Optimal conditions for protein synthesis were determined in a separate set of experiments and will be described elsewhere. Each 225 μ l of reaction mixture contained 54 μ l of mouse ascites tumor cell extract (S30), 0.75–1.0 A₂₆₀ U of rabbit reticulocyte tRNA, 30 μ l of concentrated ribosomal salt wash fraction (4.6 A₂₆₀/5.4 A₂₈₀ U/ml), and the following components in the indicated final concentration: HEPES buffer (27 mM), MgCl₂ (3.4 mM), KCl (85 mM), 19 nonradioactive amino acids minus leucine (0.13 mM each), tritiated leucine [New England Nuclear, 38.8 Ci/mmol specific activity] (47 μ Ci/ml), ATP (1.0 mM), GTP (0.11 mM), CTP (0.6 mM), creatine phosphate (12 mM), and creatine phosphokinase (100 IU/ml). Messenger RNA was added to the final concentrations indicated in the text.

The mixtures were incubated for 40 min at 37°C. Ten microliter portions were then removed, and the amount of protein synthesis assayed by measuring the incorporation of radioactivity into 5% trichloroacetic acid precipitable material as previously described.⁷

The remaining 215 μ l of the reaction mixture were mixed with 80 mg of nonradioactive human hemoglobin in the form of membrane-free hemolysate; globin was prepared from the mixture by acid-acetone precipitation. The mixture was then fractionated by carboxymethylcellulose chromatography in the presence of 8 M urea^{3,7,16}; optical density and radioactivity were then measured in each fraction to identify the nonradioactive marker globin peaks and the radioactive products of the cell-free synthesis.

Intact Cell Globin Synthesis

Globin synthesis in intact reticulocytes was measured by incubating 3 ml of peripheral blood with 6 mg D-glucose and 10 μ Ci of ¹⁴C-leucine (New England Nuclear, 266 mCi/mmol) for 2 hr at 37°C. After incubation, the red cells were washed four or five times with 0.9% NaCl, and lysed in 5 volumes of 5 mM MgCl₂; tonicity was restored by adding 1 volume of a solution of 1.5 M sucrose and 0.15 M KCl and stroma removed from the lysate by centrifugation for 30 min at 20,000 g. Globin was then prepared from the supernatant lysate and analyzed by carboxymethyl-cellulose chromatography.

RESULTS

The mRNA obtained by sucrose gradient fractionation of reticulocyte RNA from the patients with Hb H disease was tested for its capacity to direct globin synthesis in the mRNA-dependent Krebs Type II mouse ascites tumor cell lysate system. Figure 1 shows the effect of increasing amounts of Hb H disease mRNA (from patient 1) on total protein synthesis by the cell-free system. At lower concentrations of mRNA, there is a linear increase in protein synthesis with increasing concentrations of mRNA. At higher mRNA concentrations, the activity tends to reach a plateau value. At the highest mRNA concentration

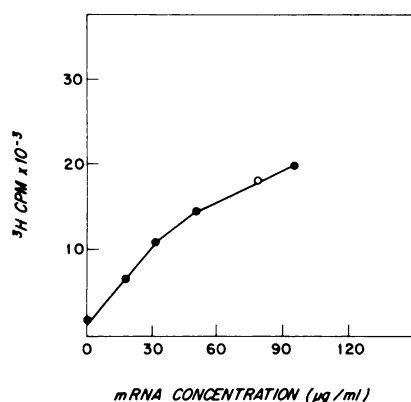


Fig. 1. Stimulation of protein synthesis in the Krebs mouse ascites cell-free system by Hb H disease mRNA. Activity is expressed as total ^3H leucine cpm incorporated into protein in a $10\ \mu\text{l}$ cell-free incubation mixture. The solid circles refer to mRNA from patient No. 1 (see Fig. 2B) and the β/α -globin synthetic ratios for each point are listed in Table 1. The open circle refers to mRNA from patient No. 2 (see Fig. 3B).

used, a 12-fold stimulation of protein synthesis was observed. Concentration curves with similar slopes were obtained with mRNA from normal (nonthalasemic) patients, sickle cell anemia patients, and patients with sickle cell β -thalassemia and homozygous β -thalassemia. The Hb H mRNA was nearly as efficient on a μg -to- μg basis as the nonthalassemic mRNA in stimulating total protein synthesis in the cell-free system; the maximum amount of protein synthesis achieved at the highest mRNA concentration tested was approximately the same with Hb H disease mRNA as with nonthalassemic mRNA.

The protein synthesized by the cell-free system in the presence of mRNA from the two patients with Hb H disease was analyzed by carboxymethyl-cellulose column chromatography. The results are shown in Figs. 2 and 3. Intact reticulocytes from these same patients synthesized less α than β chain, as expected; the β/α ratios were 2.6 (patient 1), and 2.8 (patient 2), Figs. 2A and 3A). These values are comparable to those previously obtained by others with Hb H disease reticulocytes.^{2,3} Significantly different β/α synthetic ratios were obtained in the cell-free system in the presence of mRNA from these patients (Fig. 2B and 3B). The β/α ratios obtained were 25.6 and 15.6. The figure for α -chain radioactivity used to calculate this ratio was obtained by summing the radioactivity of the fractions in the α -chain optical density peak after subtracting from each fraction an amount of radioactivity equal to the average radioactivity of the baseline fractions on either side of the α -chain peak. This figure represents a maximum estimate of α -chain radioactivity because it includes some radioactivity contributed by a small "pre- α " peak of material which elutes just prior to the α -chain peak. This radioactive pre- α peak was also present when control reaction mixtures incubated without added mRNA were chromatographed by the same method (data not shown). It is thought that this peak may represent a small amount of rabbit globin synthesis, stimulated by small amounts of residual rabbit mRNA present in the salt wash fraction; other possibilities include synthesis of nonglobin protein endogenous to the system or stimulated by the salt wash.

From these experiments, it is concluded that the imbalance of α - to β -chain synthesis observed in Hb H disease reticulocytes is associated with a deficiency in functional mRNA for α -chain synthesis in these cells. In addition, the

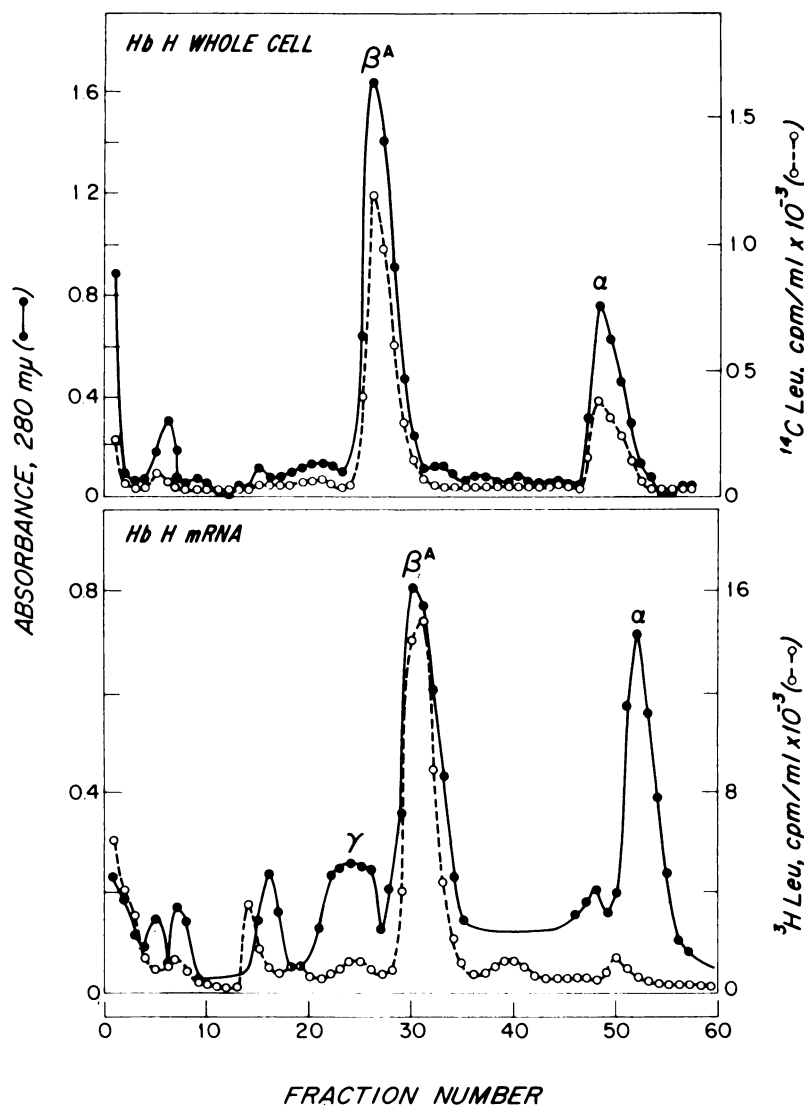


Fig. 2. (A) (Top panel). Globin synthesis by intact reticulocytes of patient No. 1 with Hb H disease. (B) (Bottom panel). Globin synthesis in the Krebs mouse ascites cell-free system directed by mRNA isolated from reticulocytes of the same patient. The cell-free incubation mixture (225 μ l) contained 0.48 A₂₆₀ U of mRNA (highest mRNA concentration shown in Fig. 1) and 80 mg of hemoglobin A and F were added as markers prior to carboxymethylcellulose column chromatography.

imbalance observed in the cell-free system is much greater than in the intact reticulocytes of the same patients. In the intact cells, there is roughly three times more β -chain synthesis than α -chain synthesis, whereas, in the cell-free system, there is 15–25 times more β -chain than α -chain synthesis.

In separate experiments, normal (nonthalassemic) human globin mRNA was incubated in the cell-free system, and analyzed by carboxymethyl-cellulose

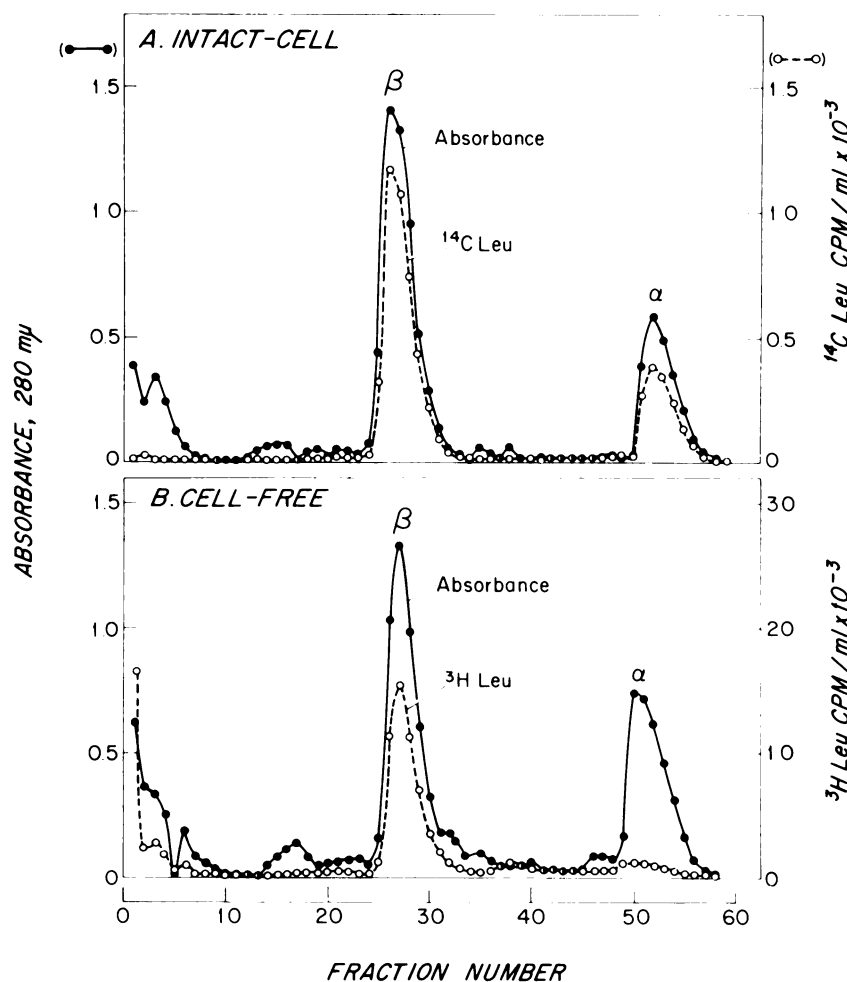


Fig. 3. (A) Globin synthesis by intact reticulocytes of patient No. 2 with Hb H disease. (B) Globin synthesis in cell-free system directed by mRNA from the same patient. The conditions were the same as in Fig. 2 except that 0.34 A₂₆₀ U of mRNA were added to the 225 μ l cell-free incubation (mRNA concentration shown by the open circle in Fig. 1).

chromatography, or by acrylamide gel electrophoresis (the latter method gave β/α ratios similar to those of the carboxymethyl-cellulose columns). The range of concentrations studied was the same as that shown in Fig. 1. The same reaction mixtures which provided the data shown in Fig. 1 for Hb H disease mRNA were also analyzed by CMC column chromatography. The β/α ratios obtained with Hb H disease mRNA at each concentration are compared to those obtained with nonthalassemic (Hb A) mRNA in Table 1. At all concentrations studied, Hb H disease mRNA directs the synthesis of much less α than β chain. The β/α ratios were greater than 22 at all concentrations studied. In contrast, the β/α ratio was 1.0 with the nonthalassemic mRNA at low mRNA concentrations, but with increasing concentrations of Hb A mRNA, preferential synthesis of β chains occurred, and the β/α ratios increased to a value of 2.2. The ratio never increased above 2.2, even at concentrations of Hb A mRNA greater than

Table 1. α - and β -Chain Synthesis in Cell-free System

mRNA Conc. ($\mu\text{g/ml}$)	$^3\text{H cpm} \times 10^{-3}/\text{ml Reaction Mix}$		β/α Ratio
	α Chain	β Chain	
Nonthalasemic mRNA ($\mu\text{g/ml}$)			
14	697	725	1.04
35	936	1092	1.16
56	1032	1809	1.80
105	1015	2235	2.20
Hemoglobin H disease mRNA (patient 1)			
14	25	591	22.9
35	49	1168	23.3
56	45	1469	24.2
105	66	1714	25.6

α - and β -globin chain synthesis directed by different concentrations of human nonthalasemic, and Hb H disease mRNA in the Krebs Type II cell-free system. The nonthalasemic mRNA was isolated from a patient with Hb A and hemolytic anemia due to paroxysmal nocturnal hemoglobinuria. The Hb H disease mRNA was from patient No. 1 (J.M.). The mRNA concentrations shown refer to total RNA of the sucrose gradient purified mRNA fraction ($1 \mu\text{g} = 0.025 A_{260} \text{ U}$) added per 1 ml of cell-free incubation mixture. The globin chains were separated by acrylamide gel electrophoresis (nonthalasemic mRNA) or by carboxymethylcellulose column chromatography (Hb H disease mRNA). The cpm shown have been corrected for differences in counting efficiency between the two systems and refer to the total $\text{cpm} \times 10^{-3}$ in α - and β -globin chains in a 1-ml cell-free incubation mixture.

those shown in Table 1. The results listed in Table 1 show that the total amount of α -chain synthesis obtained with Hb H disease mRNA is much less than that obtained with nonthalasemic mRNA at all mRNA concentrations studied, whereas the amount of β -chain synthesis was roughly the same with both types of mRNA.

DISCUSSION

In a heterologous cell-free protein synthesizing system in which the only added human component is Hb H disease mRNA, much less human α -globin chain than β chain was synthesized at all mRNA concentrations studied; in contrast, normal (nonthalasemic) human globin mRNA directed α - and β -chain synthesis in much more balanced proportions. In the presence of a low concentration of mRNA at which normal mRNA directed equal synthesis of α and β chains, Hb H disease mRNA stimulated abundant β -chain synthesis, but virtually no α -chain synthesis resulted. These results strongly suggest that the decreased α -chain synthesis which occurs in hemoglobin H disease is due to a decrease in the amount of functional globin mRNA for α chain synthesis.

Even though the cell-free system favors a 2-2.5-fold excess of β -chain synthesis in the presence of high concentrations of normal mRNA, the preponderance of β -chain synthesis obtained with Hb H disease mRNA is not due to that idiosyncrasy of the cell-free system. This is clearly shown by Table 1. First, the β/α ratio obtained in the presence of Hb H disease mRNA is greater than 20 even at the same low concentrations of mRNA which give equal amounts of α - and β -chain synthesis with nonthalasemic mRNA. Secondly, the β/α ratio stabilizes at 2.2 even at very high ($100 \mu\text{g/ml}$) concentrations of normal mRNA whereas, with Hb H disease mRNA, the β/α ratio is greater than 20 at all

mRNA concentrations tested. Finally, the high β/α ratios obtained with Hb H disease mRNA are due to a near absence of α -chain synthesis at all concentrations tested; the preferential β -chain synthesis observed with normal mRNA is due to increased β -chain synthesis and not to loss of α -chain synthesis (Table 1). Therefore, the virtual absence of α -chain synthesis observed with Hb H disease mRNA is unique and cannot be duplicated with normal mRNA in our cell-free system.

Results have been recently obtained from DNA-RNA hybridization studies,¹⁷ and from nucleotide sequence analysis of the mRNA,¹⁸ which show an actual quantitative deficiency in the amount of α -chain mRNA in Hb H disease. These studies depend on properties of the mRNA molecules which are not strictly dependent on the capacity of the messenger to direct protein synthesis. Therefore, the deficiency in the amount of functional α -chain mRNA seems to be associated with a similar deficiency in the actual chemical amount of the mRNA present in the Hb H disease reticulocytes. All of these various studies of mRNA in Hb H disease were done with mRNA isolated from total reticulocyte lysates and not polysomes. The results indicate, therefore, that the deficiency observed is not due to the presence of an abnormal mRNA which is present in the cell but unable to enter into polysomes.

The imbalance in globin synthesis produced by translation of Hb H disease mRNA in the cell-free system was much more pronounced than that observed in the intact reticulocytes of Hb H disease patients^{2,3} (Figs. 2A and 3A). The basis for this discrepancy is not clear. Possibly the α -chain mRNA present in Hb H disease reticulocytes is unstable and susceptible to breakdown during the RNA isolation procedure. If this is so, it must be a general property of Hb H disease mRNA, because the same phenomenon was observed in both of our patients and in the patient studied by Grossbard et al.⁹ using mRNA prepared in a different manner. The β/α mRNA content of the mRNA from our patients with Hb H disease was directly quantitated by the technique of RNA to DNA hybridization. The results indicated the presence of at least six times more β mRNA than α mRNA in the Hb H disease mRNA,¹⁷ and provided a second demonstration in these patients of more profound α chain mRNA deficiency than suggested by intact cell globin synthesis. The cell-free system probably exaggerates the deficiency in α -chain mRNA, perhaps by preferentially translating β -chain mRNA when α -chain mRNA is very deficient. In any case, the fact that the intact Hb H disease reticulocyte manifests less imbalance of α/β -chain synthesis than expected for its α -mRNA content (as determined by hybridization studies as well as cell-free translation) suggests that there may exist in these reticulocytes, some translational control of globin-chain synthesis resulting in decreased β -chain synthesis because of a lack of free α chains. A regulatory role for α chains in the synthesis of β chains has been demonstrated in a number of experimental systems.¹⁹⁻²³

Deficiency of functional β -chain mRNA occurs in homozygous β -thalassemia.^{7,8,24} We, as well as Grossbard et al.⁹ have now shown that a deficiency in functional α -chain mRNA exists in hemoglobin H disease. These studies suggest that a deficiency of functional chain specific globin mRNA is a general feature of the thalassemia syndromes.

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