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Further Evidence of a Quantitative Deficiency of Chain-Specific Globin mRNA in the Thalassemia Syndromes

[γ-chain messenger RNA/hemoglobin H disease/ sickle cell anemia/ formamide gel electrophoresis/oligo(dT)–cellulose]

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ABSTRACT **Formamide gel electrophoresis separates the mRNA fraction from reticulocyte polyribosomes of adult humans into two major RNA species with migratory rates identical to those of the α- and β-globin mRNAs of the rabbit. That these two RNAs of human origin are the globin mRNAs is further supported by the deficiency of the presumed β mRNA in reticulocyte polyribosomes of fetuses and premature infants, whose cells make γ chains in preference to β chains.**

The globin mRNAs of reticulocyte polyribosomes from patients with hematological disorders were estimated by scanning the stained formamide gels. In contrast to individuals with either hemolytic anemia without hemoglobinopathy or sickle cell anemia who had β mRNA to α mRNA ratios of approximately one, a patient with Hb S-β-thalassemia had a ratio of β mRNA to α mRNA of 0.75 while two subjects with homozygous β-thalassemia had severe deficiencies of β mRNA. Conversely, a patient with α-thalassemia (Hb H disease) had a ratio of β mRNA to α mRNA on reticulocyte polyribosomes of 6. These data provide further evidence of a quantitative deficiency of chain-specific globin mRNA in patients with the thalassemia syndromes.

The thalassemia syndromes are an inherited group of hemolytic, hypochromic anemias in which the synthesis of a specific hemoglobin chain is impaired (1–3). Thus, in β-thalassemia, which occurs in various ethnic groups (4), the synthesis of normal β chains is deficient relative to that of α chains. In contrast, α-thalassemia, one form of which is Hb H disease, is characterized by a deficiency of α-chain synthesis (5). Since genetic analyses have placed the β-thalassemia locus or loci close to the β-chain structural locus (6–8), recent work on the underlying defect in the thalassemias has focused on the globin mRNAs.

In the β- and α-thalassemias, assays of globin mRNA in cell-free translating systems have demonstrated a relative deficiency in the activity of β mRNA and α mRNA, respectively, commensurate with the deficiency in globin-chain synthesis in these disorders (9–12). RNA·DNA hybridization studies have suggested that the mRNA defect in these conditions is a quantitative one (13, 14), and that in β^0-thalassemia, characterized by an absence of β-chain synthesis, the deficiency of β mRNA is complete (15). However, further studies of the relative α and β mRNA concentration in the thalassemia syndromes have awaited a means to separate physically the α mRNA and β mRNA molecules.

Recently, Gould and Hamlyn used polyacrylamide electrophoresis in formamide to separate the 10S RNA of rabbit reticulocytes into two species, and they suggested that these RNAs represented the α- and β-globin mRNAs (16). We have added these two RNAs separately to the Krebs II ascites cell-free system, and by analyzing the peptide chains produced in each assay have demonstrated that the faster migrating 10S RNA is α mRNA and the slower migrating 10S RNA is β mRNA (17). Morrison *et al.* have reached the same conclusion for the mouse globin mRNAs (18). We now have applied the same electrophoretic method to globin mRNA purified from reticulocytes of human beings and have confirmed the existence of a quantitative deficiency of chain-specific globin mRNA in the thalassemia syndromes.

METHODS

The methods used for reticulocyte lysis and polyribosome isolation have been described (19, 20). In some experiments polyribosomes were treated with EDTA, and the RNA was fractionated on a 10–30% linear sucrose gradient (21). The 14S region containing 10S RNA was collected; the RNA was extracted with phenol and precipitated with ethanol (21). This RNA was then subjected to oligo(dT)–cellulose chromatography to isolate globin mRNA (22, 23). In other experiments, the polyribosomes were directly extracted two to five times with phenol–choloroform–isoamyl alcohol (50:50:1) (23). The RNA was precipitated in ethanol and was applied to an oligo(dT)–cellulose column. If the quantity of RNA was greater than 2 mg, the RNA eluted in 0.5 M KCl was reapplied to the column, the two poly(A)-rich RNA fractions were then pooled, and these fractions were then chromatographed a third time. RNA (4–8 μg) was subjected to formamide electrophoresis on 7.5% polyacrylamide gels as described (24, 16, 17); the gels were stained with Stains-All (25) and scanned at 600 nm. The quantity of an RNA was estimated from the area under the recorded curve. After electrophoresis, the two 10S RNA bands were observed in similar proportions no matter which isolation procedure was used, but an improved yield of mRNA was obtained by omitting the sucrose gradient step.

Abbreviations: Hb H, hemoglobin H composed of four β chains; cDNA, a DNA copy of most of a globin mRNA initiated at the 3′-OH end of the mRNA; β^+- (α^+-)thalassemia, a thalassemia in which the synthesis of β (α) chains is reduced, but present; β^0- (α^0-)thalassemia, a thalassemia characterized by the absence of β (α) chain synthesis.

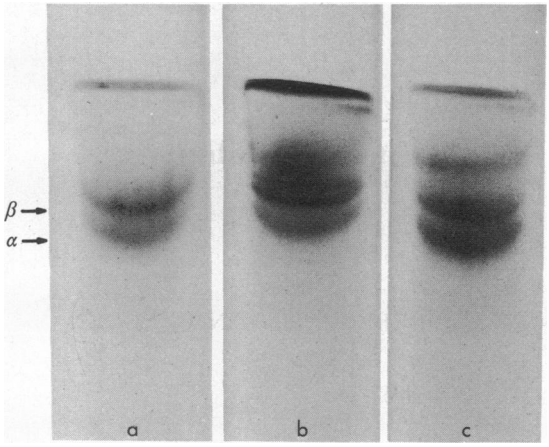


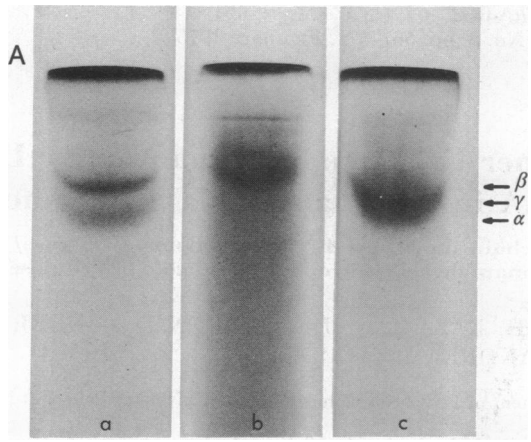
FIG. 1. Polyacrylamide gel electrophoresis in formamide of poly(A)-rich RNA from reticulocyte polyribosomes of humans. From left to right, the RNA originates from patients with (a) hemolytic anemia without hemoglobinopathy, (b) sickle cell anemia, and (c) Hb S-β-thalassemia. In Figs. 1–3, α- and β-globin mRNAs are indicated by *arrows* and were obtained as described in *Methods*. Bands of RNA closer to the top of the gel than the mRNAs were occasionally seen in samples from a variety of sources and are believed to be artifacts of the electrophoresis procedure. Scans of the mRNA regions of gels a and c are found in Fig. 4, panels a and b.

Cell-free assay of mRNA activity was performed with the Krebs II ascites cell-free system (26, 27). Reticulocytes were incubated with [^{3}H]leucine as the radioactive amino acid, and the chains formed were assayed after the addition of [^{14}C]leucine carrier globin by cellulose-acetate electrophoresis (28) or the Clegg column (29). Hemoglobins A and F were separated by Bio Rex 70 chromatography (30).

RESULTS

Separation of the Human Globin mRNAs by Formamide Gel Electrophoresis. When polyribosomal RNA from reticulocytes of adult humans was subjected to chromatography on oligo(dT)–cellulose columns, a small fraction (about 1%) of the RNA adhered in 0.5 M KCl and eluted from the column when KCl was removed from the buffer. Translational analysis of the RNA fractions indicated that more than 90% of the mRNA activity was in this poly(A)-rich fraction. After electrophoresis of the poly(A)-rich RNA on 7.5% polyacrylamide gels in formamide, two major RNA species were observed (Fig. 1). In many experiments these RNAs had the same electrophoretic mobility as the α mRNA and β mRNA of rabbit (Figs. 2 and 3).

Further evidence that these two RNAs are the α mRNA and β mRNA molecules is provided by electrophoresis of the mRNA of premature infants and midtrimester fetuses. Blood was obtained at exchange transfusion for erythroblastosis fetalis from two premature infants with gestational ages of 28 and 32 weeks and weights of 1000 and 1700 g, respectively. The poly(A)-rich RNA fraction of reticulocyte polyribosomes from both infants was deficient in the presumed β mRNA (Figs. 2A and 4) and contained a new RNA that migrated slightly more slowly than α mRNA on formamide gel electrophoresis. After incubation of the reticulocytes of one infant with [^{3}H]leucine and separation of the radioactive hemoglobins A and F, it was determined that the synthesis of Hb A ($\alpha_2\beta_2$) was 15% that of Hb F ($\alpha_2\gamma_2$). Since γ-chain synthesis



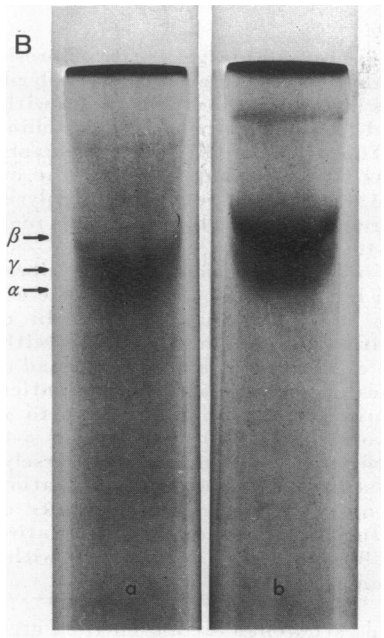


FIG. 2. (A) Formamide gel electrophoresis of RNA from the following sources: (a) 10S RNA derived from polyribosomal RNA of rabbit reticulocytes. This preparation was not subjected to oligo(dT)–cellulose chromatography. (b) Poly(A)-rich RNA of reticulocyte polyribosomes from a patient with α-thalassemia. This sample is deficient in the α-mRNA species. (c) Poly(A)-rich RNA of reticulocyte polyribosomes from a premature infant, age 32 weeks from conception. This preparation is deficient in the β mRNA species and contains γ mRNA, which appears to migrate slightly more slowly than α mRNA in this system. Scans of the mRNA regions of gels b and c are shown in Fig. 4, panels c and e. (B) Formamide gel electrophoresis of (a) poly(A)-rich RNA from reticulocyte polyribosomes of four midtrimester fetuses, ages 13–20 weeks, and (b) 10S RNA of rabbit reticulocytes prior to oligo(dT)–chromatography, as shown in panel 2A.

accounts for the bulk of non-α-chain synthesis at this time of development, the slower mRNA species is presumably the γ mRNA. This assumption is strengthened by data obtained on the globin mRNA pooled from the reticulocytes of four abortuses, aged 13–20 weeks, whose cells made 4–6% as much Hb A as Hb F. This globin mRNA preparation contained no visible β mRNA, but demonstrated the presumed γ mRNA in addition to α mRNA (Fig. 3). In addition, both γ and α chains were synthesized when this RNA was added to the Krebs ascites cell-free system. It is noteworthy that al-

TABLE 1. *Globin synthesis and globin mRNA in human reticulocytes*

Patient	β/α Synthetic ratio in reticulocytes	β mRNA/α mRNA (estimated on gel)
Hemolytic anemia (Hb A)	—	1.1
Sickle cell anemia (Hb S)	1.1 0.9	1.0
Hb S-β-thalassemia	$(\beta^S/\alpha) = 0.50$, $(\beta^A/\alpha) = 0.02$	0.72 0.77
Homozygous β-thalassemia (Black-American)	$(\beta^A/\alpha) = 0.15$, $(\gamma/\alpha) = 0.21$	< 0.2*
Hb H disease	2.7	6.0

* $\frac{\beta \text{ mRNA}}{\alpha \text{ mRNA} + \gamma \text{ mRNA}}$

though the γ chain contains the same number of amino-acid residues as the β chain (146) and is a β-chain analogue, its mRNA has a greater electrophoretic mobility than β mRNA in this system. Since formamide electrophoresis separates RNAs on the basis of size (24), γ mRNA is presumably smaller than β mRNA.

Because (*i*) the poly(A)-rich RNAs of adult human reticulocytes have the same electrophoretic mobility as the α mRNA and β mRNA molecules of rabbit, and (*ii*) the presumed β mRNA is markedly deficient in reticulocytes of human fetuses whose cells are synthesizing relatively few β chains, we conclude that the two poly(A)-rich RNAs separated from adult humans are the α mRNA and β mRNA molecules.

Quantitation of α- and β-globin mRNA in Thalassemia and Other Anemias. Cells of patients with hemolytic anemia without hemoglobinopathy, sickle cell anemia, Hb S-β-thalassemia, β-thalassemia major, and Hb H disease were studied. The synthesis of β chains relative to that of α chains in reticulocytes was compared to the ratio obtained by formamide gel electrophoresis of β mRNA to α mRNA on reticulocyte polyribosomes (Table 1). The electrophoretic mobilities of globin mRNAs in formamide suggest that β mRNA is about 10% larger than α mRNA. Thus, it may bind more Stains-All than the α mRNA molecule. However, no correction for a possible 10% overestimate of the β mRNA to α mRNA ratio was made in the data presented (Table 1), because such a correction would not significantly alter the conclusions presented in this paper.

The ratio of β mRNA to α mRNA was approximately one on the reticulocyte polyribosomes of one patient with hemolytic anemia without hemoglobinopathy and two patients with sickle cell anemia (Figs. 1 and 4 and Table 1). RNA isolated from one of the sickle cell patients by extraction of whole cells (31) had a β mRNA to α mRNA ratio nearly identical to that obtained from the subject's polyribosomal RNA (data not shown). Since the mRNA of β^S chains presumably differs from the mRNA of β^A chains by a single nucleotide substitution, no migratory difference between β^A mRNA and β^S mRNA was expected or observed.

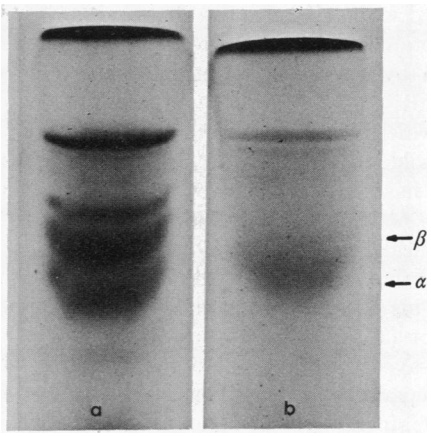


FIG. 3. Formamide gel electrophoresis of (a) 10S RNA of rabbit reticulocytes as in Fig. 2A, and (b) poly(A)-rich RNA of reticulocyte polyribosomes from a subject with homozygous β-thalassemia. Note the deficiency of the β-globin mRNA species and the similarity of the mRNA profile with that of the premature infant and midtrimester fetuses of Fig. 2. The scan of this gel is shown in Fig. 4, panel d.

The data on patients with heterozygous and homozygous β-thalassemia and Hb H disease differ significantly from the data on patients without thalassemia. When the reticulocytes of a patient heterozygous for both β^S and β-thalassemia genes

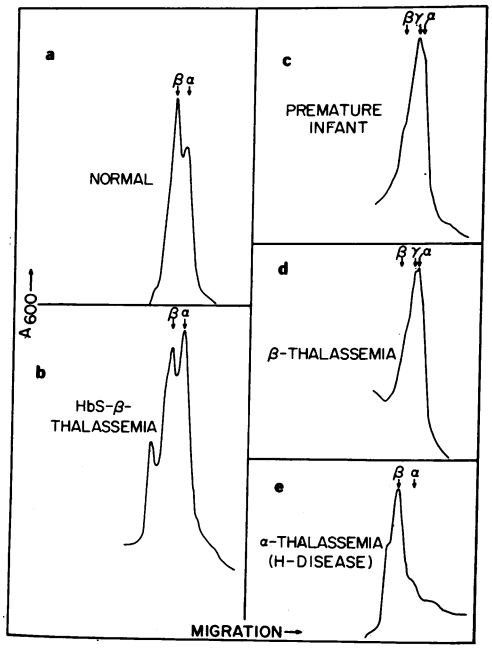


FIG. 4. Scans of gels shown in Figs. 1, 2, and 3. Sources of the RNA samples are stated in the panels. The patient without hemoglobinopathy is designated normal (panel a). In this panel, although the peak height of β mRNA is significantly greater than that of α mRNA, analysis of the area under the curves led to an estimated β mRNA to α mRNA ratio of 1.05. In other panels, the mild deficiency of β mRNA in the Hb S-β-thalassemia patient (panel b), the deficiency of β mRNA and the presence of the presumed γ mRNA in both the premature infant and one patient with homozygous β-thalassemia (panels c and d), and the deficiency of α mRNA in the patient with α-thalassemia (panel e) are seen.

were incubated with [^{3}H]leucine, a β/α chain synthetic ratio of 0.5 was obtained (Table 1). The mRNA from these cells was analyzed by formamide electrophoresis and contained 25% less β mRNA than α mRNA in two separate preparations, one of which is shown in Figs. 1 and 4.

A 43-year-old Black-American with homozygous β-thalassemia, who has had a mild course with a stable hematocrit of 30% and no transfusion requirement, was studied. A thorough clinical investigation of his family had previously demonstrated that both his parents have β-thalassemia trait and three of his 12 siblings also have a mild form of β-thalassemia major (32). Globin-chain synthesis by his reticulocytes was assayed through incubation of his cells with [^{3}H]-leucine and separation of the radioactive α, β, and γ chains by column chromatography. The ratio of β-chain synthesis to α-chain synthesis was 0.15, and the γ-chain to α-chain synthetic ratio was 0.21. After oligo(dT)–cellulose chromatography, the mRNA fraction from his reticulocyte polyribosomes contained a single diffuse band on formamide electrophoresis, the faster portion of which migrated with α mRNA (Figs. 3 and 4). Since the fetal and infant mRNA preparations indicate that γ mRNA has a mobility slightly slower than α mRNA by this technique, we presume that the diffuse mRNA band represents a mixture of α mRNA and γ mRNA. A marked deficiency of RNA migrating with the mobility of β mRNA was present (Figs. 3 and 4). The maximum estimate of the β mRNA quantity from the gel scan was less than 20% of the combined α mRNA and γ mRNA quantities in this patient.

A second subject with homozygous β-thalassemia was a 7-year-old Greek-American, one year after splenectomy, who had retained a striking transfusion requirement. In his reticulocytes, the ratio of β-chain synthesis to α-chain synthesis was 0.33, and the γ- to α-chain synthetic ratio was 0.08. The poly(A)-rich fraction of his reticulocyte polyribosomal RNA contained a sharp α mRNA band and little β mRNA, but quantitation of the β mRNA deficiency was made difficult by a background of 10S–12S RNA. Since 15% of the cells in his blood sample were nucleated erythroblasts, the RNA background was presumably due to mRNA species other than globin mRNA in erythroid precursors.

The patient with Hb H disease is 25 years old, of Greek and Italian descent, and has been essentially asymptomatic with a low-grade anemia (hematocrit 33) and 5% Hb H. The β/α chain synthetic ratio of her reticulocytes was 2.7, and she had a marked deficiency of α mRNA by gel electrophoresis (Fig. 2). When the stained gel was scanned and the area under the curve for each RNA was analyzed, the quantity of β mRNA was estimated to be six times greater than the quantity of α mRNA in her reticulocyte polyribosomes (Fig. 4).

DISCUSSION

Previous studies suggesting a quantitative deficiency of reticulocyte globin mRNA in the thalassemias have been indirect. The α- and β-globin mRNAs of rabbit were partially purified and a cDNA of about 2/3 of the nucleotide sequences of each partially purified mRNA was made (33–35). The cDNAs were then hybridized to partially purified globin mRNA of reticulocytes from normal and thalassemic human beings. In these experiments, α cDNA hybridized less well to RNA from α-thalassemic patients than did β cDNA; and β cDNA hybridized poorly compared to α cDNA when the RNA originated from patients with β-thalassemia (13–15). Although these experiments were technically satisfactory, further determination of the α- and β-globin mRNA concentrations in the thalassemias was desired. The experiments reported here support the conclusions of the RNA·DNA hybridization studies and indicate that a quantitative deficiency of globin mRNA exists in the thalassemia patients studied to date.

One relative deficiency of α mRNA in the reticulocytes of the α-thalassemic patient was greater than that of α-chain synthesis in her cells. This phenomenon has been noted previously and a downward regulation of β-chain production secondary to the impairment of α-chain synthesis has been suggested (13). A recent study has demonstrated that a mild reduction of β-chain synthesis occurs in rabbit marrow cells *in vitro* in response to a specific reduction in α-chain synthesis (36). Individuals with Hb H disease can be thankful for such regulation, whatever the mechanism, because the disease would be as severe as β-thalassemia if the imbalance in globin-chain synthesis approached the degree of imbalance observed in the globin mRNA quantities in this condition.

One individual studied here with β-thalassemia, who is deficient in β mRNA, is the first black with the disease in whom the quantity of β mRNA has been assayed. We believe that his mild course is secondary to better compensation of defective β-chain synthesis through more γ-chain production than is found in most β-thalassemics of Greek and Italian origin.

Since a number of steps are involved in globin-chain synthesis, we expect many causes for the thalassemia syndromes, and the following separate mechanisms have been described. First, data have been presented suggesting a gene deletion as the basis of α^0-thalassemia (37, 38). Second, although gene deletions may be responsible for many α^0- and β^0-thalassemias, the presence of some synthesis of the deficient globin chain in the α^+- and β^+-thalassemias discussed in this report indicates that these diseases cannot be caused by deletions of all of the structural genes for the α and β chains, respectively. A defect at any one of a number of steps may lead to the mRNA deficiencies observed in these conditions. Among these are (*a*) reduced transcription of an mRNA precursor, (*b*) defective processing of an mRNA precursor to the active 10S RNA species, (*c*) reduced transport of mRNA to the cytoplasm, or (*d*) increased degradation of cytoplasmic mRNA. In regard to the last possibility, although native globin mRNA is deficient in the cells of the thalassemic patients studied here, the presence of incomplete globin mRNA, e.g., mRNA lacking poly(A), has not been excluded. Third, in all likelihood a mutation of an α-chain terminator codon leads to an α^+-thalassemia through a defect in the synthesis of the elongated α chains, $\alpha^{\text{Constant Spring}}$, and α^{Icaria} (39, 40). Fourth, in the β^0-thalassemia of Ferrara, β-chain synthesis by reticulocyte polyribosomes in the presence of normal reticulocyte supernate has been reported, suggesting the possibility of a defect in β mRNA translation in this type of thalassemia (41). As our knowledge of the molecular mechanisms underlying the thalassemia syndromes increases, we will learn more about the normal means by which globin-chain synthesis is regulated.

Elsewhere we have reported that α mRNA exceeds β mRNA by 30–50% on polyribosomes of rabbit reticulocytes (17). When we isolated mRNA from rabbit reticulocytes by oligo(dT)–cellulose chromatography without a sucrose gra-

dient step (18), a similar excess of α mRNA on gel electrophoresis was observed. On the other hand, to date we have been unable to demonstrate an excess of α mRNA on human polyribosomes unless the α mRNA to β mRNA ratio observed in the gels is corrected for an increased uptake of stain by the β mRNA molecule. Although such a correction, which assumes that the β mRNA is larger than the α mRNA molecule, seems reasonable, its validity is unproven.

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