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Decreased Globin Messenger RNA in Thalassemia Detected by Molecular Hybridization

(reticulocytes/β chain/complementary DNA)

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ABSTRACT **In previous studies of patients with β thalassemia, mRNA extracted from reticulocytes in peripheral blood when added to cell-free systems reproduces the deficient β-chain synthesis characteristic of intact cells. The present studies with specific probes for α and β mRNA were designed to decide whether the decreased β mRNA activity is due to the presence of abnormal or reduced β globin mRNA in these cells. Purified α and β complementary DNAs (cDNAs) have been synthesized with RNA-instructed DNA polymerase; α and β mRNAs isolated from heavy (β-producing) and light (α-producing) polyribosomes of rabbit reticulocytes were used as templates. Each of the cDNAs is more than 80% pure by the criterion of biological activity. The α cDNA labeled with [^{32}P]dCTP and the β cDNA labeled with [^{3}H]dCTP have been added simultaneously to reaction mixtures containing various concentrations of mRNA from thalassemic and nonthalassemic subjects. The extent and rate of hybridization were determined, permitting a comparison of relative α and β mRNA content in the same annealing mixture. In six nonthalassemic patients, relatively equal amounts of hybridizable α and β mRNA appear to be present. In five of seven patients with β-thalassemia, significantly decreased amounts of β mRNA compared to α mRNA can be demonstrated. In two patients with Hemoglobin H disease, there is a decreased amount of α mRNA compared to β mRNA.**

The thalassemia syndromes are a group of hereditary anemias of man in which the synthesis of specific globin chains is preferentially affected (1, 2). In β thalassemias, decreased β-chain synthesis can be demonstrated when reticulocytes in peripheral blood or bone-marrow cells are incubated with radioactive amino acid (3–6). In α thalassemia, α-chain synthesis is specifically diminished (7, 8). Assays with cell-free protein-synthesizing systems have shown that mRNAs from β thalassemia cells reflect a decrease in β-chain mRNA activity (9–12). A similar decrease in α globin mRNA activity has been demonstrated in α thalassemia (Hemoglobin H) cells (13). The present studies show by molecular hybridization that there is a significant decrease in the relative amount of β to α globin mRNA in five of seven patients with β thalassemia.

The design of meaningful hybridization experiments with globin mRNA fractions from human patients must overcome several not inconsiderable technical difficulties. Due to the low number of reticulocytes present in the peripheral blood in man, it is difficult to obtain globin messenger RNA of purity comparable to that from animals in which reticulocytosis can be induced experimentally. Since there is no method of determining, with sufficient accuracy, the purity of the globin mRNA, the actual quantity of true globin message used in a hybridization reaction cannot be known with certainty.

Methods for the separation of α and β globin mRNAs have been developed for the rabbit mRNA where a particular amino-acid distribution permits the localization of the messages, respectively, on light and heavy polyribosomes (14). By use of RNA-instructed DNA polymerase to synthesize DNA complements (cDNAs) of the rabbit α and β mRNAs, labeled hybridization probes for the partially homologous human messages can be made (15). It is not, however, possible to determine the exact degree of homology among the four messages; cross-hybridization between, for example, rabbit α cDNA and human β mRNA may occur and give erroneous results. Since the separation technique does not produce messages greater than about 80% pure, it is not possible to determine to what degree the probes are specific.

Further complications arise from the nature of the hybridization reaction itself. As several authors have shown (16, 17), variations in hybridization conditions may lead to different estimates of the amount of an RNA present in a given preparation. Since these variables may affect the α and β mRNAs and cDNAs differently, it is impossible to measure the *actual amounts* of α and β globin messages present without having an authentic sample of pure material for reference.

It is possible to overcome these difficulties by designing hybridization experiments that avoid dependence on knowledge of the absolute amounts and where one of the messages serves as an internal reference for the other. The *relative amounts* of α and β mRNAs present in thalassemic and nonthalassemic patients can then be measured and the results compared.

To this end, purified α and β mRNAs were prepared from polyribosomes of rabbit reticulocytes which had been incubated with *o*-methylthreonine (14, 18). The separated messages were then used to template the synthesis of cDNAs with RNA-instructed DNA polymerase from avian myeloblastosis virus (15). The α cDNA was labeled with [^{32}P]dCTP, while the β cDNA was labeled with [^{3}H]dCTP.

The presence of both DNA probes in the same reaction mixture makes unnecessary any measure of the purity of the mRNA preparation. By comparison of the rates of hybridization of the α and β cDNAs, the *ratio* of α to β globin message can be determined. In comparing nonthalassemic and β

Abbreviation: DNA, complementary DNA.

thalassemic mRNAs, the α mRNA serves as an internal control for the amount of globin mRNA in an mRNA preparation. Thus, if the defect in β thalassemia is due to a significant decrease in the amount of β mRNA, the rate of hybridization of β thalassemia mRNA with β cDNA should be less relative to α cDNA than with nonthalassemic mRNA.

The effect of cross-contamination of the separated messages and hence the corresponding cDNA probes will be to make the probes less specific. Differences that exist in the relative amounts of α and β mRNAs will thereby be underestimated. The effect of hybridization due to partial homology between, for example, the α cDNA and the human β mRNA would also be the same. Thus, if no differences are found between the nonthalassemic and thalassemic mRNA populations, the experiment is not meaningful. Detection of a significant difference, however, will be a valid result although the magnitude of the difference may be greater than that detected in the present studies.

MATERIALS AND METHODS

Preparation and Biological Activity Assay of Human mRNAs. Globin mRNA was prepared from polyribosomes by treatment with sodium dodecyl sulfate (12, 19) or from whole cells by phenol extraction (20). The mRNAs were separated from ribosomal and tRNAs by sucrose density-gradient centrifugation (12, 19). The 6–15S region of the gradients (predominantly 10S) was pooled and purified as described (12). The mRNAs were assayed in a Krebs-ascites-tumor cell-free system supplemented by a rabbit ribosomal salt-wash fraction (21). The products of the cell-free system were analyzed for human α and β chain synthesis by carboxymethyl (CM) cellulose chromatography (22). The cell-free system was incubated with [^{3}H]leucine, and cochromatography of globin chains was performed with ^{14}C-labeled globin. Assays of α and β globin in intact cells were as described (5).

Preparation of Specific DNAs Complementary to α and β Globin mRNAs. Polyribosomes from rabbit reticulocytes were separated into heavy (β-chain synthesizing) and light (α-chain synthesizing) fractions after incubation of the cells with *o*-methylthreonine (18). The 10S mRNA was isolated from these fractions as described (14) and assayed in the Krebs ascites tumor system with analysis of the synthesized protein by CM-cellulose chromatography (23). The mRNA was used to template the synthesis of complementary DNA as described (15).

Hybridization of Globin mRNAs and cDNAs. Hybridization reactions (10-μl final volume) contained 20 mM sodium phosphate (pH 7.0), 0.1% sodium dodecyl sulfate, 0.3 M NaCl, 2 mM EDTA, about 1000 cpm each of ^{3}H and ^{32}P cDNAs, and 1.5×10^{-4}–0.3 μg of RNA. The components were sealed in borosilicate glass capillary tubes that had been soaked in a solution of *Escherichia coli* DNA (100 μg/ml in 0.3 M NaCl–0.02 M EDTA) for several hours, rinsed thoroughly in distilled water, and allowed to air dry. This treatment prevents absorption of the cDNA onto the glass. After heating at 100° for 2 min to denature the nucleic acids, the reaction mixtures were incubated at 68° for 4–8 hr. Usually, the reaction mixtures were not analyzed immediately, but were frozen at −20° until they could be conveniently processed. No difference was observed between samples treated in this manner and those processed immediately at the conclusion of incubation.

For analysis, the capillary was opened, and two 4-μl aliquots were removed for assay. One sample was treated with micrococcal nuclease under conditions in which the enzyme specifically degrades single-stranded regions of nucleic acids (manuscript in preparation); the other sample was incubated in the assay mixture without nuclease. Reactions were terminated by the addition of Cl_3CCOOH. Acid-precipitable counts were collected on nitrocellulose filters and counted in 0.4% BBOT (2,5-*bis*-2(5-*tert*-Butylbenzoxazolyl)-Thiophene) in toluene. The percent hybridization was determined as the counts recovered from the nuclease-treated sample divided by the counts from its companion aliquot, multiplied by 100. The percent recovery in the aliquot not treated with nuclease was always greater than 95% of the input radioactivity.

RESULTS AND DISCUSSION

Biological activity of human globin mRNAs

Human mRNAs isolated from whole cells and polyribosomes synthesized amounts of α and β chains comparable to those previously reported when assayed in the Krebs-ascites-tumor lysate system (12, 13, 21). Ratios of α- to β-chain synthesis are shown in Table 1. The mRNAs from nonthalassemic cells syn-

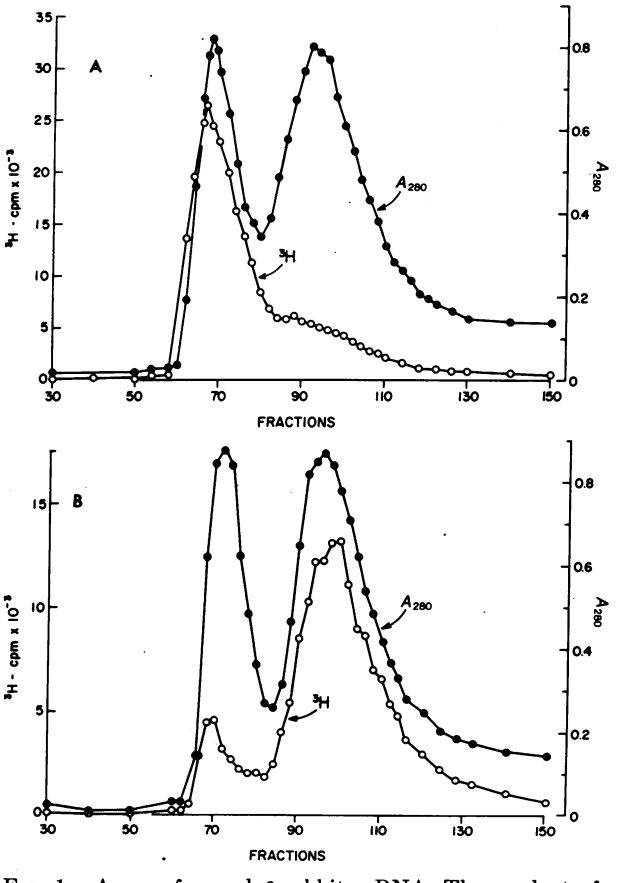

FIG. 1. Assay of α and β rabbit mRNA. The product of a Krebs-ascites-tumor cell-free assay labeled with [^{3}H]leucine was mixed with unlabeled rabbit hemolysate and globin prepared. CM-chromatography was performed as described in *Methods* (23). Elution pattern when the cell-free assay contained (*A*) 2 μg of α mRNA; (*B*) 2 μg of β mRNA. The first peak is the α chain; the second, the β chain.

TABLE 1.

Patient	Source	Hybrid % Min*	Hybrid % α Max†	Hybrid % β Max†	α $C_0t_{1/2}$‡	β $C_0t_{1/2}$	α/β Ratio Hybridization§	α/β Ratio Intact cell	α/β Ratio Cell-free
Nonthalassemia:									
1	Polyribosomes	16	64	54	1.8×10^{-3}	1.8×10^{-3}	1.0	1.0	—
2	Polyribosomes	16	62	47	2.7×10^{-2}	2.7×10^{-2}	1.0	—	0.79
3	Polyribosomes	14	63	50	3.9×10^{-2}	3.7×10^{-2}	0.9	0.95	—
4	Polyribosomes	16	60	50	1.8×10^{-2}	2.2×10^{-2}	1.2	1.0	0.90
5	Total cell	13	49	39	2.4×10^{-2}	1.4×10^{-2}	0.5	—	0.97
6	Total cell	17	63	50	5.0×10^{-2}	4.0×10^{-2}	0.8	1.0	0.90
7	Total cell	15	60	50	4.5×10^{-3}	6×10^{-3}	1.3	1.12	0.88
β Thalassemia:									
1	Polyribosomes	15	60	50	4×10^{-2}	1.4×10^{-1}	3.5	3.8	2.2
2	Polyribosomes	14	60	50	1.8×10^{-1}	6×10^{-1}	3.3	5.3	3.3
3	Polyribosomes	16	60	40	2.4×10^{-2}	3.4×10^{-2}	1.4	3.5	4.5
4	Total cell	16	60	50	3.9×10^{-1}	1.9×10^{0}	4.9	5.5	3.0
5	Total cell	16	56	46	7.6×10^{-2}	2.5×10^{-1}	3.3	>20	6.2
6	Total cell	18	64	50	8×10^{-2}	3.7×10^{-1}	4.6	7.7	3.8
7	Total cell	14	55	42	1.4×10^{-2}	2.4×10^{-2}	1.7	>20	>20
α Thalassemia:									
1	Polyribosomes	15	50	50	8.5×10^{-2}	5.5×10^{-2}	0.6	0.55	0.17
1	Total cell	14	58	58	1.2×10^{-1}	8×10^{-2}	0.7	0.55	0.17
2	Total cell	12	50	50	4×10^{-1}	2.2×10^{-1}	0.6	0.45	—

* Min is the plateau value for the percentage hybridization at low C_0t and was the same for α and β cDNAs.
† α Max and β Max are the respective percentage hybridizations at saturation for α and β cDNAs.
‡ $C_0t_{1/2}$ = (RNA concentration in moles/liter) × (time of incubation in seconds) at the mid-point between Min and α or β Max.
§ The α/β ratio = β $C_0t_{1/2}$/α $C_0t_{1/2}$ since the relative concentrations of α and β mRNA are inversely proportional to the C_0t value.

thesized approximately equal amounts of α and β globin, and the α to β ratios were similar whether the mRNA was isolated from polyribosomes or whole cells. mRNAs from cells of patients with β thalassemia directed decreased β chain synthesis compared to that of α chains. In virtually all patients, the α to β ratio in the cell-free system was lower than that in intact cells. This may reflect a greater efficiency in translation of β than α globin mRNA in the Krebs-ascites-tumor cell-free system.

Synthesis and characterization of DNA probes complementary to rabbit α and β globin mRNAs

Fig. 1 shows the CM-cellulose column profiles of globin protein made when the separated rabbit mRNAs were used to stimulate protein synthesis in the Krebs ascites cell-free system. The α globin mRNA preparation directs the synthesis of 5 times more α chain than β chain; the β mRNA preparation stimulates 5.5-fold greater synthesis of β chain than α chain.

The separated messages were used to template the synthesis of complementary DNA as described in *Methods*, and the DNAs were analyzed for their ability to hybridize to each of the separate mRNA fractions. As is shown in Fig. 2, the α cDNA hybridizes 4.5 times faster to the α mRNA preparation than to the β preparation, while the β cDNA hybridizes 3.3 times more rapidly to the β than to the α mRNA. There is evident correlation between the biological activity and hybridization rate of the separated messages. The absence of absolute specificity may be explained by some cross contamination.

It should be noted that complexes between poly(dT) and

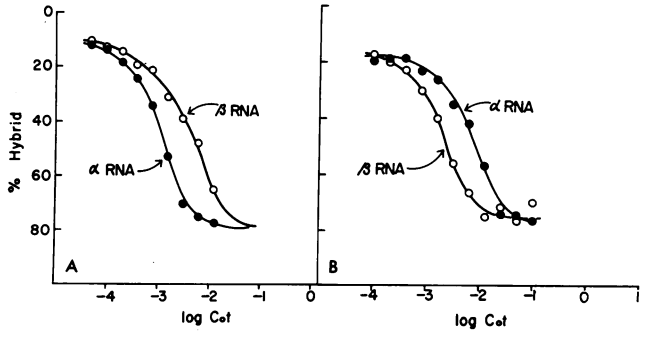

FIG. 2. Hybridization of rabbit α and β cDNAs to rabbit α and β mRNAs. Hybridizations were performed as described in *Methods*. (*A*) α cDNA and (*B*) β cDNA.

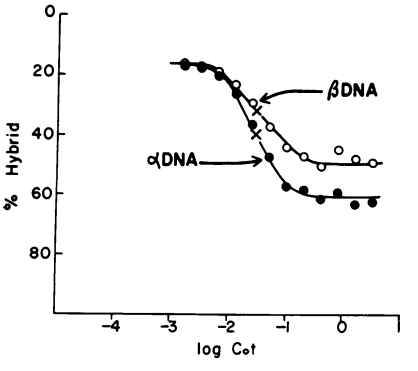

FIG. 3. Hybridization of nonthalassemic mRNA isolated from polyribosomes of patient 2 (Table 1) with α and β cDNAs. "×" indicates $C_0t_{1/2}$.

poly(rA) stretches cannot play a quantitatively significant role in the hybrids detected by the present procedure. Since the label is present in cytosine, poly(dT)·poly(rA) duplexes are not scored. Further, the micrococcal nuclease technique used here specifically degrades poly(dT)·poly(rA) duplexes while leaving more strongly paired regions intact.

The degree of specificity exhibited by the α and β cDNA probes is sufficient to detect all but rather small differences in relative α and β mRNA ratios. Differences of the magnitude suggested by the cell-free system should present no difficulties.

Hybridization of α and β cDNA probes to human globin mRNAs

The relative amounts of α and β mRNA isolated from polyribosomes and whole cells of thalassemic and nonthalassemic patients were determined by simultaneous hybridization to α and β cDNAs (Table 1). Of the seven nonthalassemic patients studied, two (patients 4 and 6) had sickle cell disease, while the other five had hemolytic anemias, either autoimmune in origin or due to congenital spherocytosis. The mRNA associated with polyribosomes in four of these patients hybridized at approximately equal rates with α and β cDNAs (Table 1, Fig. 3). The mean value of α/β by hybridization was 1.1; the range was 0.9–1.2. In the other three nonthalassemic subjects, mRNA isolated from total cells yielded approximately the same results (Table 1) (mean α/β: 0.9; range: 0.5–1.3). By contrast, five of the seven β thalassemic mRNA preparations studied hybridized at a significantly reduced rate with β cDNA compared to α cDNA (Table 1, Fig. 4). This finding was demonstrated with both polyribosomes (mean α/β: 3.4; range: 3.3–3.5) and total cell mRNA (mean α/β: 4.3; range: 3.3–4.9). Of these patients, two were of Italian, two of Iranian, and one of Spanish descent. In two other thalassemic patients, both of Italian descent (patients 3 and 7, Table 1), hybridization with β cDNA was slightly less than with α cDNA, but the difference from the nonthalassemic patients was small.

The percentage of the total DNA hybridized at saturation was significantly lower than when rabbit mRNA was used, probably reflecting interspecies differences in the nucleic acids. The average percent hybridization at saturation between rabbit α cDNA and total human mRNA was 60%, while with β cDNA the average was 50% (Table 1). Amino-acid sequence data show greater homology between the β chains (90%) than between the α chains (82%) of the two species. The discrepancy may be due to less homology at the nucleotide level between the β mRNAs than the α mRNAs or to peculiarities in the distribution of nucleotide differences which magnify the effects of base changes by altering the structure of regions of the nucleic acids.

In the majority of cases, there were no significant differences in the percentage hybridization at saturation between the thalassemic and nonthalassemic mRNAs, indicating the absence of qualitative differences sufficiently large in magnitude to alter the hybridization kinetics (Table 1). In those patients for whom atypical values for relative α and β mRNA content were obtained, one nonthalassemic (patient 5) and two β thalassemic (patients 3 and 7), lower percentage hybridizations at saturation were obtained. These results may be due to the presence of qualitatively different α and β mRNAs or to the presence of competing messages, especially γ-chain mRNA in the β thalassemic patients.

Results with two patients with hemoglobin H disease, a form of α thalassemia, demonstrate a relative decrease in α globin mRNA compared to β globin mRNA when mRNA from either total cells or polyribosomes was used (Table 1). The relative amounts of α and β mRNA found by hybridization are comparable to those in intact cells (Table 1). Further studies with additional patients are needed to determine the extent to which decreased α mRNA content is correlated with the presence of hemoglobin H disease.

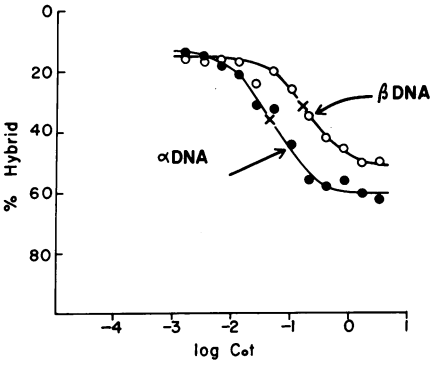

FIG. 4. Hybridization of β-thalassemic mRNA isolated from polyribosomes of patient 1 (Table 1) with α and β cDNAs. "×" indicates $C_0t_{1/2}$.

Our results indicate that in five of the seven β thalassemia patients studied here, there is a decreased amount of β globin mRNA relative to α mRNA, both in the total cell and in association with polyribosomes. In the five patients in whom decreased β mRNA was demonstrated by hybridization, the good correlation between the relative α/β ratio obtained by biological assay and by hybridization (Table 1) suggests that the decreased β mRNA activity in the cell-free assay is due to decreased β mRNA content. The comparable decreases in β mRNA relative to α mRNA, when either polyribosomal or total cell mRNA is used, indicate that there is no significant amount of nonpolyribosomal β mRNA in the supernatant fraction of the cells in the patients studied.

Although there were no gross qualitative differences detected between the majority of nonthalassemic and thalassemic mRNAs, as measured by the percentage hybridization at saturation, we cannot rule out the possiblity of small alterations in the nucleotide sequence of the β mRNAs of the β thalassemic patients. Such changes might express themselves by causing decreased transcription or increased degradation of the message. We cannot, therefore, eliminate a qualitative defect in the mRNA as the cause of the quantitative defect that we observe.

Other possible causes of decreased levels of β message are (*a*) deletion of specific globin genes; (*b*) specific repression of transcription; or (*c*) abnormal processing of the products of transcription before the appearance of mRNA in the cytoplasm. Each of these possibilities can be examined by the methodology used here.

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