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Relative Numbers of Human Globin Genes Assayed with Purified α and β Complementary Human DNA

(thalassemia)

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ABSTRACT. **Purified α and β globin complementary DNAs (cDNAs) have been separated from total radioactively labeled human globin cDNA using mRNA purified from liver of a hydrops fetalis (α thalassemia). The β cDNA hybridizes to the hydrops fetalis mRNA while the α cDNA remains single-stranded. The purified α and β cDNAs were assayed for their purity by their hybridization to mRNA prepared from reticulocytes of nonthalassemia, α thalassemia, and β thalassemia subjects. The results indicate that the separated cDNAs are selective in hybridization to α or β globin mRNAs, respectively. The previously reported deficiency of globin mRNA in thalassemia cells has been confirmed with these purified cDNAs. The purified α and β cDNAs were hybridized to cellular DNA to determine the relative number of α- and β-like genes in non-thalassemia, β^+ thalassemia, and hydrops fetalis (α thalassemia) DNA. The α cDNA hybridized to hydrops fetalis liver DNA to a much lower extent than β cDNA, confirming the previously reported deletion of α globin genes in hydrops fetalis. By contrast, both the α and β cDNA probes hybridized to the same extent to spleen DNA from non-thalassemia and from β^+ thalassemia patients. Between two and five globin genes in non-thalassemia and β^+ thalassemia DNA hybridize to β cDNA and one to five to α cDNA. These studies indicate that in β^+ thalassemia, there is no detectable deletion in β globin genes. The genetic defect in β^+ thalassemia appears to be due to either repression of transcription of β globin genes or abnormal processing of β globin mRNA.**

Using radioactively labeled complementary DNA (cDNA) as a probe, we have recently reported that there are less than 10 copies of globin genes present per haploid genome in human DNA (1). In these studies, β^+ thalassemia spleen DNA was found to have a complement of globin genes similar to that of non-thalassemia DNA when cDNA containing both α and β sequences were used. We report here the measurement of the relative numbers of α- and β-like globin genes in human DNA using purified human α and β cDNA. Hydrops fetalis (α thalassemia) mRNA which contains no α mRNA has been used to separate α and β cDNA from total normal human cDNA (2). Hybridization of total globin cDNA (α plus β cDNA) with hydrops fetalis mRNA leads to β cDNA·β mRNA hybrids, whereas α cDNA remains single stranded. The single stranded and double stranded species were separated using hydroxylapatite chromatography (1), and β mRNA removed from β mRNA·β cDNA hybrids by alkaline hydrolysis. The specificity of each of the purified α and β cDNAs has been determined by hybridization to globin mRNA from normal, β^+ thalassemia, and α thalassemia cells. Previous studies using cell-free systems (3–7) and hybridization to cDNA (8, 9) have shown that mRNA from α thalassemia cells contains decreased α mRNA, and mRNA from β thalassemia cells decreased amounts of β mRNA. In the present experiments, hybridization of the purified α and β cDNA probes to β thalassemia mRNA shows a 10-fold excess of α compared to β mRNA content, whereas, in mRNA of a patient with hemoglobin H disease (a form of α thalassemia), there is a 3-fold excess of β over α mRNA content. These results indicate that the α and β cDNAs are highly selective in their interaction with α and β mRNAs.

Hybridization of the purified cDNAs to non-thalassemia spleen DNA indicates that there are similar numbers of globin genes capable of hybridizing to α and β cDNA; there are between one and five genes hybridized to α cDNA, and two to five genes hybridized to β cDNA in human haploid genomes. Similar numbers of α and β genes are present in both non-thalassemia and β^+ thalassemia spleen DNA; thus there is no detectable deletion of β genes in β^+ thalassemia. In addition, the previously reported deletion of α gene material in hydrops fetalis DNA has been confirmed (2, 10). The results suggest that the basic genetic defect in β^+ thalassemia is due to either repression of transcription of β globin genes or a defect in processing of β globin mRNA, whereas, in α thalassemia, there is gene deletion.

MATERIALS AND METHODS

Hydrops Fetalis. A newborn Thai infant with hydrops fetalis died within 30 min after delivery. Liver tissue was frozen immediately and shipped in dry ice by air from Bangkok to New York and used in the experiments described below. Cord blood from this infant contained no hemoglobin A on hemoglobin electrophoresis. Only γ and β globin and no α globin was present when cord blood was analyzed using globin chain chromatography (11).

Preparation and Characterization of Human DNA. Portions of spleens were obtained from patients with and without β thalassemia undergoing splenectomy for therapeutic indications and were frozen immediately after cutting them into small slices (1). DNA was isolated from spleens of non-thalassemia, β thalassemia, and the hydrops fetalis liver as previously reported (1).

Isolation and Characterization of Globin mRNA. Human globin mRNAs were prepared from reticulocytes or bone marrow cells of patients with and without thalassemia as

Abbreviation: C_0t, the concentration of DNA in moles per liter × the time of hybridization (in sec).

described previously (5, 6). The mRNAs used were tested for their translatable activity in a Krebs ascites tumor cell-free system (12). Globin mRNA was isolated from the hydrops fetalis liver by a method previously described for the isolation of mRNA from mouse fetal liver (13) with the following modifications: 1 g of frozen liver was ground to a coarse powder in a mortar cooled with dry ice and suspended at room temperature in one liter of a buffer containing 0.1 M Na acetate; 0.1 M NaCl; 6 mM $MgCl_2$; 10 μg/ml of polyvinylsulfate (PVS); 0.5% sodium dodecyl sulfate; and 10 units/ml of heparin sulfate. An equal volume of phenol preheated to 55° was added within 5–10 sec and the mixture shaken for 3 min at 55°. The mixture was rapidly cooled in a dry ice–acetone bath (−15°) until the first crystals of phenol appeared. The mixture was then centrifuged at 2000 rpm for 3 min and the supernatant collected. An equal volume of phenol was added to the aqueous phase which was re-extracted after shaking for 15 min at room temperature. The aqueous phase was again collected and RNA precipitated with NaCl (0.3 M, final concentration) and 2 volumes of 95% ethanol. Undegraded RNA showing a 2:1 ratio of 28S to 18S RNA was obtained. The 6–16S RNA fraction was isolated by a second sucrose density gradient centrifugation, salt-ethanol precipitation, and washed prior to use as described previously (13).

Hybridization of Tritiated cDNA with Human mRNA and Human DNA. Globin cDNA labeled with tritiated dCTP (26C/mM, NEN) was prepared using non-thalassemia human globin mRNA isolated from the reticulocytes of patients with either auto-immune hemolytic anemia or sickle cell disease (14). Hybridizations of globin cDNA with human globin mRNAs were under conditions described previously (8). Hybridization of globin cDNA with human spleen or liver DNA was also as previously described (1).

Separation of α and β cDNAs from Total Human cDNA. Globin mRNA prepared from hydrops fetalis liver was hybridized to 1×10^6 cpm of tritiated globin cDNA under the usual conditions (see legend to Fig. 1) except that the reaction mixture was scaled up to 1 ml and the reaction carried out to C_0t of between 1 and 10, insuring maximal hybridization. Under these conditions, only 55% of the cDNA is hybridized (Fig. 1). Increasing the amount of hydrops fetalis mRNA or the time of hybridization does not lead to an increase in the percent hybridization obtained. Since hydrops fetalis mRNA contains only δ, γ, and β mRNA, the hybridized cDNA was presumed to be β cDNA, whereas, the non-hybridized cDNA was α cDNA. The hybridized cDNA was separated from the single stranded cDNA by hydroxylapatite chromatography under the following conditions: The salt concentration of the hybridization mixture was adjusted to 0.12 M sodium phosphate at pH 6.5 and 0.4% sodium dodecyl sulfate and passed through a column containing 500 mg hydroxylapatite at 68°. The hydroxylapatite was extensively washed with 0.12 M sodium phosphate at pH 6.5, 0.4% sodium dodecyl sulfate prior to layering the hybridization mixture. The single stranded cDNA was eluted at low salt (0.12 M sodium phosphate, 0.4% sodium dodecyl sulfate), the double stranded cDNA at high salt (0.4 M sodium phosphate, 0.4% sodium dodecyl sulfate). The samples were then passed separately through a column of Sephadex G-50 (30 × 0.9 cm), in 10 mM Tris·HCl at pH 7.6; 0.6 NaCl; 1 mM EDTA; 0.1% sodium dodecyl sulfate. The excluded volume was pooled and precipitated overnight with 0.3 M NaCl and 2 volumes of ethanol.

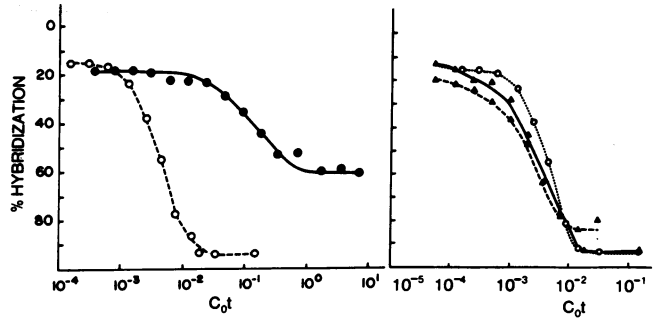

FIG. 1 (*left*). Hybridization of normal unfractionated cDNA with normal and hydrops fetalis mRNA. Unfractionated ^{3}H-labeled cDNA was dissolved in 50 mM Tris·HCl at pH 8.3, 0.6 M NaCl, 3 mM EDTA at a concentration of approximately 2500 cpm/μl. Hybridization was performed in a 10 μl reaction mixture at pH 7.0, containing (final concentration): 0.02 M sodium phosphate; 0.1% sodium dodecyl sulfate; 0.3 M NaCl; 0.002 M EDTA; 1 μl of [^{3}H]cDNA and between 2.6×10^{-3} and 2.4 μg/ml for normal mRNA and between 8×10^{-4} and 160 μg/ml for hydrops fetalis mRNA. Glass capillaries containing the reaction mixtures were sealed by heat, boiled at 100° for 2 min, and then incubated at 68° for 4 hr. The amount of mRNA was varied in the range indicated above to achieve the C_0t values shown (17). The percent hybridization was determined after digestion of single-stranded cDNA with micrococcal nuclease (18): 4 μl aliquots of each reaction mixture were treated with micrococcal nuclease and 4 μl was used as control for recovery of cDNA in individual experiments. O---O, 6–16S normal human reticulocyte mRNA; ●——●, 6–16S hydrops fetalis liver mRNA.

FIG. 2 (*right*). Hybridization of normal human mRNA to unfractionated and purified α and β cDNA. Conditions of hybridization and assay are described in legend to Fig. 1. Between 2000 and 2500 cpm of cDNA wasuse d in each hybridization. O——O, unfractionated cDNA; ▲——▲, β cDNA; △---△, α cDNA.

The samples were then centrifuged at 10,000 rpm for 1 hr at −15° in a Sorvall HB4 rotor. The pellet was suspended at a concentration of 1000 to 2500 cpm/μl and treated with alkali (14).

RESULTS

Characterization of Separated α and β cDNA. The cDNA recovered from hydroxylapatite as double stranded DNA (hybridized to hydrops fetalis mRNA) will be referred to subsequently as β cDNA, while the nonhybridized cDNA will be referred to as α cDNA. Both the α and β cDNA sedimented with a coefficient corresponding to 6–7S cDNA in alkaline sucrose gradients (1). The purified α and β cDNAs hybridized to unfractionated non-thalassemia human globin mRNA with similar kinetics (Fig. 2). This indicates that comparable amounts of α and β mRNA are present in total human globin mRNA. In order to determine the specificity and purity of the α and β cDNAs, we hybridized each of the cDNAs to β^+ thalassemia and α thalassemia mRNA. The C_0t 1/2s were compared to measure the relative amounts of α and β mRNA (9). When β^+ thalassemia mRNA from the reticulocytes of a patient homozygous for β^+ thalassemia was used (Fig. 3A), the C_0t 1/2 required to protect β cDNA was 10-fold that required to protect an equal amount of α cDNA. When mRNA from the peripheral blood of a patient with heterozygous β thalassemia was used, the C_0t 1/2 required to hybridize β cDNA was 3-fold greater than that needed to protect α cDNA. When the bone marrow of a patient with homozy-

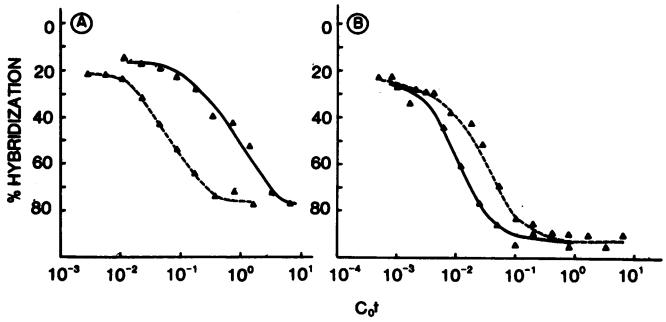

FIG. 3. Hybridization of homozygous β^+ thalassemia and hemoglobin H disease mRNA to purified α and β cDNA. Conditions are as described in legend to Fig. 1. ▲——▲, β cDNA; Δ- - -Δ, α cDNA. A, homozygous β^+ thalassemia mRNA; B, hemoglobin H mRNA.

gous β thalassemia was tested, the C_0t 1/2 for β cDNA was 5-fold that with α cDNA (Table 1). When mRNA from a patient with hemoglobin H disease, a form of α thalassemia, was used, the C_0t 1/2 was 3-fold greater with α cDNA than with β cDNA (Fig. 3B). Previous studies have shown that β thalassemia mRNA contains a decreased amount of β mRNA by cell-free assay (3–7), and by molecular hybridization when α and β cDNA probes synthesized from rabbit globin mRNAs are used (8, 9). A decrease in α mRNA has also been demonstrated in hemoglobin H disease using cell-free assay and cDNA hybridization (7–9). The present results with human α and β cDNA probes confirm these findings, and simultaneously, demonstrate the selectivity of the purified α and β cDNAs.

The α cDNA hybridized to hydrops fetalis mRNA to 20% above background at C_0ts of greater than 10^1 (Fig. 4), whereas, the β cDNA hybridized almost completely (Fig. 4). These results are consistent with either a 20% contamination of α cDNA by β cDNA, or homology of 20% of the sequences of α cDNA with sequences of β or γ mRNA.

The Relative Numbers of α- and β-Like Globin Genes in DNA from Normal Subjects and Patients with Thalassemia. In three separate experiments, α and β cDNAs were hybridized to hydrops fetalis DNA (Table 2, Fig. 5). The hybridization obtained with α cDNA was 24–33%; when the same amount of β cDNA was added to hydrops DNA, 48–60% hybridization occurred (Table 2). These results confirm experiments reported previously (2, 10) and are consistent with a relative decrease in the number of α globin gene sequences in hydrops fetalis DNA. These data also suggest that the α and β cDNA used in these studies distinguish between α- and β-like nucleotide sequences in human DNA

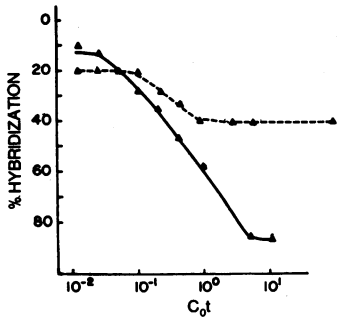

FIG. 4. Hybridization of hydrops fetalis mRNA to purified α and β cDNA. Conditions are as described in legend to Fig. 1. ▲——▲, β cDNA; Δ- - -Δ, α cDNA.

TABLE 1. *Comparison of relative amounts of α and β globin synthesis by intact cells and α and β mRNA content by hybridization*

Source	Intact cell*	Hybridization†
Normal		
Peripheral blood (1)‡ (Fig. 2)	1.1	1.0
Bone marrow (2)	1.0	1.0
Peripheral blood (3)	1.0	1.0
Homozygous β⁺ thalassemia		
Peripheral blood (4) (Fig. 3)	5.5	12.0
Bone marrow (5)	—	5.0
Heterozygous β⁺ thalassemia		
Peripheral blood (6)	2.0	3.8
Bone marrow (6)	—	3.7
Hemoglobin H disease		
Peripheral blood (7) (Fig. 4)	0.55	0.29
Bone marrow (7)	0.55	0.29

* The ratio of α to β globin synthesis by intact cells determined by column chromatography (6–8).

† The C_0t $^1/_2$ using purified β cDNA divided by the C_0t $^1/_2$ using α cDNA (ref. 1, Figs. 3, 4, and 5).

‡ The numbers in parentheses are used to identify different patients.

and can be used to detect globin gene deletions or translocations or nucleotide changes of some length. In three separate experiments, spleen DNA from a patient without β thalassemia hybridized with similar kinetics and to similar extents with both α and β cDNA (Table 2, Fig. 6A). Approximately 50–60% hybridization was obtained with the purified α and β cDNAs. Spleen DNA from a patient with β^+ thalassemia hybridized with the purified α and β cDNA with similar kinetics and to similar extents at three different levels of cDNA input (Table 2, Fig. 6B). The pattern of hybridization using β^+ thalassemia and non-thalassemia spleen DNA to each of the probes was similar.

Numbers of α- and β-Like Globin Genes in Human DNA. The number of globin genes hybridizing with the purified α and β cDNAs can be calculated from the relative amounts of cDNA and cellular DNA added to hybridization mixtures, and the percent hybridizations obtained (ref. 1 and legend to Table 2). Using these calculations, there are between one and four copies of α genes in normal and β^+ thalassemia hybridizing to α cDNA, and between 0.5 and 2 genes hybridizing in hydrops fetalis DNA. This suggests that there is significant deletion of gene material capable of hybridizing to α cDNA in hydrops DNA. At all levels of cDNA·cellular DNA tested, a decrease in the amount of gene material in hydrops fetalis DNA capable of hybridization to α cDNA was reproduced. There are between one and five copies of gene material hybridizing with β cDNA in non-thalassemia, β^+ thalassemia, and hydrops fetalis DNA at three different levels of cDNA input (Table 2). Thus, there appears to be no detectable loss of β globin genes in β^+ thalassemia DNA, and the number of β globin genes in hydrops fetalis DNA is similar to that of non-thalassemia DNA.

Stability of cDNA·Cellular DNA Hybrids. Hybrids of non-thalassemia, β^+ thalassemia, and hydrops fetalis DNA with purified α and β cDNAs were tested for their stability by measurement of their melting temperatures (T_m) using

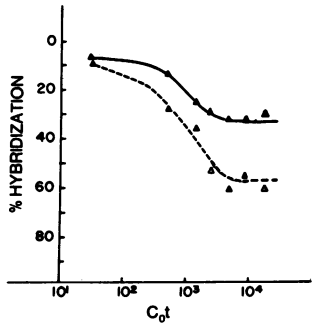

FIG. 5. Hybridization of hydrops fetalis liver DNA to purified α and β cDNAs. 50 μl aliquots of a 400 μl incubation mixture containing 1.2 mg of DNA and 2500 cpm of either α or β cDNA in 0.12 M sodium phosphate (pH 6.8) and 0.4% dodecyl sulfate were heated in a boiling H_2O bath for 15 min and incubated at 68° for various times in order to attain the desired C_0t values shown (17). The samples were assayed by hydroxylapatite as described previously (1). ▲——▲, α cDNA; △- - -△, β cDNA.

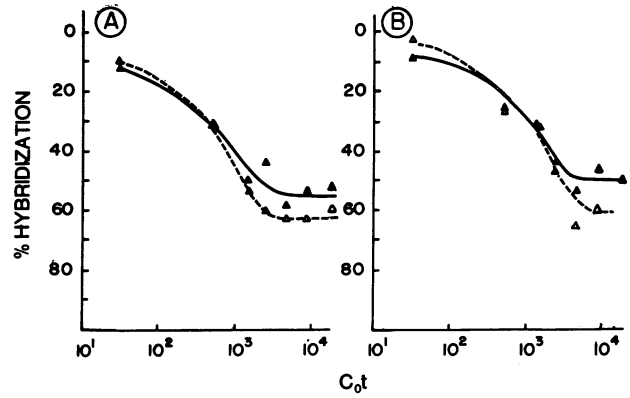

FIG. 6. Hybridization of normal and β^+ thalassemia spleen DNA to purified α and β cDNA. The hybridization mixtures and conditions of assay are as described in the legend to Fig. 5. ▲——▲, α cDNA; △- - -△, β cDNA. A, normal spleen DNA; B, β^+ thalassemia spleen DNA.

hydroxylapatite analysis as previously described (1). The T_m in hydrops DNA·α cDNA hybrids was 84.5° which was lower than that of hydrops DNA·βcDNA, 86.5°. The T_m of α and β cDNA with non-thalassemia and β^+ thalassemia DNAs was 87–88° (Table 3).

DISCUSSION

Purified α and β cDNAs have been prepared and used to assay the relative amounts of α and β mRNA in total human mRNA, and to determine the relative number of α and β globin genes in human DNA. The hybridization of these purified probes to non-thalassemia, β thalassemia, and α thalassemia mRNA are consistent with their being highly selective in their interaction with α or β globin-specific nucleotide sequences. The plateau of hybridization at 55% between hydrops fetalis mRNA and total human globin cDNA suggests that there is limited cross hybridization between α cDNA and non-α mRNAs (Fig. 2). The selectivity of the probes is further indicated by their hybridization to normal, β thalassemia, and α thalassemia mRNA (Table 1).

Hybridization of the purified α and β cDNAs with cellular DNA indicates that there are between one and five β-like genes and one to four α-like genes in nonthalassemia DNA. The results show that hydrops fetalis DNA contains numbers of globin genes hybridized with β cDNA that are similar to that for non-thalassemia DNA. There is no detectable deletion of β genes in the cellular DNA of the β^+ thalassemia patient analyzed. On the other hand, there is deficiency in the number of genes hybridizing to α cDNA in the hydrops fetalis as compared to normal or β^+ thalassemia DNA. These results are consistent with the existence of a deletion of at least part of the α globin genes in patients with hydrops fetalis (2, 10).

TABLE 2. *Calculation of gene copy number and saturation hybridization values to α and β cDNA*

Source of DNA:	Exp.	α cDNA (ng)	% Hybrid	No. of α gene copies	β cDNA (ng)	% Hybrid	No. of β gene copies
Normal spleen	1	0.328	54	3	0.328	61	4
Normal spleen	2	0.150	47	1	0.143	59	2
Normal spleen	3	0.750	39	3–4	0.714	45	4–5
β^+ thalassemia	1	0.328	49	2–3	0.328	59	3–4
β^+ thalassemia	2	0.150	47	1	0.143	58	2
β^+-thalassemia	3	0.750	35	3–4	0.714	40	4
Hydrops fetalis liver	1	0.328	33	1	0.328	60	3–4
Hydrops fetalis liver	2	0.150	30	0.5	0.143	55	1–2
Hydrops fetalis liver	3	0.750	24	1–2	0.714	48	5

Each hybridization contained 1.2 mg of DNA in a 400 μl reaction mixture except for Exp. 3, in which β^+ thalassemia DNA which contained 1.13 mg was used. The number of gene copies was calculated as previously described (1). The specific activity of cDNA is 1.4 × × 10^7 cpm/μg, and between 2000 and 10,500 cpm were added to each hybridization. The fraction of a single globin gene in the human genome was calculated as 1.1 × 10^{-7} (1); thus, a single globin gene represents 1.1 × 10^{-7} × 1.2 mg = 0.132 ng. The number of globin genes was calculated by the equation:

$$\frac{0.132\ (N)}{0.132\ (N) + \text{ng of cDNA}} = \%\ \text{hybridization of input cDNA}$$

where N is the number of globin genes. For normal DNA, Exp. 1:

$$\frac{0.132\ (N)}{0.132\ (N) + 0.328} = 54\%,\ N = 3.$$

The hybridization kinetics of Exp. 1 (hydrops fetalis DNA) is shown in Fig. 5, while Exp. 1 in which normal and β^+ thalassemia DNA were used is shown in Fig. 6.

TABLE 3. *Melting temperature of DNA·cDNA hybrids**

DNA	cDNA	T_m (°C)
Non-thalassemia	—	87.5
Non-thalassemia	α	87.5
Non-thalassemia	β	88.0
β thalassemia	—	88.0
β thalassemia	α	88.0
β thalassemia	β	87.5
Hydrops fetalis	—	87.5
Hydrops fetalis	α	84.5
Hydrops fetalis	β	86.5

* The T_m is the temperature at which 50% of the cDNA or cellular DNA retained by hydroxylapatite in 0.14 M sodium phosphate at 64° becomes single stranded and is eluted from the column.

There is some hybridization of α cDNA to hydrops fetalis DNA (Fig. 5), the nature of which is unclear. This hybridization may represent the continued presence of some undeleted α globin structural gene material, or hybridization of α cDNA to either untranscribed α chain genes or to zeta genes (15). Zeta globin chains have significant homology with α chains (15). The precise nature of the globin genes hybridizing to β cDNA is also not defined. This genetic material may represent β genes alone, β and δ genes, or β, δ, and γ genes. From genetic studies, there appears to be one β and one δ gene per human haploid genome (16). Since the δ chains are structurally quite similar to β chains, it is likely that β cDNA will cross hybridize to δ genes. There is evidence that there are multiple numbers of γ chain genes in human genomes (16). The extent of hybridization of γ chain genes to the β cDNA is not as yet defined.

It is interesting that at all levels of cDNA input, the amount of gene material hybridizing to β cDNA is somewhat greater than that hybridizing to α cDNA. This may be due to a greater number of β-like genes than α-like genes in the human DNA. Alternatively, it is possible that these differences reflect different levels of contamination of one cDNA with the other or greater cross hybridization of the β cDNA with non-β globin gene sequences.

The inability to detect a difference in the relative amounts of α- and β-like genes present in the DNA of β^+ thalassemia patients and normal DNA studied here suggest that there is no detectable deletion of β globin genes in β^+ thalassemia. The sensitivity of these experiments to detect a deletion in β globin gene material, of course, depends on the nature of the gene sequences hybridizing to the β globin cDNA. Extrapolating from the results obtained using hydrops fetalis DNA, the deletion of one type of globin gene (α genes, in this case) can be detected. If, on the other hand, the major percent of the hybridization of the β cDNA is to δ and γ genes as compared to β genes, it may be possible that a small deletion in the amount of β globin gene material in β^+ thalassemia genomes could be obscured. The melting temperature profiles of purified α and β cDNA with normal and β^+ thalassemia spleen DNA are similar and indicate comparable base pairing between the separated α and β cDNAs and cellular DNAs. The T_m of the hybrids of α cDNA with hydrops fetalis DNA is 2° less than with β cDNA (Table 3). These data, however, do not permit quantitation of the differences in the homology of the hybridizing cellular DNA sequences.

The data to date suggest that in β^+ thalassemia there is no deletion of β globin genes. At the same time, there is a significant reduction in the amount of β globin mRNA detectable in the cytoplasm of these cells. These results indicate that the decreased amount of β mRNA is due to either repression of transcription of β globin genes in the nucleus or abnormal processing of β globin mRNA resulting in rapid destruction of mRNA either in the nucleus or cytoplasm. By contrast, in the α thalassemias, a deletion of α gene material appears to account for the decreased α mRNA. It will be interesting and important to examine the DNA of other patients with α, β^+, and β^0 thalassemia in order to determine the spectrum of genetic defects which account for the thalassemia syndromes.

Note Added in Proof. In an experiment in which a large excess of hydrops fetalis liver DNA was added to α cDNA (3 mg of liver DNA and 0.03 ng of α cDNA), only 34% hybridization was obtained. Thus, α structural gene material in hydrops liver is either largely deleted or absent.

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