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Human Globin Gene Analysis for a Patient with $\beta^0/\delta\beta^0$-Thalassemia

(gene number/gene linkage/hemoglobin synthesis)

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ABSTRACT **Complementary DNA (cDNA) was prepared with RNA-dependent DNA polymerase from human globin messenger RNA (mRNA). Annealing and translation experiments with total mRNA from circulating cells from a patient with heterozygous beta^0/heterozygous beta-delta^0 thalassemia ($\beta^0/\delta\beta^0$-thalassemia) demonstrated no detectable mRNA for β-globin. cDNA enriched in sequences homologous to β-globin mRNA was prepared by hydroxylapatite fractionation of hybrids formed between $\beta^0/\delta\beta^0$-thalassemic mRNA and cDNA made from mRNA from a patient with α-thalassemia (hemoglobin H disease). The rate of annealing of this β-enriched cDNA to normal human nuclear DNA was that of a sequence present as only a single copy per haploid genome. The β-enriched cDNA annealed to the β^0-$\delta\beta^0$-thalassemia total DNA with approximately the same kinetics as to normal DNA, indicating that no total gene deletion of β-globin genes from the diploid genome has occurred, although the accuracy of the technique could not exclude with certainty a partial deletion or a deletion of a β-globin gene from only one of the haploid genomes. This demonstrates that at least one of the β^0- or the $\delta\beta^0$-thalassemia haploid genomes in this case contains a substantially intact β-globin gene.**

The common genetic disease β-thalassemia (β-thal) is characterized by a reduced production of hemoglobin A (HbA, $\alpha_2\beta_2$), which results from the reduced synthesis of β-globin chains relative to α-globin chains, thus causing an imbalance in globin chain production and hence abnormal erythropoiesis (1). The β-thalassemias show heterogeneity, and there are now several well-defined conditions that form this group. In some cases there is a partial deficiency of β-chain production (β^+-thal), in others a total deficiency of β-chain production (β^0-thal), and finally there is a condition in which both the δ chains of HbA_2 ($\alpha_2\delta_2$) and the β-chains of HbA are not produced ($\delta\beta^0$-thal). It has been demonstrated previously that in many of these forms of β-thalassemia there is a reduction in the amount of β-globin messenger RNA (mRNA). This is seen by the lack of mRNA translation activity *in vivo* and in homologous cell-free systems (2, 3), in heterologous cell-free systems (4–6), and by hybridization with complementary DNA (cDNA) prepared from globin mRNA with viral RNA-dependent DNA polymerase (7–9). There is evidence, however, that in at least one variant of β-thalassemia, that originating in the Ferrara region of Italy, inactive globin mRNA for β-globin chains is present (10, 11). In all other types studied there appears to be a direct correspondence between the amount of β-mRNA present and the amount of β-globin chain synthesized, both in homozygotes and heterozygotes.

The β, δ, and γ-globin genes are thought to be adjacent to one another in a small region of a single chromosome (1, 12). The likely order of genes is γ^{gly}, γ^{ala}, δ, β. It is possible, therefore, that the β^0 and $\delta\beta^0$-thalassemias represent gene deletions of either the β, or the β and δ, globin genes, in whole or in part. On the other hand, it is possible that single base mutations in the DNA prevent synthesis of globin mRNA or allow synthesis but prevent translation. Such mutations could be in the coding gene or in the intergene region of the genome. Other types of remote mutations may be postulated, involving control elements (nonhistone proteins, transfer RNAs, etc.) which might cause the loss of specific gene function.

Recently it has been shown by cDNA/DNA hybridization techniques that in at least some cases the severe form of α-thalassemia is the result of a gene deletion involving all or a considerable part of the α-globin genes (13, 14). In the present study we have applied similar techniques to see if the β-globin genes are present in an individual heterozygous for both β^0- and $\delta\beta^0$-thalassemia ($\beta^0/\delta\beta^0$-thalassemia). In the course of this study we have also made an estimate by two independent methods of the number of genes for β-globin and the total number of human globin genes.

MATERIALS AND METHODS

Patient Studied. The patient was an $18^1/_2$-year-old Italian man who presented the clinical picture of thalassemia intermedia. He was splenectomized at the age of four and since then has been relatively well, with a hemoglobin level in the 8 g/dl range. He presented recently with symptoms and signs of iron overload and was further investigated at this time. The blood findings were as follows: Hb, 9.5 g/dl; erythrocytes, $3.64 \times 10^6/\mu l$; mean corpuscular Hb, 26.5 pg; mean corpuscular volume, 80 fl; and mean corpuscular Hb concentration, 32.9%. The blood film showed typical thalassemic changes with many nucleated red cells. Starch gel electrophoretic analysis of his hemoglobin (1) revealed Hbs F and A_2 only, and no HbA was detectable. The HbA_2 value was 1.5%. The absence of HbA was confirmed by column chromatography on Amberlite IRC50 (15).

Both parents were available for study. In each case there was a mild thalassemic blood picture, and the following elec-

Abbreviations: D_0t, product of D_0 (initial concentration of cDNA in mol of nucleotide $\times$ liter^{-1}) and time (sec); C_0t, product of C_0 (initial concentration of total DNA in mol of nucleotide $\times$ liter^{-1}) and time (sec).

trophoretic data were obtained: father, HbA_2 3.3%, HbF 11.4%; mother, HbA_2 6.5%, HbF 1.8%. These findings indicate that the patient is heterozygous for both β^0- and $\delta\beta^0$-thalassemia, the β^0-thalassemia gene having come from the mother and the $\delta\beta^0$-thalassemia gene from the father.

Because the patient showed marked iron loading, a program of repeated venesections was started, thus enabling us to obtain relatively large amounts of nucleated red cells from the peripheral blood for the isolation and purification of DNA. For this purpose, whole blood was centrifuged and the cells were lysed with 1.5 volumes of distilled water for nuclear isolation. DNA was prepared from the fraction pelleting at 2000 *g* for 10 min (16). In addition, blood was obtained for RNA isolation, in which case the cells were washed three times and the buffy coat (white cells) was discarded.

Normal human DNA was prepared from the spleen of a patient with a nonthalassemic disorder (16). Similarly, adult human reticulocytes from patients with nonthalassemia hemolytic anemias were obtained for the preparation of α, β-globin mRNA and cord blood reticulocytes were obtained at exchange transfusion of infants with Rh incompatibility for the preparation of α, β, γ-globin mRNA. In order to obtain reticulocytes containing enriched β-globin mRNA, peripheral blood was obtained from an individual with HbH disease.

Messenger RNA Isolation and Translation. Messenger RNA was prepared by affinity chromatography with oligo(dT)-cellulose (G. D. Searle, High Wycombe, Bucks, England) from total RNA prepared with phenol/chloroform from whole blood cells (17, 18). Approximately 1% of the total RNA was retained by the column. The mRNA was assayed for translational activity in a wheat germ cell-free system containing [^{14}C]leucine (19). After incubation for 90 min, 30 mg of carrier human hemoglobin (prepared from neonatal exchange transfusion blood) was added, and the globin was extracted and chromatographed on carboxymethyl-cellulose (20).

Total DNA Isolation. DNA was prepared from normal spleen and thalassemic circulating blood cell nuclei by hydroxylapatite chromatography (16) and sonicated in 0.1 M NaOH to an average size of approximately 300 nucleotides.

Complementary DNA Preparation. cDNAs were prepared with reticulocyte mRNAs as template and oligo(dT)$_{12-18}$ as primer in the presence of actinomycin D with RNA-dependent DNA polymerase prepared from avian myeloblastosis virus (a gift from Dr. Beard, Duke University, North Carolina), with [^{3}H]dCTP [Radiochemical Centre, Amersham, U.K., specific activity 13.3 Ci/mmol ($cDNA_{\alpha,\gamma,\beta}$) or 25 Ci/mmol ($cDNA_{\alpha,\beta}$, $cDNA_{HbH}$)] as the only labeled deoxynucleotide (21). The normal adult mRNA used directed only the synthesis of α and β globins (ratio 57:43) ($mRNA_{\alpha,\beta}$); the mRNA from cord exchange blood, α, β, and γ globins (55:13:32) ($mRNA_{\alpha,\gamma,\beta}$); and the mRNA from hemoglobin H disease blood, α and β globins (17:83) ($mRNA_{HbH}$). The cDNAs from these templates were purified on alkaline sucrose gradients, and fractions sedimenting at greater than 5 S were taken. The mean molecular weight of the $cDNA_{\alpha,\gamma,\beta}$ is approximately 130,000; that of $cDNA_{\alpha,\beta}$ is approximately 100,000; and that of $cDNA_{HbH}$, 80,000–90,000. The specific activity of the $cDNA_{\alpha,\gamma,\beta}$ is 7,000 cpm/ng and that of both $cDNA_{\alpha,\beta}$ and $cDNA_{HbH}$ is 14,000 cpm/ng, at a counting efficiency of 30%. For some experiments the cDNA was freed of the low level of sequences behaving as double-stranded DNA by passage through hydroxylapatite; the nonbound fraction at 0.16 M phosphate was desalted by dialysis, decalcified by Dowex X8 resin, and lyophilized.

cDNA Hybridization to mRNA. Thirty-five picograms of cDNA in 2.5 μl were incubated with varying amounts of mRNA in a hybridization solution containing 0.5 M NaCl, 25 mM Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 10 mM EDTA (pH 6.8), 50% formamide, and 500 μg/ml of *Escherichia coli* RNA for 12 days at 43° (21, 22). Under these conditions all hybridization of globin cDNA to mRNA will go to completion. Some hybridizations were performed at 50°, as indicated in the text, to determine whether mismatched hybrids of low stability are formed at the lower temperature.

Preparation of β-Enriched cDNA. Twenty nanograms of $cDNA_{HbH}$ were hybridized to 200 μg of total $\beta^0/\delta\beta^0$-thalassemia RNA in 0.5 ml of hybridization buffer at 43°. After 4 days the mixture was diluted with 20 volumes of 150 mM NaCl and the nonhybridized cDNA (enriched in $cDNA_\beta$ sequences) was separated from the hybrid by hydroxylapatite fractionation at 60°. Approximately 30% of the cDNA was eluted by 0.14 M sodium phosphate, pH 6.8. This fraction was treated with 0.3 M NaOH to remove the RNA, neutralized, desalted by dialysis, decalcified by Dowex X8 resin treatment, and lyophilized ($cDNA_{\beta\text{-enriched}}$).

cDNA Hybridization to Excess Total DNA. Total DNA fragments were neutralized, precipitated with ethanol, redissolved in distilled water, and dialyzed three times against 20 volumes of water. $cDNA_{\beta\text{-enriched}}$ (0.16 ng, 2240 cpm) was mixed with excess (40×10^6:1) normal or β-thalassemic DNA at a concentration of 5 mg of DNA per ml in 0.12 M sodium phosphate, pH 6.8. Ninety-microliter aliquots of the solution were sealed in sterile silicone-treated capillaries; the mixtures were denatured for 10 min at 100°, then annealed at 60°. At the appropriate times, samples were removed and fractionated on hydroxylapatite columns (13, 21). Reannealing of total DNA was followed by measuring spectrophotometrically the proportion of DNA eluted as single-stranded material (at 0.16 M sodium phosphate, pH 6.8) and double-stranded material (at 0.4 M sodium phosphate). Radioactivity of the 0.16 M and 0.4 M phosphate fractions was determined directly in Instagel (Packard). In all cases, at least 95% of the radioactivity, or of the total DNA, was recovered as single-stranded or reannealed DNA by hydroxylapatite. Zero time backgrounds of approximately 5–10% were subtracted, and the results are normalized for this.

Hybridization of cDNA to Total DNA in cDNA Excess. Nine hundred micrograms of normal or β-thalassemic total DNA were mixed with 165 or 190 pg of $cDNA_{\beta\text{-enriched}}$ (approximately 2000 cpm) in 0.14 M sodium phosphate, pH 6.8, at a concentration of 5 mg of DNA/ml. The scintillation counter background for a control sample was 9–11 cpm. Other cDNA:DNA ratios were also used, as indicated in *Results*. Twenty-microliter aliquots were sealed in capillaries, denatured at 100° for 10 min, and annealed at 66°. At appropriate times, samples were diluted with 500 μl of S1 nuclease assay buffer (50 mM Na acetate, pH 4.5, 2 mM $ZnSO_4$, 0.1 M NaCl, 10 μg of denatured mouse DNA/ml) and treated with S1 nuclease (21, 23). An aliquot of this solu-

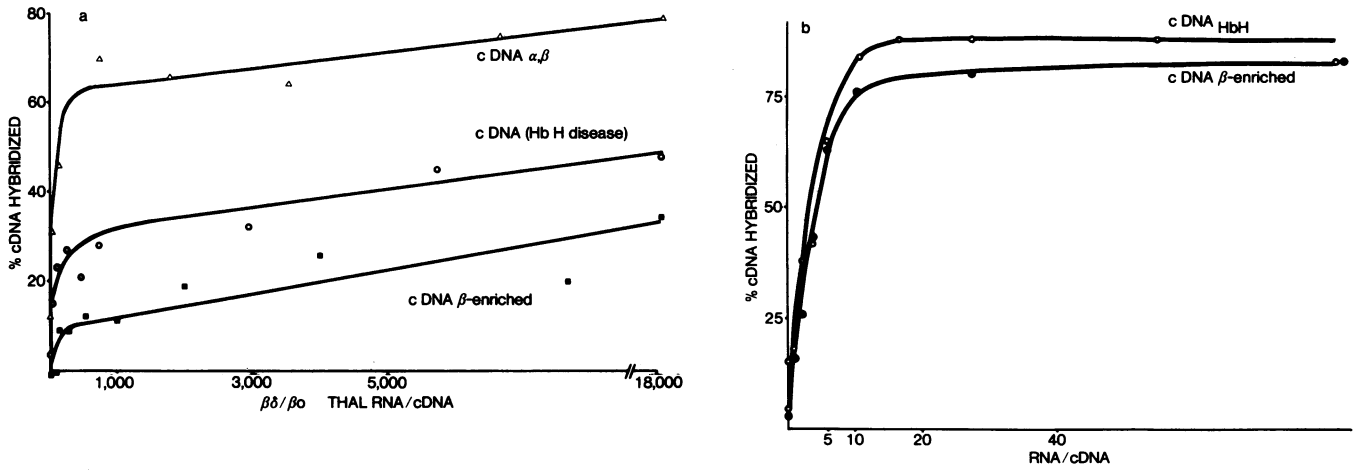

FIG. 1. Hybridization of cDNAs to $\beta^0/\delta\beta^0$-thalassemic total RNA and normal adult globin mRNA. (a) $\beta^0/\delta\beta^0$-Thalassemic total RNA hybridized to $cDNA_{\alpha,\beta}$ (△), $cDNA_{HbH}$ (○), and $cDNA_{\beta\text{-enriched}}$ (■). (b) Adult $mRNA_{\alpha,\beta}$ hybridized to $cDNA_{HbH}$ (○) and $cDNA_{\beta\text{-enriched}}$ (●).

tion was removed for measurement of total radioactivity; the remainder was precipitated with 1.0 M perchloric acid in the presence of carrier bovine serum albumin (200 μg per sample). The pellets were washed once with 1.0 M perchloric acid, dissolved in 5 M NaOH, and neutralized; radioactivity was then determined.

Normal or β-thalassemic total DNA (450 μg) was mixed with 600 pg of $cDNA_{\alpha,\gamma,\beta}$ (previously freed of double-stranded sequences) (5600 cpm) and hybridized and assayed as above.

RESULTS

Cell-Free Protein Synthesis. One microgram of mRNA from circulating erythroid cells from the patient with $\beta^0/\delta\beta^0$-thalassemia was added to a wheat germ cell-free system at a concentration where linear incorporation with mRNA is obtained, and the globin chains were fractionated. Only α- and γ-globin synthesis occur in measurable amounts, at a ratio of 76:24. The radioactivity recovered as completed globin chains was 40% of the total isotope incorporated. Less than 2% of the radioactivity incorporated into globin was under the β-globin peak.

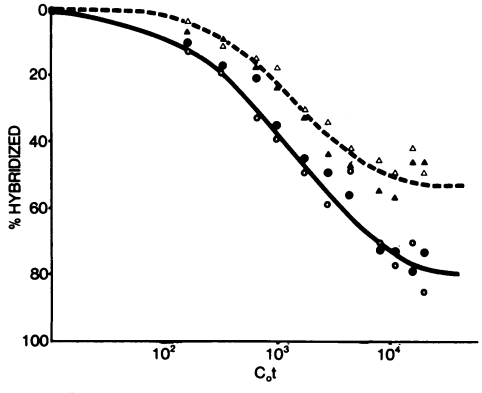

FIG. 2. Hybridization of $cDNA_{\beta\text{-enriched}}$ to excess total normal DNA (△) and $\beta^0/\delta\beta^0$-thalassemic DNA (▲). The optical reannealing of the same sample of normal DNA (○) and $\beta^0/\delta\beta^0$-thalassemic DNA (●) are also shown.

Saturation Hybridization of cDNA to mRNA Prepared from the $\beta^0/\delta\beta^0$-Thalassemia Blood. cDNA was prepared with RNA-dependent DNA polymerase to mRNA from normal adult reticulocytes ($mRNA_{\alpha,\beta}$) and to mRNA from reticulocytes from a patient with α-thalassemia (hemoglobin H disease) ($mRNA_{HbH}$). The $mRNA_{HbH}$ is already enriched in mRNA for β-globin, and cDNA prepared from it was further enriched in sequences complementary to β-globin by a cycle of hybridization to mRNA from the circulating erythroid cells of the patient with $\beta^0/\delta\beta^0$-thalassemia. The nonhybridizing cDNA ($cDNA_{\beta\text{-enriched}}$), as well as $cDNA_{\alpha,\beta}$ and $cDNA_{HbH}$, was hybridized to total RNA from the $\beta^0/\delta\beta^0$-thalassemia blood at varying RNA/DNA ratios, and the proportion hybridized was estimated by resistance to S1 nuclease digestion (Fig. 1a). This hybridization was repeated at 50° and gave identical results (data not shown). The hybridization of the $cDNA_{HbH}$ and $cDNA_{\beta\text{-enriched}}$ to purified $mRNA_{\alpha,\beta}$ goes to completion (85–90%), a necessary control to demonstrate that the thalassemic cDNAs can hybridize to mRNA containing β-globin sequences (Fig. 1b). The cDNA prepared from mRNA from nonthalassemic reticulocytes gives a plateau value with β^0-thalassemic mRNA of approximately 60–65% $cDNA_{\alpha}$, confirming the excess of α-chain synthesis (57%) found with the cell-free protein-synthesis system.

Hybridization of cDNA to Excess Normal and Thalassemic DNA. The reannealing kinetics of $cDNA_{\beta\text{-enriched}}$ to normal and $\beta^0/\delta\beta^0$-thalassemic total nuclear DNAs at a DNA:cDNA ratio of 4×10^6:1 are shown in Fig. 2. The $C_0t_{1/2}$ values for the optical reannealing of the total normal and thalassemic DNAs were indistinguishable at 1200; that for $cDNA_{\beta\text{-enriched}}$ is 1350 with normal DNA and 1750 with thalassemic DNA.

cDNA Excess Hybridization to Total DNA. The hybrid formed between $cDNA_{\beta\text{-enriched}}$ and 50 μg of total normal and $\beta^0/\delta\beta^0$-thalassemic DNAs in cDNA excess is shown as a function of time of hybridization (Fig. 3a). The counts obtained as hybrid have been converted to both percentage cDNA as hybrid and also picograms of DNA forming hybrid (pg of gene) per 50 μg of total DNA. The complexity of a

TABLE 1. *Complexities of human globin genes determined by cDNA saturation*

	cDNA/DNA	% cDNA as hybrid	Complexity (saturation values)
			βδ genes
Normal	38 pg cDNA$_\beta$/	18.2	131,500
Thal	100 μg DNA	16.4	118,600
Normal	21 pg cDNA$_\beta$/	25.4	122,000
Thal	100 μg DNA	22.2	108,900
Normal	12.6 pg cDNA$_\beta$/	32.2	103,600
Thal	100 μg DNA	27.6	83,800
			αβγ genes
Normal	132 pg cDNA$_{\alpha,\gamma,\beta}$/	19.8	544,000
Thal	100 μg DNA	23.6	679,000
Normal	264 pg cDNA$_{\alpha,\gamma,\beta}$/	14.6	759,000
Thal	100 μg DNA	15.6	821,000
Normal	396 pg cDNA$_{\alpha,\gamma,\beta}$/ 100 μg DNA	9.5	747,000
Normal	800 pg cDNA$_{\alpha,\gamma,\beta}$/ 100 μg DNA	7.5	1,006,000

Gene complexity is calculated according to equation 2 of Bishop and Freeman (23). The complexity of the cDNAs is 75,000 for cDNA$_\beta$ and 175,000–300,000 for cDNA$_{\alpha,\gamma\,\beta}$ (eq. 3, ref. 23).

DNA is defined as the total molecular weight of the sum of its nucleotide sequences, as determined experimentally by hybridization. From the data a complexity of 122,000 and 109,000 (averages of two experiments) can be calculated for normal and $\beta^0/\delta\beta^0$-thalassemic β-genes, respectively (22). The value of approximately 25% cDNA as hybrid obtained at saturation shows, retrospectively, that the cDNA excess over the β-globin genes was approximately 3:1 in these experiments. Similar results were obtained at different cDNA/DNA ratios (Table 1). The $D_0t_{1/2}$ of the hybridization was estimated from the initial slope as described by Bishop and Freeman (23), and is 48.7 μmol liter^{-1} sec and 58.5 μmol liter^{-1} sec for the hybridization of cDNA$_{\beta\text{-enriched}}$ to normal or $\beta^0/\delta\beta^0$-thalassemic DNA respectively. These two values are nearly identical, and an average complexity of the cDNA of approximately 75,000 can be calculated after correction for the salt concentration (24).

A similar experiment with cDNA$_{\alpha,\gamma,\beta}$ is shown in Fig. 3b. The complexity of the cDNA is between 175,000 and 300,000; it is more difficult to obtain an accurate $D_0t_{1/2}$ value because the cDNAs for α, β, and γ globin are not present in equal amounts. The amount of DNA sequence hybridized at saturation (complexity of the α, γ, β genes) is from 540,000 to 680,000 or higher, according to the cDNA/DNA ratio (Table 1).

DISCUSSION

In $\beta^0/\delta\beta^0$-thalassemia, no β-globin is synthesized, and from published data, it was anticipated that β-globin mRNA would be absent, as judged by heterologous cell-free translation (4–6) and by cDNA·mRNA hybridization (7–9). The absence of detectable β-globin mRNA was shown in the wheat germ cell-free protein-synthesis system and by hybridization of cDNA$_{\alpha,\beta}$ and cDNA$_{HbH}$ (Fig. 1).

The hybridization of cDNA$_{\alpha,\beta}$ and cDNA$_{HbH}$ to mRNA$_{\beta\text{-thal}}$ also demonstrates that at a very high RNA$_{\beta\text{-thal}}$/

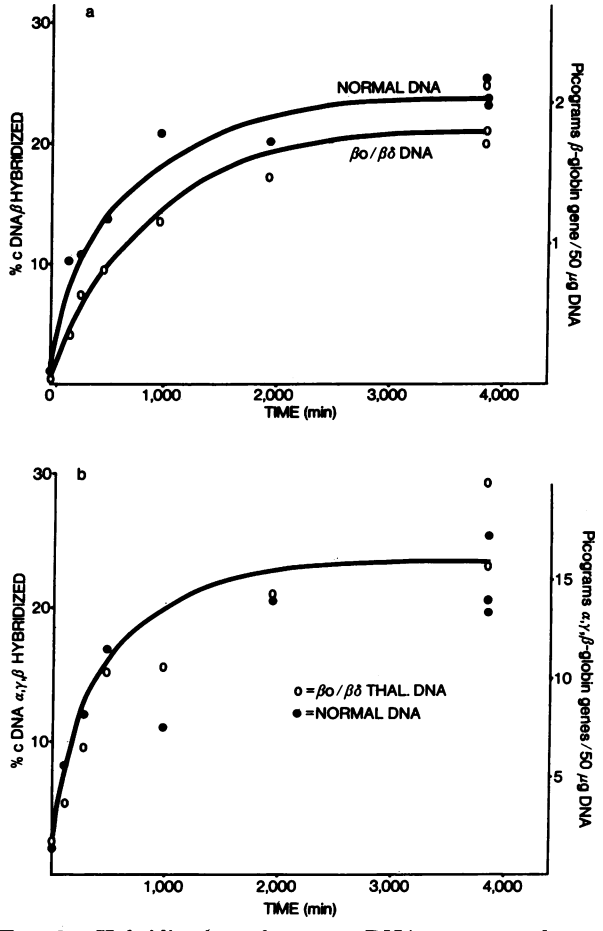

FIG. 3. Hybridization of excess cDNA$_{\beta\text{-enriched}}$ and excess cDNA$_{\alpha,\gamma,\beta}$ to total normal DNA and $\beta^0/\delta\beta^0$-thalassemic DNA. (a) cDNA$_{\beta\text{-enriched}}$ to normal DNA (●) and $\beta^0/\delta\beta^0$-thalassemic DNA (○). (b) cDNA$_{\alpha,\gamma,\beta}$ to normal DNA (●) and $\beta^0/\delta\beta^0$-thalassemic DNA (○).

cDNA ratio a small additional hybridization of cDNA$_\beta$ is observed. We interpret this slow rise as being due to the low level of mRNA$_\delta$ present, in agreement with Forget *et al.* (7). The fact that the slope of this slow rise is identical for the three types of cDNA supports this hypothesis. However, it is also possible that a very low level of mRNA$_\beta$ is present.

There is no annealing between mRNA$_\gamma$ and cDNA$_\beta$ under the hybridization conditions used (25). Possible cross-hybridization between sequences coding for β-globin and δ-globin is harder to estimate. The difference in amino acid sequence between the proteins is approximately 5%, but this gives no direct estimate of the divergence between nucleotide sequences, as already shown for globin genes during evolution (26). Nucleotide sequence mismatching between the two genes might be sufficient to affect the rate of hybridization significantly in a saturation analysis, where all components have hybridized to completion (27, 28). Thus, one would anticipate that cross-hybridization between β-globin and δ-globin coding sequences would be less significant in the rate analysis given in Fig. 2 and the initial slopes of Fig. 3a than in the plateau values obtained in Figs. 1a and 3a. The δ-globin coding sequences in these experiments arise from the mRNA isolated from the thalassemic erythroblasts and are, of course, present in the normal genome; δ-globin mRNA is almost completely absent from the normal mRNA$_{\alpha,\beta}$ from

TABLE 2. *Number of each human globin gene*

Globin chain	Total DNA excess	cDNA excess	Genetic analysis
α	2	—	2
β	1	1	1
γ	3 ± 1	—	2–4
δ	—	1	1
ϵ	—	—	2
ζ	—	—	1
Total	Not possible for total	10 ± 2 ($\alpha,\gamma,\beta,\delta$)	9–11

which the cDNA used in these experiments was prepared (29).

Both total normal and $\beta^0/\delta\beta^0$-thalassemic DNA unique fractions reannealed with a $C_0t_{1/2}$ (optical) of 1200, as previously reported for our conditions (13). The $C_0t_{1/2}$ for $cDNA_\beta$ was 1350 with total normal DNA, demonstrating that the β-globin gene sequence is present as a single copy per haploid genome. The DNA:cDNA ratio for the β-globin gene in the DNA excess experiment (Fig. 2) can be calculated for a single gene and is approximately 1.6:1; this lack of great DNA excess, together with enrichment of nonhybridizing material on hydroxylapatite to 20% of the total (Fig. 1b), explains the relatively low final level of hybridization of $cDNA_{\beta\text{-enriched}}$ obtained after optical reannealing is complete. This plateau level also supports the calculation of one β-globin gene sequence per haploid genome.

In our hands under similar reannealing conditions, $cDNA_\alpha$ hybridizes with a $C_0t_{1/2}$ of approximately 850 and $cDNA_\gamma$ with a $C_0t_{1/2}$ of approximately 350. These values are estimated from Fig. 5 of ref. 13, assuming in the case of $cDNA_\gamma$ that the contaminating cDNA does not hybridize to the α-thalassemic DNA. The $C_0t_{1/2}$ value for $cDNA_\alpha$ is double that for $cDNA_\beta$, and that for $cDNA_\gamma$ is four times that of $cDNA_\beta$; this suggests there are two copies of the α-globin gene and approximately four copies of the γ-globin gene.

The $C_0t_{1/2}$ value for $cDNA_\beta$ with total excess $\beta^0/\delta\beta^0$-thalassemic DNA was 1750, slightly greater than for normal DNA; a similar plateau value of 50% was observed. There are four genes per diploid genome, two for β-globin and two for δ-globin, which could hybridize to $cDNA_\beta$, although mismatching for $\delta:\beta$ hybrids could affect the rate of hybridization. Our results demonstrate that at least 75% (and perhaps all) of the β-globin hybridizing sequences (which may include δ-globin genes as well as β-globin genes, especially in saturation analyses) are present; we would suggest that at least one and perhaps both substantially intact β-globin genes are present in this patient with thalassemia per diploid genome. Therefore, either the β^0 or the $\delta\beta^0$ lesion cannot be due to a complete gene deletion. The technique used would be less sensitive in detecting a partial deletion towards the 5′-end of the coding strand of the DNA, since cDNA synthesis with RNA-dependent DNA polymerase proceeds from the 3′-end of the mRNA.

A detailed analysis of the use of hybridization of cDNA excess to determine globin gene number in duck has recently been published (23); we are indebted to them and have followed their treatment in this case. Since the rate of hybridization is measured, nucleotide sequences present at low concentrations contribute relatively little to the analysis, as the kinetics are dictated by the components present in greatest amounts. Since more than 90% of the hybridizable $cDNA_{\beta\text{-enriched}}$ is complementary to $mRNA_\beta$, and more than 90% of the $cDNA_{\alpha,\gamma,\beta}$ is complementary to $mRNA_{\alpha,\gamma,\beta}$ (13), the initial slope gives the rate of reannealing for these sequences and the plateau their saturation value. The rate of reannealing is inversely proportional to the nucleotide sequence length, or complexity, of all the molecular sequences present in the cDNA.

For $cDNA_{\beta\text{-enriched}}$, the complexity with normal DNA is 80,000–90,000 (100,000 by gradient sedimentation for the length of the cDNA, minus 10,000 for the poly(dT) primer sequence). This demonstrates that there is only a single major molecular species, as judged by hybridization kinetics present in $cDNA_{\beta\text{-enriched}}$. The plateau of 2–3 pg of β-globin gene sequence per 50 μg of DNA corresponds to a molecular weight of total DNA hybridizing of 100,000–120,000 per haploid genome. Since this is no more than one to two times the complexity of the $cDNA_{\beta\text{-enriched}}$, the β-like genes are represented once or twice only by a complementary sequence in the haploid genome. It is likely that under the experimental conditions used the $cDNA_\beta$ would hybridize to the δ-gene as well as to the β-gene; the complexity of the gene determined above demonstrates both the β and δ genes are unique.

The roughly equal saturation plateaus in hybridization in cDNA excess for normal and $\beta^0/\delta\beta^0$-thalassemic DNAs confirms that a major β-gene deletion has not occurred in at least one of the two thalassemic haploid genomes.

A similar analysis for $cDNA_{\alpha,\gamma,\beta}$ gives a cDNA complexity of 175,000–300,000 (demonstrating two to three major molecular species of cDNA present, as the average molecular weight of the cDNA is slightly greater) and a saturation value of molecular weight between 600,000 and 1,000,000 per haploid genome, depending on the cDNA/DNA ratio. This suggests the presence of approximately three gene groups, each reduplicated approximately 3-fold. Even at five times higher cDNA:DNA ratios, the saturation values and complexities increase less than 2-fold, demonstrating the purity of the cDNA probes used.

One can suggest gene numbers for each of the globin genes from DNA excess (high C_0t), from cDNA excess data, and from genetic analysis (Table 2). The number of globin genes per haploid genome has also been estimated by genetic analysis of hemoglobinopathies (1). All of these results are in general agreement with the molecular determination reported above.

The β^+-thalassemias, which can occur in homozygotes or heterozygotes, and in which there can be very low (1–10%) β-chain synthesis, cannot be due to gene dosage alone, since a single gene must be present in the genome in one or two copies, or completely absent. Therefore, since at least one copy (50%) of the structural gene must be present, β^+-thalassemia must be a regulatory mutation.

The fact that this case of $\beta^0/\delta\beta^0$-thalassemia does not involve a total or substantial β-gene deletion in both haploid genomes demonstrates that either β^0- or $\delta\beta^0$-thalassemia, or both, occurs with the presence of the β-globin gene. This contrasts with the cases of homozygous α-thalassemia recently reported (13, 14) and again demonstrates the complexity of the levels at which thalassemia may arise. Other cases of β-thalassemia may have quite a different molecular origin.

Since this work was completed, a paper has appeared (30) indicating fewer than 20 globin genes in the human haploid genome. There was no difference in the hybridization of total human cDNA nor rabbit $cDNA_{\beta\text{-enriched}}$ to total normal human DNA and β^+-thalassemia DNA, as expected.

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1. Weatherall, D. J. & Clegg, J. B. (1972) *The Thalassaemia Syndromes* (Blackwell, Oxford), 2nd Ed.
2. Weatherall, D. J., Clegg, J. B. & Naughton, M. A. (1965) *Nature* **208**, 1061–1065.
3. Nathan, D. G., Lodish, H. F., Kan, Y. W. & Housman, D. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 2514–2518.
4. Dow, L. W., Terada, M., Natta, C., Metafora, S., Grossbard, E., Marks, P. A. & Bank, A. (1973) *Nature New Biol.* **243**, 114–116.
5. Nienhuis, A. W. & Anderson, W. F. (1971) *J. Clin. Invest.* **50**, 2458–2460.
6. Benz, E. J. & Forget, B. G. (1971) *J. Clin. Invest.* **50**, 2755–2760.
7. Forget, B. G., Benz, E. J., Jr., Skoultchi, A., Baglioni, C. & Housman, D. (1974) *Nature* **247**, 379–381.
8. Housman, D., Forget, B. G., Skoultchi, A. & Benz, E. J., Jr. (1973) *Proc. Nat. Acad. Sci. USA* **70**, 1089–1813.
9. Kacian, D. L., Gambino, R., Dow, L. W., Grossbard, E., Natta, C., Ramirez, F., Spiegelman, S., Marks, P. A. & Bank, A. (1973) *Proc. Nat. Acad. Sci. USA* **70**, 1886–1890.
10. Conconi, F., Rowley, P. T., DelSenno, L., Pontremoli, S. & Volpato, S. (1972) *Nature New Biol.* **238**, 83–87.
11. Rowley, P. T. & Kosciolek, B. (1972) *Nature New Biol.* **239**, 234–235.
12. Huisman, T. H. J., Wrightstone, R. N., Wilson, J. B., Schroeder, W. A. & Kendall, A. G. (1972) *Arch. Biochem. Biophys.* **153**, 850–853.
13. Ottolenghi, S., Lanyon, W. G., Paul, J., Williamson, R., Weatherall, D. J., Clegg, J. B., Pritchard, J., Pootrakul, S. & Boon, W. H. (1974) *Nature* **251**, 389–392.
14. Taylor, J. M., Dozy, A., Kan, Y. W., Varmus, H. E., Lie-Injo, L. E., Ganeson, J. & Todd, D. (1974) *Nature* **251**, 392–393.
15. Weatherall, D. J., Clegg, J. B., Na-Nakorn, S. & Wasi, P. (1969) *Brit. J. Haematol.* **16**, 251–259.
16. Hell, A., Birnie, G. D., Slimming, T. K. & Paul, J. (1972) *Anal. Biochem.* **48**, 369–377.
17. Perry, R. P., LaTorre, J., Kelley, D. E. & Greenberg, J. R. (1972) *Biochim. Biophys. Acta* **262**, 220–229.
18. Aviv, H., & Leder, P. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 1408–1412.
19. Roberts, B. E. & Paterson, B. M. (1973) *Proc. Nat. Acad. Sci. USA* **70**, 2330–2334.
20. Clegg, J. B., Naughton, M. A. & Weatherall, D. J. (1966) *J. Mol. Biol.* **19**, 91–108.
21. Harrison, P. R., Birnie, G. D., Hell, A., Humphries, S., Young, B. D. & Paul, J. (1974) *J. Mol. Biol.* **84**, 539–554.
22. Young, B. D., Harrison, P. R., Gilmour, R. S., Birnie, G. D., Hell, A., Humphries, S. & Paul, J. (1974) *J. Mol. Biol.* **84**, 555–568.
23. Bishop, J. O. & Freeman, K. B. (1974) *Cold Spring Harbor Symp. Quant. Biol.* **38**, 707–716.
24. Britten, R. J. (1969) in *Problems in Biology: RNA in Development*, ed. Hanly, E. W. (University of Utah Press, Salt Lake City), p. 187.
25. Lanyon, W. G., Ottolenghi, S. & Williamson, R. (1975) *Proc. Nat. Acad. Sci. USA* **72**, 258–262.
26. Gummerson, K. W. & Williamson, R. (1974) *Nature* **247**, 265–267.
27. Southern, E. M. (1971) *Nature New Biol.* **232**, 82–83.
28. Britten, R. & Bonner, T. (1971) *Annual Report of the Carnegie Institution, Department of Terrestrial Magnetism* **70**, 373–374.
29. Roberts, A. V., Weatherall, D. J. & Clegg, J. B. (1972) *Biochem. Biophys. Res. Commun.* **47**, 81–87.
30. Gambino, R., Kacian, D., O'Donnell, J., Ramirez, F., Marks, P. A. & Bank, A. (1974) *Proc. Nat. Acad. Sci. USA* **71**, 3966–3970.