Proc. Natl. Acad. Sci. USA
Vol. 75, No. 3, pp. 1222–1226, March 1978
Biochemistry

Changes in restricted human cellular DNA fragments containing globin gene sequences in thalassemias and related disorders

(β thalassemia/restriction enzyme digestion/"blot" hybridization/cellular DNA)

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Communicated by Paul A. Marks, January 3, 1978

ABSTRACT **Human cellular DNA fragments from cells of normal subjects and patients with thalassemia obtained by restriction enzyme digestion were analyzed for their globin gene content. The fragments were separated on agarose gels, transferred to nitrocellulose filters, hybridized to globin [^{32}P]cDNA, and radioautographed. One to ten picograms of globin gene sequences were detectable. With *Eco*RI digestion, eight to nine cellular DNA fragments were found to contain globin genes. Three of these contained β-like gene sequences assayed with β globin cDNA probe. One β-like fragment was absent in DNA from a homozygous subject for hemoglobin Lepore. Two of the three β gene-containing fragments present in normal DNA were absent in DNA from a patient with hereditary persistence of fetal hemoglobin. The same two fragments containing β-like genes were absent from δβ thalassemic DNA and one new fragment containing β-like genes was found. Together with results obtained by hybridization of these DNAs in solution, the data are consistent with deletion of specific restriction human DNA fragments in subjects with these disorders and a greater deletion of β-like gene sequences in subjects with hereditary persistence of fetal hemoglobin than in those with δβ thalassemia.**

Genetic and biochemical studies of mutant hemoglobins have demonstrated linkage of the δ, β, and γ globin genes in human beings (1). The β thalassemias and related disorders provide models for studying the regulation of this group of linked genes. In the homozygous state of β^+ and β^0 thalassemia, in which the structural β and δ globin genes are present (2, 3), γ globin gene expression is limited and anemia is severe. In δβ thalassemia and hereditary persistence of fetal hemoglobin (HPFH), no β or δ synthesis is detectable, while there is a marked increase in γ globin synthesis; there is a mild anemia in δβ thalassemia and no anemia n HPFH. In both these disorders, there is deletion of β-like globin genes assayed by hybridization in solution to β globin complementary DNA (cDNA) (4–7). We have previously reported (6) greater deletion of β-like genes in DNA from subjects with HPFH than in DNA from those with δβ thalassemia by hybridization studies in solution.

In order to further investigate the extent of deletion and to define more precisely the structural changes in DNA in these disorders, we have analyzed the globin gene-containing restriction fragments of these cellular DNAs and compared them with those from normal DNA. Human DNA fragments obtained after cleavage with the restriction enzyme *Eco*RI were separated by agarose gel electrophoresis, transferred to nitrocellulose filters, and hybridized to ^{32}P-labeled human globin cDNA. The globin gene-containing fragments were analyzed by radioautography. Between 1 and 10 pg of cellular globin gene sequences can be detected by this technique. The results indicate that in normal human DNA, eight to nine *Eco*RI cleavage products contain globin gene sequences. Of the three β-like bands found in normal DNA, homozygous Lepore DNA lacks one. HPFH DNA lacks two bands containing β-like globin gene sequences that are present in normal DNA; this is consistent with deletion of β-like gene material. δβ thalassemic DNA lacks the same two bands as HPFH and has one new β-like band, consistent with less deletion of β-like gene sequences than in HPFH. These results indicate that restriction enzyme analysis can be used to detect deletions of specific globin gene fragments in human cellular DNA. More extensive analysis of this type can be expected to define further the molecular defect in the DNA of patients with these and related disorders.

MATERIALS AND METHODS

Source and Preparation of Cellular DNA. Lymphocyte cell lines from normal subjects and from previously described patients homozygous for HPFH (8), δβ thalassemia (6), and hemoglobin Lepore (9) were established by Epstein–Barr virus transformation of mononuclear cell fractions of peripheral blood (10). Cellular DNA was also obtained from normal human spleens and from liver of a patient homozygous for hydrops fetalis (2). DNA was extracted and samples were hybridized in solution as described (11).

Purified *Hha* I-digested PMB9 plasmid DNA, containing full-length β cDNA, was kindly provided by John Wilson of Yale University (J. T. Wilson, L. B. Wilson, B. F. Forget, and S. M. Weissman, unpublished data). Fragments of plasmid DNA were handled under P_1 containment conditions in accordance with the National Institutes of Health Guidelines for Recombinant DNA Research, and with the approval of the Columbia University Biosafety Committee. Salmon testes DNA (Worthington) was sheared by boiling in 0.3 M NaOH/10 mM EDTA for 20 min. The DNA was neutralized with HCl and precipitated with ethanol; it was redissolved in 1.5 mM NaCl/0.05 mM Na_2EDTA, pH 7.0.

Preparation of Globin [^{32}P]cDNA. Poly(A)-containing mRNA was obtained from the reticulocytes of patients with SS disease as described (12). Purified α and β mRNA fractions were obtained by polyacrylamide gel electrophoresis and elution as described (13). The procedure for cDNA preparation was essentially that of Kacian and Myers (14): the 100-μl reaction mixture contained 50 mM Tris (pH 8.3), 8 mM $MgCl_2$, 50 mM KCl, 4 mM sodium pyrophosphate, 0.4 mM dithiothreitol, 5 μg of oligo$(dT)_{10}$ per ml, 0.1 mM of each de-

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Abbreviations: HPFH, hereditary persistence of fetal hemoglobin; cDNA, complementary DNA; SSC, 0.15 M NaCl/15 mM trisodium citrate, pH 6.8; kb, kilobases.

oxynucleotide, 15 units of reverse transcriptase (RNA-directed DNA nucleotidyltransferase), and 0.6–2.5 μg of mRNA. Either [^{32}P]dCTP or [^{32}P]dGTP (New England Nuclear) was used as the labeled nucleotide and was diluted to a specific activity of approximately 100 Ci/mmol with unlabeled dCTP or dGTP. Reverse transcriptase was generously contributed by J. Beard. cDNA (calculated specific activity 1.2–1.5 × 10^8 cpm/μg) was prepared in this reaction.

Restriction Endonuclease Cleavage. *Eco*RI was obtained from either New England Biolabs or Boehringer Mannheim. The *Eco*RI digestions were at 37°C for 1 hr in 0.1 M Tris·HCl (pH 7.5)/50 mM NaCl/10 mm $MgCl_2$. To ensure complete digestion, 1.5-fold more enzyme was added than that required to completely digest viral DNA. After digestion, the DNA was extracted with phenol and precipitated with ethanol. The pellet was washed with 70% ethanol and dried under reduced pressure. The DNA was redissolved in 1.5 mM NaCl/0.05 mM Na_2EDTA, pH 7.0.

Gel Electrophoresis. Vertical slab gels (EC Corp., Hialeah, FL) 6 mm thick were formed with 1.0% agarose (Seakem) with eight slots (10 × 5 × 8 mm). The agarose was dissolved by boiling in 30 mM NaCl/2 mM EDTA (pH 7.0)/0.5 μg of ethidium bromide per ml. Aliquots (30 μl) of DNA were added to 20 μl of 0.2% agarose beads and allowed to sit for 15 min at room temperature (15). The samples were then applied to the gel slots and allowed to settle for 5 min. They were run at 4°C at 3 V/cm for 15–16 hr in 30 mM NaOH/2 mM EDTA (15). *Hin*dIII digests of λ DNA were run concomitantly in an outer slot as size markers.

DNA Transfer and Hybridization. After electrophoresis, the gels were sliced with a razor blade (usually two channels of the gel per slice), and neutralized in 1 M Tris (pH 7.0) by gently shaking for 1 hr. The gels were then soaked in 6× SSC (SSC: 0.15 M NaCl/15 mM trisodium citrate, pH 6.8) for 0.5 hr. Nitrocellulose strips (HAWP, Millipore Corp.) were cut slightly larger than the size of the gel and soaked in 6× SSC for 5 min.

DNA fragments were transferred to the nitrocellulose by a modification of the technique of Southern (16, 17). The gel was placed on top of a wet nitrocellulose strip which in turn was placed on 10 layers of dry Whatman no. 1 filter paper. A strip of Whatman no. 1 paper wet with 6× SSC, was placed on the gel. This Whatman strip was kept well moistened with 6× SSC for 1 hr. The layers of Whatman paper under the nitrocellulose filter were changed every 15 min, or when excessively moist. The Whatman strip atop the gel was then removed and the gel was covered with Saran Wrap. The gel was allowed to dry to a thin membrane-like layer for at least 4 hr. The underlying Whatman papers were changed every 30 min and the gels were weighted with a glass plate during this process; for the final 30 min, the gel was weighted with an empty 1-liter flask. The gels were then peeled off and the nitrocellulose strips were briefly washed in 2× SSC and both sides firmly rubbed by polyethylene-gloved fingers to remove any adherent pieces of gel or filter paper. The strips were air-dried at room temperature for 30 min and then placed in a vacuum oven at 80°C for 8–15 hr. Some filters were stored under reduced pressure at room temperature until hybridization. Filters were then preincubated prior to hybridization for 6–8 hr at 68°C in 6× SSC, 0.02% Ficoll, 0.02% bovine serum albumin, 0.02% polyvinylpyrollidone, 0.25 M NaH_2PO_4, 1.5% sodium pyrophosphate (pH 6.5), and 50 μg of sheared salmon testes DNA per ml.

Hybridizations were performed in sealed plastic 20-ml scintillation vials (2.4 cm in diameter) which contained an inner siliconized glass vial (1.9 × 4.8 cm). Generally, three nitrocellulose filters, each 11 × 3 cm, were wrapped while wet with preincubation solution around the inner glass vial. They were added to the plastic cylinder, which contained 2 ml of hybridization solution consisting of 1.5× SSC, 0.02% bovine serum albumin, 0.02% Ficoll, 0.02% polyvinylpyrollidone, 0.1% sodium dodecyl sulfate ($NaDodSO_4$), 1 mM EDTA (pH 7.0), sheared salmon testes DNA (5 μg/ml), and globin [^{32}P]cDNA (25 ng/ml). This concentration of cDNA was used to obtain a C_0t of 5 × 10^{-3} after 24 hr of hybridization (18). The capped cylinder was rotated slowly at 60° from the horizontal in a water bath at 68°C for 24 hr. After hybridization, the filters were washed eight times in 2× SSC/0.5% $NaDodSO_4$ at 68°C for 10 min each. A final wash in 0.2× SSC/0.5 % $NaDodSO_4$ was performed at 68°C for 10 min. The filters were then rinsed in 2× SSC at room temperature and dried under a heat lamp. Radioautography was performed for various times in Kodak cassettes with Kodak film and Dupont "Lighting Plus" intensifying screens.

RESULTS

General Considerations. In initial experiments, the restriction enzyme-digested DNA was run in neutral gels. A large amount of DNA that hybridized to globin [^{32}P]cDNA was found close to the origin under these conditions. The use of alkaline gels resolved this problem. The high background of filters seen in initial experiments was eliminated by several modifications which included preincubation with phosphate-containing solutions (*Materials and Methods*), low salt concentration in the hybridization mixture, adequate and continuous wetting of the filters during hybridization by rotating the hybridization vessel, and extensive washing of the filters after hybridization.

Sensitivity and Specificity of Hybridization. An experiment was performed to analyze the sensitivity and specificity of the system using *Hha* I-digested plasmid DNA containing full-length β globin cDNA. The digested plasmid DNA was added in decreasing amounts to 60-μg aliquots of *Eco*RI-digested normal human cellular DNA. After electrophoresis and transfer to filters, the mixtures of plasmid and cellular DNA fragments were hybridized to ^{32}P-labeled total (α- and β-containing) globin cDNA. The results indicate that by this technique between 1 and 10 pg of structural globin gene material can be detected in the presence of 60 μg of human cellular DNA. In another experiment, decreasing amounts of restriction enzyme-digested plasmid DNA were electrophoresed in the presence of sheared salmon testes DNA. Hybridization occurred only with the known digested plasmid fragments and with the same sensitivity as before.

Globin Gene-Containing Restriction Fragments in Normal DNA. *Eco*RI-digested DNA fragments of several transformed lymphocyte cell lines obtained from normal individuals as well as from splenic tissue of patients with nonthalassemic disorders were hybridized to total (α- and β-containing) globin [^{32}P]DNA. The globin [^{32}P]cDNA was largely full length (about 700 nucleotides) when analyzed by polyacrylamide gel electrophoresis in 98% formamide (19). Between eight and nine cellular DNA fragments containing globin gene material were identified (Fig. 1). There is moderate variability in the intensity of different globin gene-containing DNA fragments. Six fragments were consistently seen as intense bands. The smallest bands [1.5, 2.0, and 2.3 kilobases kb)] are seen inconsistently but are present in the majority of experiments.

The migration of the fragments relative to each other was reproducible in four different nonthalassemic samples and in multiple experiments with the same sample (Figs. 1–4). Mi-

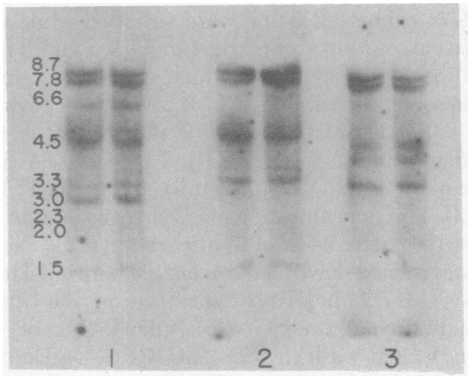

FIG. 1. Hybridizations of total [^{32}P]cDNA to *Eco*RI restriction fragments of human DNAs. Digestion of DNA, agarose gel electrophoresis, transfer to nitrocellulose filters, and hybridization and radioautography are described in *Materials and Methods*. Duplicate DNA samples are run in adjacent slots. The numbers indicate the kilobases of fragments estimated from λ DNA markers. (*1*) Normal lymphocyte cell line (130) DNA; (*2*) homozygous HPFH DNA; (*3*) homozygous δβ thalassemic DNA.

gration of *Hin*dIII digests of λ DNA, used as approximate size markers for the cellular DNA fragments, is linear over the 2- to 10-kb range when the logarithmic length of the fragments is plotted against mobility. The following length human cellular DNA fragments containing globin gene material were identified in order of decreasing size (in kb): 8.7, 7.8, 6.6, 4.5, 3.3, 3.0, 2.3, 2.0, and 1.5.

To determine which DNA bands contain β-like globin genes, experiments were performed with purified β globin [^{32}P]cDNA as the probe. With DNA from four nonthalassemic subjects, three bands with sizes of 6.6, 4.5, and 3.0 kb were identified that hybridized intensely to the β globin cDNA probe (Figs. 2 and 3). Less intense bands, corresponding to other fragments seen with total globin cDNA, were present and were variable in their relative intensity. *Eco*RI fragments from hydrops fetalis–α thalassemic liver DNA were also analyzed with β globin [^{32}P]cDNA; the three intense bands seen with nonthalassemic DNA were identified, while other bands were not clearly distinguishable (Fig. 3).

Globin DNA-Containing Fragments in Homozygous Lepore, HPFH, and δβ Thalassemia. *Eco*RI-digested DNA fragments obtained from transformed lymphocyte cell lines established from patients homozygous for HPFH and δβ thalassemia were analyzed in an identical fashion with total globin cDNA and β globin cDNA. With both total and β globin cDNA, the 6.6- and 3.0-kb fragments seen in normal DNA were absent, while the 4.5-kb band persisted (Figs. 1 and 2). In addition, a new band, 4.2 kb in size, was found in digested DNA from the δβ thalassemic cell line. Less intense bands, corresponding to the 8.7-, 7.8-, and 3.3-kb bands seen with total globin cDNA, were present with β globin cDNA. *Eco*RI-digested DNA fragments of a transformed lymphocyte cell line established from a patient homozygous for hemoglobin Lepore were analyzed with total globin cDNA. The 6.6-kb band was absent (Fig. 4).

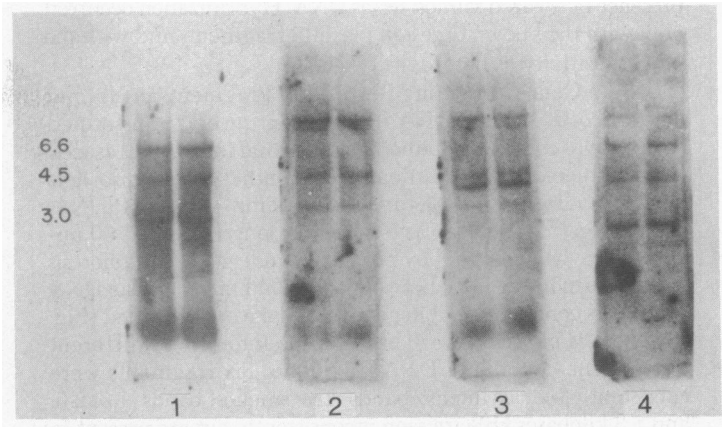

FIG. 2. Hybridizations of β globin [^{32}P]cDNA to *Eco*RI restriction fragments of human DNAs. Conditions are as described in the legend to Fig. 1. (*1*) Normal splenic DNA; (*2*) homozygous HPFH DNA; (*3*) homozygous δβ thalassemic DNA; (*4*) normal lymphocyte cell line (130) DNA.

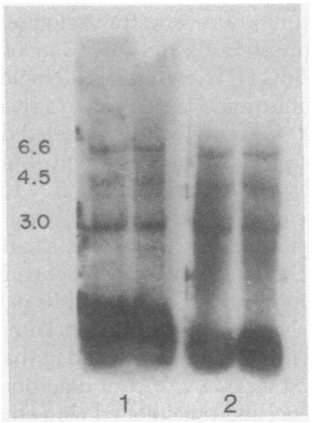

FIG. 3. Hybridizations of β globin [^{32}P]cDNA to *Eco*RI restriction fragments of human DNAs. Conditions are as described in the legend to Fig. 1. (*1*) Normal lymphocyte cell line (131) DNA; (*2*) hydrops fetalis–α thalassemic DNA.

Hybridization in Solution. Normal, homozygous δβ thalassemic, and homozygous HPFH DNA used in the restriction enzyme digest analyses described above were hybridized in solution to purified ^{3}H-labeled α and β globin cDNAs, which were full length or nearly full length (700 nucleotides long) by acrylamide gel electrophoretic analysis (19). 3.3×10^{-7} as much

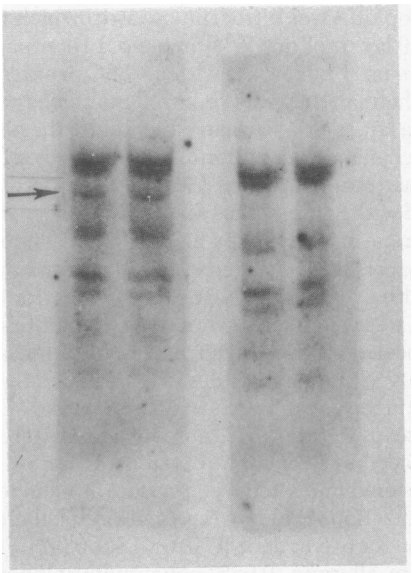

FIG. 4. Hybridizations of total [^{32}P]cDNA to *Eco*RI restriction fragments of human DNAs. Conditions are as described in *Materials and Methods* and legend to Fig. 1. (*Left*) Normal lymphocyte cell line (130) DNA; (*Right*) homozygous Lepore DNA. The arrow points to the 6.6-kb fragment present in normal DNA and absent in Lepore DNA.

globin cDNA as cellular DNA was used and assayed as described (2, 6). Forty-five to 50% of the β globin cDNA hybridized to normal cellular DNA at saturation. By contrast, 12–16% of the β globin cDNA hybridized to homozygous HPFH DNA, and 21–22% to the δβ thalassemic DNA. All of the DNAs hybridized to the same extent with α globin cDNA (50–60%). These data are consistent with those previously reported from this laboratory (6).

DISCUSSION

The data in this paper indicate that a modification of the Southern "blotting" technique (16) can be used to study the organization of human globin genes in cellular DNA and to detect structural abnormalities in DNA containing human globin genes. The theoretical sensitivity of this method for detecting a single unique gene has been demonstrated in practice in these experiments in a manner analogous to that previously reported for viral sequences (17, 20) and for the rabbit β globin gene (21). Since a unique human gene represents approximately 1.1×10^{-7} of the total human genome (11), 60 μg of cellular DNA can be expected to contain about 7 pg of any single copy gene. Since 1–10 pg of globin gene are detectable in the experiments described in this paper, single copies of human genes can be measured by this method in unfractionated cellular DNA. The ^{32}P hybridization results from this study are consistent with genetic data which indicate the presence of one β, one δ, and two α globin genes per human haploid genome. The precise numbers of these genes, however, cannot be accurately assessed.

Eight to nine *Eco*RI restriction fragments were found to contain globin gene sequences when total human globin cDNA was used as a probe. The *Eco*RI restriction patterns from two different normal cell lines and two nonthalassemic spleens are identical. In preliminary studies of splenic DNA from one patient with a myeloproliferative disorder, the largest band was absent; the *Eco*RI pattern is otherwise identical to a normal pattern. The sizes of the restriction fragments obtained from the four different nonthalassemic samples are identical. This indicates conservation of the DNA sequences, at least at the *Eco*RI sites within and adjacent to the globin structural genes. Since *Eco*RI has relatively few restriction sites in DNA, the extent of variations in DNA sequence either within or adjacent to the globin structural genes cannot be accurately defined until experiments with enzymes that cleave DNA with greater frequency are performed. Both lymphocyte cell line DNA and splenic DNA give similar results, suggesting that infection of the lymphocyte cell lines with Epstein–Barr virus has not altered the relationship of the *Eco*RI restriction sites to the globin structural genes.

Recent reports demonstrate that *Eco*RI cleaves human β globin cDNA once (22) and does not cleave human globin α cDNA (23). It is, however, difficult to compare the fragments obtained from globin cDNA and cellular DNA restriction digestion directly. First, the sequences adjacent to the structural genes will alter the size of the cellular DNA fragments at the 5′ and 3′ ends of the gene. Second, the possibility that there are inserted nucleotide sequences of considerable length within the known structural globin gene sequences makes comparison with globin cDNA cleavage products less meaningful, because these inserted sequences may contain restriction sites absent in globin cDNA. Such inserted sequences have been demonstrated in mouse (24) and rabbit (25) cellular β globin genes.

Three of the *Eco*RI fragments definitely contain β-like globin structural gene sequences. These three fragments hybridize specifically and intensely with β globin cDNA; in addition, they are all present in hydrops fetalis–α thalassemic DNA, which involves deletion or near-deletion of all α but no β globin genes (2, 26, 27). It is unlikely that these fragments contain γ structural gene sequences since, under stringent hybridization conditions, γ cDNA does not crosshybridize with β-like gene sequences (6). It is not possible at present to distinguish the δ and β globin gene-containing fragments since there is significant crosshybridization between these sequences (28). We, therefore, cannot determine which β-like fragments are β and which are δ. However, it is known that hemoglobin Lepore is a δβ fusion gene product. The finding of two normal β-like restriction fragments (4.5 and 3.0 kb) in this disorder suggests that one contains δ gene sequences and the other β gene sequences. Also, we do not know the extent to which the β-like fragments contain structural genes, inserted sequences, and sequences adjacent to the structural genes. Comparisons of restriction digests obtained with different enzymes alone and in combination may resolve this latter question. The globin gene sequences contained in the non-β-like bands that hybridize with total globin cDNA are not well characterized. The 8.7-, 7.8-, and 3.3-kb bands are the most intense non-β-like bands and may contain α-like gene sequences. The 8.7- and 7.8-kb bands also are absent in hydrops fetalis DNA when hybridized with total globin cDNA. Since α globin gene sequences are deleted in this disorder, the absence of these bands suggests that they contain α-like gene sequences. The small amount of γ mRNA (5–10%) present in the globin mRNA used to synthesize the total globin cDNA may be leading to detection of γ globin gene-containing fragments in the less intense bands. In addition, embryonic globin gene sequences (1) may be crosshybridizing with cDNA components.

With these reservations in mind, we have compared the β-like cellular DNA fragments of normal, homozygous Lepore, HPFH, and δβ thalassemic DNA. In homozygous Lepore DNA, the 6.6-kb β-like gene-containing DNA fragment is absent; this is consistent with the known fusion gene product formed (1, 9) due to the deletion of gene material between the δ and β structural genes. This result also indicates that 6.6 kb is the minimum distance between the δ and β structural genes. In HPFH and δβ thalassemic DNA, two of the three *Eco*RI restriction fragments containing β-like sequences are absent and are compatible with the deletion of *Eco*RI restriction sites within β-like structural genes. Deletion of nonstructural sequences alone would be inconsistent with solution hybridization data showing extensive deletion of β-like structural gene sequences in these disorders (6). Preliminary data with *Pst* I-digested DNA hybridized to total globin cDNA also indicate distinct differences in the fragments obtained with normal cellular DNA compared to Lepore, HPFH, and δβ thalassemic DNA.

One new β-like *Eco*RI restriction fragment is clearly demonstrable in δβ thalassemic DNA (Figs. 1 and 2) which is not present in normal or HPFH DNA. This finding indicates that the deletion of β-like genes in HPFH is different and more extensive than that in δβ thalassemic DNA; these results confirm and extend our previous findings using hybridization of these DNAs in solution (6). The variable appearance of the 8.7-, 7.8-, and 3.3-kb bands in experiments with β globin cDNA as probe may be due to contamination of the β globin cDNA with 10–15% α globin cDNA. The 8.7- and 7.8-kb bands are the most intense bands seen in all experiments. The possibility that these bands contain intact α structural genes, which may be present in multiple copies, may explain their appearance in experiments with β globin cDNA.

Several limitations in the present experiments make it dif-

ficult to present a detailed or unique model for the organization of the β-like globin genes in normal DNA and for rearrangements in δβ thalassemic and HPFH DNA. First, fragments containing globin genes that are less than 1.0 kb cannot be seen well in these gels and may be missed. Second, small amounts of structural gene, even within large fragments, may not hybridize well to the labeled globin cDNA due to the hybridization conditions. Several different models can explain the data presented in this paper. If it is presumed that the δ and β genes are colinear, unequal crossing-over events could result in deletion of structural gene sequences in a manner analogous to that seen in the generation of hemoglobins Lepore (δβ), Miyada (βδ), and Kenya (γβ). The sites of the crossover, whether they are single or multiple, and the extent of deletion of δ and β gene sequences are unknown. Alternatively, other genetic mechanisms, such as translocation or other rearrangements of parts of the δ and/or β structural genes (29), could lead to the results obtained. Whatever the mechanism, the results in this paper are clearly consistent with deletion of β-like structural gene sequences in Lepore, HPFH, and δβ thalassemic DNA.

A more detailed understanding of the genetic defects in HPFH and the thalassemias will require further restriction fragment analysis and eventual sequence determination of cloned human cellular DNA fragments. However, the experimental approach presented contributes to a better definition of the genetic defects in these and other thalassemias and related disorders. If differences in the restriction fragment patterns of β^+ and/or β^0 thalassemia can be identified, they might be used to establish the prenatal diagnosis of these disorders with fetal DNA extracted from amniocentesis fluid cells. Also, these techniques may provide a general method of analyzing possible deletions and rearrangements of unique structural genes and their neighboring sequences in a variety of inherited and acquired disorders.

Note Added in Proof. We have recently found that the 4.5-kb fragment contains the 5′ end of the β structural gene and the 3.0-kb fragment the 3′ end of the δ structural gene. In addition, we have evidence for an inserted intragenic nucleotide sequence not represented in β cDNA within the δ, β, and Lepore globin genes.

We thank Dr. Giuseppe Russo for the sample of blood from the homozygous δβ thalassemia patient and Dr. Nevio Quattrin for the sample from the homozygous Lepore patient. Also, we thank Joyce O'Donnell, Deborah Starkman, and Judy Banks for their excellent technical assistance. We are indebted to Dr. Arthur Greene and Marian Manduka of the Mutant Cell Repository, Camden, NJ for establishing and providing lymphocyte cell lines and to Drs. J. Beard and G. Chirigis for supplying avian myeloblastosis virus reverse transcriptase through the Office of Program Resources and Logistics, Viral Cancer Program, Viral Oncology, Division of Cancer Cause and Prevention, National Cancer Institute. We are grateful to our colleagues Richard Axel, Raymond Sweet, Michael Weiss, Saul Silverstein, and Paul Marks for many useful discussions, and Richard Flavell for making a preprint of his work available to us. This work was supported by National Institutes of Health Grants GM-14552, GM-19153, GM-22052, CA-03526, and CA-13696, National Science Foundation Grant 27388, National Foundation-March of Dimes Grant 1-304, and a grant from the Cooley's Anemia Foundation. J.G.M. is a trainee on National Heart, Lung and Blood Institute Grant HL-07148.

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