Hemolytic Disease of the Newborn Caused by a New Deletion of the Entire \( \beta \)-Globin Cluster

MARIO PIRASTU and YUET WAI KAN, Howard Hughes Medical Institute Laboratory and the Division of Medical Genetics and Molecular Hematology, Department of Medicine, University of California, San Francisco, California 94143

C. C. LIN, Department of Pediatrics and Medical Biochemistry, University of Calgary, Calgary, Alberta, Canada T2N 4NI

ROSALIE M. Baine, Department of Health and Human Services, Centers for Disease Control, Atlanta, Georgia 30333

C. TATE HOLBROOK, Department of Pediatrics, East Carolina University School of Medicine, Greenville, North Carolina 27834

ABSTRACT We describe a new type of \( \gamma \delta \beta \)-thalassemia in four generations of a family of Scotch-Irish descent. The proposita presented with hemolytic disease of the newborn, which was characterized by a microcytic anemia. Initial restriction endonuclease analysis of the DNA showed no grossly abnormal patterns, but studies of polymorphic restriction sites and gene dosage revealed an extensive deletion that removed all the \( \beta \)- and \( \beta \)-like globin genes from the affected chromosome. In situ hybridization of chromosome preparations with radioactive \( \beta \)-globin gene probes showed that only one 11p homolog contained the \( \beta \)-globin gene cluster in the affected family members.

INTRODUCTION

The human \( \beta \)-globin gene cluster lies on the short arm of chromosome 11 (1, 2) in the order \( \epsilon -\gamma ^{*}-\gamma -\psi \beta -\delta -\beta \) (3). Many different DNA deletions, varying in length from a single nucleotide to thousands of base pairs, affect the globin genes (4). In the \( \beta \)-globin cluster, deletion produces either the genotypes of \( \beta \)-thalassemia (5), \( \delta \beta \)-thalassemia (6, 7), hereditary persistence of fetal hemoglobin (HPFH) (6–9), or \( \gamma \delta \beta \)-thalassemia (10, 11).

\( \gamma \delta \beta \)-Thalassemia is an uncommon clinical syndrome that produces hemolytic disease in the newborn (10, 12). Unlike the common form of \( \beta \)-thalassemia that has no clinical manifestations at birth, newborns heterozygous for \( \gamma \delta \beta \)-thalassemia present with microcytic hemolytic anemia and normoblastemia. The acute hemolytic episodes usually subside after the neonatal period, but the erythrocyte morphology of thalassemia trait persists. Adults with this disorder have normal Hb A2 and F levels and an \( \alpha /\beta \) globin chain synthetic ratio of \( \sim 2.0 \). Two extensive deletions involving more than 60,000 base pairs (60 kb) of DNA on chromosome 11 have been associated with this syndrome. Both involve the \( \epsilon - , \gamma ^{*}, \gamma , \psi \beta , \) and \( \delta \)-globin loci; one stops just upstream from the \( \beta \)-locus and leaves the entire \( \beta \)-globin gene intact (10) and the other includes the 5' portion of the \( \beta \)-globin gene but leaves the 3' portion (11). Southern analysis of genomic DNA from these syndromes using a \( \beta \)-globin gene probe revealed abnormal patterns with many restriction enzymes.

This report describes an extensive new DNA deletion that also produces the \( \gamma \delta \beta \)-thalassemia syndrome. The deletion removes the entire \( \beta \)-globin gene cluster from the affected chromosome.

Dr. Kan is an investigator of the Howard Hughes Medical Institute.

Received for publication 27 December 1982 and in revised form 6 April 1983.

1 Abbreviations used in this paper: HPFH, hereditary persistence of fetal hemoglobin; IVS, intervening sequence.
METHODS

The proposita, the second child of Scotch-Irish parents, was delivered by repeat Caesarian section at the 37th week of gestation. Bile-stained amniotic fluid was noted. The mother had type B blood, Rh\(^+\), and the infant had type O, Rh\(^+\), with negative direct and indirect Coomb's test. The proposita weighed 2,660 g and was 49 cm long. Head circumference was 31 cm. Multiple purpuric lesions were noted on the skin and mucous membranes. The liver was 3 cm and the spleen 2 cm below the costal margin. Hemoglobin was 8.6 g/dl; hematocrit, 29.6%; reticulocyte count, 12.2%; and mean corpuscular volume 90 fl. The peripheral blood had 1,580 nucleated erythrocytes/100 lymphocytes, and erythrocytes were microcytic and hypochromic with extreme anisocytosis, poikilocytosis, and targeting. Platelet count was 85,000/\mu l; serum bilirubin, 6.4 mg/dl; serum glutamic oxalacetic transaminase, 286 mg/dl; serum glutamic pyruvic transaminase, 26 mg/dl; and hepatitis B antigen was negative. Electrophoresis showed Hb F and A, but no Hb Bart's. Serological tests and cultures for bacterial, protozoan, and viral infections were negative. Antiplatelet antibodies (isotype and autoimmunne) were not detected. The infant received a single transfusion of 10 ml/kg with type-specific, washed, packed erythrocytes. After transfusion, the hospital course was uneventful and the patient was discharged on the 8th hospital day with persistent microcytic anemia. Subsequent studies demonstrated normal iron stores, absence of unstable hemoglobin, and resolution of thombocytopenia. The infant, now 2½ yr old, has persistent mild hypochromic and microcytic anemia, but continues to grow normally.

The proposita's sister had a similar history and required prolonged hospitalization during the neonatal period. Her father, paternal aunt, uncle, grandmother, and great-grandmother have histories of anemia.

Hematologic data were obtained with the Coulter counter, model S (Coulter Electronics, Hialeah, FL). Globin chain synthesis studies were performed as previously described (13). DNA was prepared from the peripheral blood lymphocytes and digested with the enzymes EcoRI, BamHI, BglII, PstI, HpaI, HindII, AatI, and HindIII (New England Biolabs, Beverly MA; Bethesda Research Laboratories, Gaithersburg, MD). The \(\epsilon\)-globin gene fragment used as hybridization probe was the EcoRI-BamHI-digested plasmid p\(\epsilon\)3; the \(\gamma\)-probe was JW151; and \(\beta\)-probe was the XbaI-BglII fragment of plasmid \(\psi\)B1-5 containing the entire \(\psi\)\(\beta\)-globin gene; and the \(\beta\)-probes were plasmids JW102 and the BamHI-EcoRI-digested \(H\beta\)-IS containing the intervening sequence of the \(\beta\)-globin gene. In addition, we used three cloned, unique sequence DNA probes: one derived from \(\gamma\)S-1 plasmid was located more than 100 kb 5' to the \(\beta\)-globin cluster; the second, from \(\delta\)HPFH plasmid, was located at least 60 kb 3' to the gene cluster; and the third, pRK28, was 17 kb 3' from the \(\beta\)-globin gene. The first two were isolated from the region near the break points of DNA from the patients with \(\gamma\delta\beta\)-thalassemia (10) and HPFH (8), respectively. The third was isolated from a Charon 4A library (14). For comparison, DNA was also digested with SstI and hybridized with a human insulin gene probe (from plasmid pH11). The \(\alpha\)-globin genotypes were determined by hybridization with \(\alpha\)- and \(\gamma\)-globin probes derived from plasmids JW101 and pPRf1.

In situ hybridization was also used to detect the \(\beta\)-globin gene complex on chromosome preparations. The probes, which were derived from plasmids p\(\epsilon\)3, JW151, \(\psi\)B1-5, H\(\beta\)-IS, and RIH, were nick-translated to 10\(^6\) cpm/\mu g with \([\text{H}]\)dCTP and [\(\text{H}\)]TTP (New England Nuclear, Boston, MA). Hybridization was performed according to the conditions described by Harper and Saunders (15). The slides were coated with Kodak NTB\(_2\) emulsion (Eastman Kodak Co., Rochester, NY) and exposed for 12 d. Chromosomes were subsequently identified by Q-banding (16) and examined for the presence or absence of grains on the short (p) and long (q) arms. 30 consecutive cells were analyzed from each subject and each arm labeled either positive or negative. No attempt was made to correct for the number of grains on each arm or the length of the arms of the individual chromosomes.

RESULTS

The pedigree of the family and their hematologic data are presented in Fig. 1 and Table I. Members with a history of anemia have microcytic hypochromic erythrocytes, normal Hb A\(_2\) and F levels, and increased \(\alpha\)/non-\(\alpha\) globin chain synthetic ratios. This clinical picture and the occurrence of hemolytic anemia at birth strongly suggest a diagnosis of \(\gamma\delta\beta\)-thalassemia.

Our analysis of DNA from this family produced different results from those in the two previously reported cases of \(\gamma\delta\beta\)-thalassemia, in which abnormal \(\beta\)-globin gene-containing fragments were detected.
In this family, the DNA from the affected subjects produced normal-sized restriction fragments that contained the β-globin gene when digested with EcoRI, HpaI, PstI, and BamHI, and hybridized with the β-globin probe. The α-globin genotypes were normal and additional hybridizations with γ- and ε-globin gene probes were also normal. However, the intensity of hybridization with the β- and β-like globin gene probes was lighter than normal and this raised the possibility of an extensive deletion involving the entire β-globin gene cluster. Such a deletion would produce normal globin gene restriction patterns because the DNA fragments detected would be derived from the unaffected chromosome.

Several restriction sites in the β-globin gene cluster are polymorphic; a given individual is usually heterozygous for some of these sites because the paternal and maternal chromosomes are different in them (17). We studied seven of these polymorphic sites: the HindIII site 5′ to the ε-globin gene (17), the HindIII sites in the second intervening sequence (IVS) of the εγ- and δγ-globin genes (18), the two HindIII sites in and 3′ to the ψβ-globin gene (17), the AvaII site in the second IVS of the β-globin gene (17), and the BamHI site 3′ to the β-globin gene (Table II; 19). Whereas the normal-mucytic family member was heterozygous for several of these sites, all members with microcytosis exhibited a single restriction pattern at each of the sites. This suggested that the β-globin gene cluster was present on one homologous chromosome only and that the affected members were hemizygous for these sites.

Detailed analysis of several sites supported this contention. Results from the BamHI analysis are shown in Fig. 2. The DNA digest from the propoita’s father (III-3) showed only a 22-kb fragment which contained the 3′ portion of the β-globin gene due to the absence

<table>
<thead>
<tr>
<th>Subject</th>
<th>Age</th>
<th>Hct</th>
<th>RBC</th>
<th>MCV</th>
<th>MCH</th>
<th>MCHC</th>
<th>Retic</th>
<th>Hb A2</th>
<th>Hb F</th>
<th>α/μα-α ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>I-1</td>
<td>76</td>
<td>12.8</td>
<td>39.6</td>
<td>6.08</td>
<td>65</td>
<td>21.1</td>
<td>32.4</td>
<td>2.0</td>
<td>2.9</td>
<td>0.5</td>
</tr>
<tr>
<td>II-2</td>
<td>50</td>
<td>11.6</td>
<td>35.8</td>
<td>6.00</td>
<td>60</td>
<td>19.3</td>
<td>32.4</td>
<td>1.0</td>
<td>2.9</td>
<td>0.9</td>
</tr>
<tr>
<td>III-3</td>
<td>30</td>
<td>13.2</td>
<td>41.1</td>
<td>6.92</td>
<td>59</td>
<td>19.1</td>
<td>32.1</td>
<td>2.2</td>
<td>3.0</td>
<td>1.3</td>
</tr>
<tr>
<td>III-4</td>
<td>29</td>
<td>13.0</td>
<td>38.6</td>
<td>4.08</td>
<td>90</td>
<td>30.5</td>
<td>33.8</td>
<td>1.0</td>
<td>2.9</td>
<td>0.6</td>
</tr>
<tr>
<td>IV-1</td>
<td>4</td>
<td>10.6</td>
<td>32.5</td>
<td>5.53</td>
<td>58</td>
<td>19.2</td>
<td>32.7</td>
<td>2.5</td>
<td>3.2</td>
<td>0.6</td>
</tr>
<tr>
<td>IV-2</td>
<td>2</td>
<td>9.0</td>
<td>29.0</td>
<td>4.65</td>
<td>62</td>
<td>19.4</td>
<td>31.3</td>
<td>5.0</td>
<td>2.9</td>
<td>2.2</td>
</tr>
</tbody>
</table>

* Globin chain synthesis study performed at 2 mo of age.

**TABLE II**

<table>
<thead>
<tr>
<th>HinfI</th>
<th>HindIII</th>
<th>HinfI</th>
<th>AvaII</th>
<th>BamHI</th>
</tr>
</thead>
<tbody>
<tr>
<td>5′γ</td>
<td>3′γ</td>
<td>3′γ</td>
<td>3′γ</td>
<td>δγ</td>
</tr>
<tr>
<td>I-1</td>
<td>−/−</td>
<td>−/−</td>
<td>−/−</td>
<td>−/−</td>
</tr>
<tr>
<td>II-2</td>
<td>+/−</td>
<td>+/−</td>
<td>+/−</td>
<td>+/−</td>
</tr>
<tr>
<td>III-3</td>
<td>/−/</td>
<td>/−/</td>
<td>/−/</td>
<td>/−/</td>
</tr>
<tr>
<td>III-4</td>
<td>−/+</td>
<td>−/+</td>
<td>−/+</td>
<td>−/+</td>
</tr>
<tr>
<td>IV-1</td>
<td>−/+</td>
<td>−/+</td>
<td>−/+</td>
<td>−/+</td>
</tr>
<tr>
<td>IV-2</td>
<td>+/−</td>
<td>+/−</td>
<td>+/−</td>
<td>+/−</td>
</tr>
</tbody>
</table>

**Figure 2** Autoradiographs of DNA digested with BamHI and SstI and hybridized with the β-globin and insulin gene probes, respectively. The 1.8-kb fragment contains the 5′ portion of the β-globin gene, while the polymorphic BamHI site 3′ to the β-globin gene results in a 9.3- or 22-kb fragment containing the 3′ portion of the gene. (1) III-4, (2) III-3, (3) IV-1, (4) IV-2.
of the BamHI site 3' to the gene. The mother's (III-4) DNA produced a 9.3-kb fragment with this enzyme because the BamHI site is present. If both parents are homozygous for these patterns, DNA from the proposita (IV-2) and her sister (IV-1) should show both the 9.3- and 22-kb fragments. In fact, only the 9.3-kb fragment from the mother was observed, indicating that neither child inherited a β-globin gene from the father. Analysis of the HincII site at the γβ-globin gene points to a similar conclusion. In fact, haplotype analysis of all seven restriction sites showed that the patterns carried by the two children were inherited from the two different maternal chromosomes (Table II).

As a control, we studied the inheritance of the human insulin gene. The insulin gene is also located on the short arm of chromosome 11 (20). Restriction analysis has shown a high degree of polymorphism due to variations in the length of DNA sequences 5' to it (21). This polymorphism is detectable by digestion with SstI. When we digested the DNA with this enzyme, the proposita's father had a 4.8-kb SstI fragment, the mother had a 6.0-kb fragment, and the proposita and her sister inherited the 4.8- and 6.0-kb fragments from the father and mother, respectively. These results demonstrate that both offspring inherited one insulin gene from each parent, but only one β-globin gene cluster from the mother.

We confirmed the decrease in β-globin gene dosage by quantitating the intensity of hybridization of the globin gene bands. A filter containing BglII-digested DNA was blotted with mixed α- and β-globin probes. In all family members, the β-globin gene resided in a 5.2-kb fragment and the two α-globin loci, α1 and α2, were on 7.5-kb and 12-kb fragments, respectively. Densitometric scans of the autoradiographs supported our conclusion that only one β-globin locus is present per diploid genome in the microcytic family members. The intensity of the β-globin band relative to the two α-globin bands in these individuals was similar to that found in a heterozygote with the deletion type of δββ0-thalassemia (6, 7) and was half that of a normal subject (Fig. 3).

We estimated the extent of the deletion by hybridization with probes situated 5' and 3' to the β-globin gene cluster. Hybridization of TaqI-digested DNA with the γβ-I probe located over 100 kb 5' to the β-globin gene cluster yielded a 3.6-kb fragment from the proposita's mother and a 2.3-kb fragment from her father (Fig. 4). The proposita inherited only the 3.6-
FIGURE 4 Autoradiographs of DNA digested with TaqI and HindIII. The TaqI-digest was hybridized with a γβ-l probe located at least 100 kb 5' to the β-globin cluster; the HindIII digest was hybridized with a pRK28 probe located 17 kb 3' to the β-globin gene. (1) III-4, (2) III-3, (3) IV-2.

kb fragment from her mother, demonstrating that the deletion extends from at least 100 kb 5' to the β-globin gene cluster on the affected chromosome. Then, with a probe located at least 60 kb 3' to the β-globin gene cluster, we hybridized to DNA digested with BamHI, EcoRI, BglII, or HindIII, and detected no differences in restriction patterns or intensity of hybridization between the normal and affected members. This indicates that this region of the DNA is not deleted (data not shown). We also used a probe (pRK28) located 17 kb from the β-globin gene to determine the end point of the 3' deletion. A polymorphic HindIII site 7 kb 3' to the β-globin gene results in 11.5- or 13.3-kb HindIII fragments with the pRK28 probe (22). The father has an 11.5-kb fragment and the mother has both the 11.5- and 13.3-kb fragments. The proposita has both fragments and must have inherited the 11.5-kb fragment from her father and the 13.3-kb fragment from her mother (Fig. 4). These results indicate that the HindIII site 7 kb from the β-globin gene is intact and that the 3' breakpoint of the deletion lies between this site and the 3' end of the β-globin gene.

We also documented the absence of the β-globin gene cluster on one chromosome 11 homolog in the affected individuals by in situ hybridization (Fig. 5). The short arm of chromosome 11 (11p) from the proposita's mother (III-4) showed the most frequent hybridization to the β-like probes when compared with the other chromosome arms. In contrast, the 11p from the proposita (IV-2) and her father (III-4) hybridized half as frequently (Fig. 6). We analyzed 30 cells from

FIGURE 5 Representative metaphase spread showing labeled chromosomes after hybridization with 3H-globin gene cluster probes. (a) Partial metaphase spread from the normal individual (III-4). The short arms of both chromosomes 11 were labeled (indicated by arrows). (b) Q-banding chromosome preparation from the same spread as a obtained by sequential staining with quinacrine dihydrochloride. The two chromosomes 11 are indicated. (c) Partial metaphase from proposita (IV-2) with the short arm of a chromosome 11 labeled. (d) Q-banding chromosome preparation from the same spread as c.
each of these three individuals and counted the number of cells in which both 11p homologs were labeled. In III-4, 8 cells (26.7%) showed hybridization in both homologs, whereas no such cells were found in III-3 or IV-2 (Table III). We were able to obtain such a high frequency of hybridization by using five probes along the β-globin cluster.

**DISCUSSION**

We have described four generations of a family in which a new type of gene deletion involves a 100-kb region encompassing the entire β-globin cluster, and produces a γδβ-thalassemia syndrome. Heterozygosity for this deletion produces hypochromia, microcytosis, normoblastemia, and hemolytic anemia at birth. Affected adults exhibit microcytic hypochromic erythrocytes, normal Hb A₂ and F levels, and a decreased

**TABLE III**

*In Situ Hybridization of the β-Globin Gene Probes in 30 Consecutive Cells*

<table>
<thead>
<tr>
<th></th>
<th>Both 11p</th>
<th>One 11p</th>
<th>Neither 11p</th>
<th>No. of positive chromosomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>III-4</td>
<td>8 (26.7)</td>
<td>18 (60.0)</td>
<td>4 (13.3)</td>
<td>34</td>
</tr>
<tr>
<td>III-3</td>
<td>0</td>
<td>15 (50.0)</td>
<td>15 (50.0)</td>
<td>15</td>
</tr>
<tr>
<td>IV-2</td>
<td>0</td>
<td>17 (53.3)</td>
<td>13 (46.7)</td>
<td>17</td>
</tr>
</tbody>
</table>
\(\beta/\alpha\)-globin chain synthetic ratio. Homozygosity for this gene is probably lethal since the fetus would not produce any embryonic or fetal hemoglobin.

Because fragments containing the \(\beta\)-like globin genes were all derived from the unaffected chromosome, initial Southern analysis did not reveal any rearrangement in the DNA. The \(\beta\)-like globin genes from the affected chromosome were all deleted and therefore not represented on the blot analysis. We confirmed the existence of only one \(\beta\)-globin gene cluster per diploid genome in four different ways. Firstly, none of the microcytic individuals were heterozygous for any of the restriction endonuclease sites commonly known to be polymorphic in human populations. Secondly, a comparison of the polymorphic restriction patterns of two sets of genes on the short arm of chromosome 11 revealed that the two children inherited only the maternal genes for the \(\beta\)-globin and \(\beta\)-like globin genes, but both paternal and maternal patterns for the insulin gene. Thirdly, the intensity of hybridization of the \(\beta\)-globin probe with the DNA from affected individuals was half the normal level. Hybridization with DNA probes upstream and downstream from the \(\beta\)-globin gene cluster demonstrated that the deletion starts over 100 kb 5' to the \(\beta\)-globin gene cluster and extends to within 7 kb 3' to it.

Finally, we studied the abnormal chromosomes in this family by in situ hybridization. Five different unique sequence probes were used to increase the intensity of hybridization. With this method, positive signals were obtained in 12 d instead of the 6-wk exposure time reported in other in situ studies of globin genes (23). Under these hybridization conditions, \(\sim\)50% of the 11p are labeled in a normal individual. The expected percentage of cells in which both homologs in the cell are labeled is 25%, which approximates the percentage found in the normal family member. In contrast, the two thalassemic individuals had no cells in which both 11p were labeled. These data directly demonstrate that the \(\beta\)-globin cluster is deleted from one 11p homolog.

Many different molecular lesions cause thalassemia (4). A single phenotype can arise through several different mutational events. Thus, in addition to the two lesions that have already been described for \(\gamma\delta\beta\)-thalassemia, this study defines a new gene deletion that produces the same syndrome. Recently, Kazazian et al. (24) described a similar extensive deletion producing this syndrome in a family of Mexican origin. It is not known whether these two extensive deletions are similar.

The frequency of the deletion described has not been determined. In view of the increasing number of cases reported, differential diagnosis of hemolytic disease of the newborn should include \(\gamma\delta\beta\)-thalassemia when the erythrocytes show microcytosis. As this study illustrates, a normal DNA restriction pattern does not necessarily exclude this diagnosis.

ACKNOWLEDGMENTS

We thank Dr. Tom Maniatis for the pBR3, pel.3, H\(\beta\)-IS, and RH1 probes; Dr. Bernard Forget and Dr. Edward J. Benz, Jr. for the JW101, JW102, JW151, and \(\psi\beta\)-S probes; Dr. Elio Vanin for the \(\gamma\beta\)-1 plasmid and 3' HPFH probes; Dr. Russell Kaufman for the pRK28 probe; Dr. Howard Goodman and Dr. Barbara Cordell for the insulin gene probe; and Jennifer Gampell for editorial comments.

This work was supported in part by grant AM 16666 from the National Institutes of Health and a grant from UNICO National, Inc.

REFERENCES


