Hemolytic Disease of the Newborn Caused by a New Deletion of the Entire β -Globin Cluster

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ABSTRACT We describe a new type of $\gamma\delta\beta$ -thalassemia in four generations of a family of Scotch-Irish descent. The probanda 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 β - and β -like globin genes from the affected chromosome. In situ hybridization of chromosome preparations with radioactive β -globin gene probes showed that only one 11p homolog contained the β -globin gene cluster in the affected family members.

INTRODUCTION

The human β -globin gene cluster lies on the short arm of chromosome 11 (1, 2) in the order ϵ - γ - γ - $\psi\beta$ - δ - β (3). Many different DNA deletions, varying in length from a single nucleotide to thousands of base pairs, affect the globin genes (4). In the β -globin cluster, deletion produces either the genotypes of β -thalassemia (5), $\delta\beta$ -thalassemia (6, 7), hereditary persistence

of fetal hemoglobin (HPFH)¹ (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 β -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 α/β globin chain synthetic ratio of ~ 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 ϵ -, γ -, γ -, $\psi\beta$ -, and δ -globin loci; one stops just upstream from the β -locus and leaves the entire β -globin gene intact (10) and the other includes the 5' portion of the β -globin gene but leaves the 3' portion (11). Southern analysis of genomic DNA from these syndromes using a β -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 β -globin gene cluster from the affected chromosome.

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

METHODS

The probanda, 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 probanda 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/ μ l; serum bilirubin, 6.4 mg/dl; serum IgM, 16 mg/dl; serum glutamic oxalacetic transaminase, 286 mg/dl; serum glutamic pyruvic transaminase, 26 mg/dl; and hepatitis Bs 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 autoimmune) 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 thrombocytopenia. The infant, now 2½ yr old, has persistent mild hypochromic and microcytic anemia, but continues to grow normally.

The probanda'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 *Eco*RI, *Bam*HI, *Bgl*II, *Pst*I, *Hpa*I, *Hinc*II, *Ava*I, and *Hind*III (New England Biolabs, Beverly MA; Bethesda Research Laboratories, Gaithersburg, MD). The ϵ -globin gene fragment used as hybridization probe was the *Eco*RI-*Bam*HI-digested plasmid p ϵ 1.3; the γ -probe was JW151; and $\psi\beta$ probe was the *Xba*I-*Bgl*II fragment of plasmid $\psi\beta$ 1-5 containing the entire $\psi\beta$ -globin gene; and the β -probes were plasmids JW102 and the *Bam*HI-*Eco*RI-digested H β -IS containing the intervening sequence of the β -globin gene. In addition, we used three cloned, unique sequence DNA probes: one derived from $\gamma\beta$ -I plasmid was located more than 100 kb 5' to the β -globin cluster; the second, from 3'HPFH plasmid, was located at least 60 kb 3' to the gene cluster; and the third, pRK28, was 17 kb 3' from the β -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 *Sst*I and hybridized with a human insulin gene probe (from plasmid pHuI). The α -globin genotypes were determined by hybridization with α - and ζ -globin probes derived from plasmids JW101 and pPR ζ .

In situ hybridization was also used to detect the β -globin gene complex on chromosome preparations. The probes, which were derived from plasmids p ϵ 1.3, JW151, $\psi\beta$ 1-5,

H β -IS, and RIH, were nick-translated to 10⁸ cpm/ μ g with [³H]dCTP and [³H]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 NTB₂ 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 A₂ and F levels, and increased α /non- α 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 β -globin gene-containing fragments were detected

FIGURE 1 Pedigree of the family. The black symbols indicate family members who have a history of anemia. II-1, III-1, and III-2 (denoted by asterisks) were not available for study. The arrow indicates the probanda.

TABLE I
Hematologic Data of the Family

Subject	Age	Hb	Hct	RBC	MCV	MCH	MCHC	Retic	Hb A ₂	Hb F	$\alpha/\text{non-}\alpha$ ratio
		g/dl	%	$\times 10^6/\mu\text{l}$	fL	pg	g/dl	%	%	%	
I-1	76	12.8	39.6	6.08	65	21.1	32.4	2.0	2.9	0.5	1.8
II-2	50	11.6	35.8	6.00	60	19.3	32.4	1.0	2.9	0.9	2.3
III-3	30	13.2	41.1	6.92	59	19.1	32.1	2.2	3.0	1.3	2.1
III-4	29	13.0	38.6	4.08	90	30.5	33.8	1.0	2.9	0.6	1.0
IV-1	4	10.6	32.5	5.53	58	19.2	32.7	2.5	3.2	0.6	1.8
IV-2	2	9.0	29.0	4.65	62	19.4	31.3	5.0	2.9	2.2	2.1*

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

(10, 11). 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 *HincII* site 5' to the ϵ -globin gene (17), the *HindIII* sites in the second intervening sequence (IVS) of the $\epsilon\gamma$ - and $\epsilon\gamma$ -globin genes (18), the two *HincII* sites in and 3' to the $\psi\beta$ -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 nor-

mocytic 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 proposita'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 II
Haplotypes of the Family

	<i>HincII</i> 5' ϵ	<i>HindIII</i>		<i>HincII</i>		<i>AvaII</i> β	<i>BamHI</i> 3' β
		$\epsilon\gamma$	$\epsilon\gamma$	$\psi\beta$	3' $\psi\beta$		
I-1	-/-	-/-	-/-	-/-	+/+	-/-	-/-
II-2	+/+	+/+	-/-	+/+	+/+	+/+	+/+
III-3	-/-	+/+	-/-	+/+	+/+	+/+	-/-
III-4	-/+	+/+	+/+	-/-	+/+	-/+	+/+
IV-1	-/-	+/+	+/+	-/-	+/+	-/-	+/+
IV-2	+/+	-/-	-/-	-/-	+/+	+/+	+/+

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 *Bam*HI site 3' to the gene. The mother's (III-4) DNA produced a 9.3-kb fragment with this enzyme because the *Bam*HI 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 *Hinc*II site at the $\psi\beta$ -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 *Sst*I. When we digested the DNA with this enzyme, the proposita's father had a 4.8-kb *Sst*I 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 dem-

onstrate 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 *Bgl*II-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, $\alpha 1$ and $\alpha 2$, 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 $\delta^0\beta^0$ -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 *Taq*I-digested DNA with the $\gamma\beta$ -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 3 Densitometric scanning of autoradiographs from DNA digested with *Bgl*II and hybridized with mixed α - and β -globin probes. The arrow shows the direction of electrophoresis. The sizes of the DNA fragments are: β , 5.2 kb; $\alpha 1$, 7.5 kb; and $\alpha 2$, 12 kb. (A) normal (III-4), (B) proposita (IV-2), 2, and (C) a heterozygote for the deletion type of $\delta^0\beta^0$ thalassemia.

FIGURE 4 Autoradiographs of DNA digested with *TaqI* and *HindIII*. The *TaqI*-digest was hybridized with a $\gamma\beta$ -I 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 *Bam*HI, *Eco*RI, *Bgl*II, or *Hind*III, 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 *Hind*III site 7 kb 3' to the β -globin gene results in 11.5- or 13.3-kb *Hind*III 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 probanda 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 *Hind*III 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 probanda'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 probanda (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 spread from probanda (IV-2) with the short arm of a chromosome 11 labeled. (d) Q-banding chromosome preparation from the same spread as c.

FIGURE 6 Frequency distribution of labeled specific chromosome arms from 30 consecutive metaphase spreads examined from the proposita (IV-2), her father (III-3), and her mother (III-4).

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 $\gamma\delta\beta$ -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

	No. (and percentage) of cells showing positive hybridization			No. of positive chromosomes
	Both 11p	One 11p	Neither 11p	
III-4	8 (26.7)	18 (60.0)	4 (13.3)	34
III-3	0	15 (50.0)	15 (50.0)	15
IV-2	0	17 (53.3)	13 (46.7)	17

β/α -globin chain synthetic ratio. Homozygosity for this gene is probably lethal since the fetus would not produce any embryonic or fetal hemoglobins.

Because fragments containing the β -like globin genes were all derived from the unaffected chromosome, initial Southern analysis did not reveal any rearrangement in the DNA. The β -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 β -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 β -globin and β -like globin genes, but both paternal and maternal patterns for the insulin gene. Thirdly, the intensity of hybridization of the β -globin probe with the DNA from affected individuals was half the normal level. Hybridization with DNA probes upstream and downstream from the β -globin gene cluster demonstrated that the deletion starts over 100 kb 5' to the β -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, ~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 β -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.

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