A Distant Gene Deletion Affects β-Globin Gene Function in an Atypical γδβ-Thalassemia

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Abstract

We describe an English family with an atypical γδβ-thalassemia syndrome. Heterozygosity results in a β-thalassemia phenotype with normal hemoglobin A_2. However, unlike previously described cases, no history of neonatal hemolytic anemia requiring blood transfusion was obtained. Gene mapping showed a deletion that extended from the third exon of the $^{G}\gamma$-globin gene upstream for ~100 kilobases (kb). The $^{A}\gamma$-globin, ψβ-, δ-, and β-globin genes *in cis* remained intact. The malfunction of the β-globin gene on a chromosome in which the deletion is located 25 kb away suggests that chromatin structure and conformation are important for globin gene expression.

Introduction

Studies with recombinant DNA technology have uncovered many different molecular mechanisms that can cause defective globin chain synthesis in the thalassemia syndromes. The common defects in β-thalassemia are point mutations that result in abnormal globin mRNA transcription or processing, and in nonsense or frameshift mutations affecting globin chain translation (1–3). Less commonly, β-thalassemia is caused by gene deletion. Deletions affecting the β-globin gene cluster result in the syndromes of β-thalassemia (4), hereditary persistence of fetal hemoglobin (Hb)[1] (5–8), δβ-thalassemia (7, 8), and γδβ-thalassemia (9–15). The structural genes are usually inactivated by the deletion, although an exception has been described in one case of γδβ-thalassemia (11, 12).

γδβ-Thalassemia is a rare syndrome that has been described only in the heterozygous state. The carriers have a clinical course marked by neonatal hemolytic anemia, followed by the hematologic picture of β-thalassemia minor in adult life. Globin synthetic studies reveal a β to α ratio of ~0.5, but unlike the usual β-thalassemia heterozygote, the levels of HbA_2 (and HbF) are normal. To date, the molecular defects responsible for this disorder have all involved extensive deletions of most or all of the β-globin gene cluster. In three of the previously described cases, the deletion encompasses the entire β-globin gene cluster, including all or part of the β-globin gene itself, and inactivates all the β-globin-like genes *in cis* (9, 10, 14, 15). In a fourth and perhaps most interesting case, the ε-, $^{G}\gamma$-, $^{A}\gamma$-, and δ-globin genes are deleted, whereas the β-globin gene and 2.5 kilobases (kb) of 5′ flanking sequence remain intact. Although the β-globin gene is present and structurally normal, it is not expressed (11, 12).

This report describes an English family with a syndrome resembling γδβ-thalassemia. Restriction endonuclease mapping revealed a large deletion that included the ε-globin gene and part of the $^{G}\gamma$-globin gene, but spared the $^{A}\gamma$-, δ-, and β-globin genes. Although the breakpoint of the deletion was located more than 25 kb from the β-globin gene, the gene malfunctioned.

Methods

Hematologic values were determined by routine methods (16, 17). The $^{A}\gamma$ and $^{G}\gamma$ ratios were determined by high pressure liquid chromatography (18). DNA was prepared from peripheral blood lymphocytes from two of the subjects heterozygous for the disorder, and from normal controls. Extensive restriction mapping was performed in one subject (I-1), whereas selected enzymes were used in the second (II-1) to ascertain whether the same lesion was inherited in the family. The DNA was digested as recommended by the manufacturers with the restriction endonucleases *Apa*I, *Ava*II, *Bam*HI, *Bcl*I, *Bgl*II, *Eco*RI, *Hin*dIII, *Hin*fI, *Hpa*I, *Msp*I, *Taq*I, and *Xba*I (Bethesda Research Laboratories, Gaithersburg, MD; New England Biolabs, Beverly, MA; Boehringer Mannheim Biochemicals, Indianapolis, IN). Digested DNAs were size-fractionated on horizontal 0.8–1% agarose gels, transferred to nitrocellulose filters, and hybridized to specific ^{32}P-radiolabeled probes. Bound radioactivity was visualized by autoradiography on X-ray film (19).

Two β-globin gene probes derived from the plasmid Hβ-1S were used. One was the 0.9-kb *Bam*HI-*Eco*RI fragment that contains the second intervening sequence of the β-globin gene. The other was the 1.8-kb *Bam*HI fragment containing the 5′ portion and flanking sequence of the β-globin gene. The ψβ probe was the *Xba*I-*Bgl*II fragment of plasmid ψβ1-5 which contains the ψβ-globin gene. The γ-complementary DNA (cDNA) probe was from plasmid JW151. The 3′ γ-globin gene probe was a 1.6-kb *Eco*RI fragment excised from a cloned $^{G}\gamma$-globin gene. The ε-globin gene probe was the *Eco*RI-*Bam*HI fragment of plasmid pε1.3. Two cloned unique sequence probes flanking the β-globin gene cluster were used. One was derived from the γβ-1 plasmid, which is homologous to a locus more than 90 kb upstream from the β-globin cluster, and was

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1. *Abbreviations used in this paper:* Hb, hemoglobin; kb, kilobase.

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isolated from the breakpoint of a large deletion in a case of $\gamma\delta\beta$-thalassemia (11). The other was from the pRK28 plasmid, which is homologous to a locus 17 kb downstream from the β-globin gene and was isolated from a Charon 4A library (20).

Results

Clinical and hematologic data. A 20-yr-old pregnant English woman (II-1 in Table I) was found to have a microcytic hypochromic anemia in the absence of iron deficiency. The HbA_2 level was normal and the HbF was slightly elevated. Her father (I-1) and one sibling (II-3) also have hypochromic microcytic anemia with normal HbA_2 and HbF. Her husband is hematologically normal. A male infant (III-1) was delivered spontaneously and experienced an uneventful neonatal period, according to the history. Hematologic data were not obtained until the infant was 1 yr old, when he was found to have a hypochromic and microcytic anemia. The HbA_2 level was normal and the HbF was elevated. Similar values were obtained at age 2 yr. At 2 yr the $^{G}\gamma$ to $^{A}\gamma$ ratio of the fetal hemoglobin was 1 to 1. We also determined the $^{G}\gamma$ and $^{A}\gamma$ ratios for subjects I-1 and II-1, both of whom had lower fetal hemoglobin levels. The high pressure liquid chromatography showed predominantly $^{A}\gamma$-globin chains.

Intact map of the $\psi\beta$-, δ-, and β-globin gene loci. Extensive restriction endonuclease mapping was performed on DNA from subject I-1. Digestion with the enzymes *Ava*II, *Bam*HI, *Bcl*I, *Bgl*II, *Eco*RI, *Hin*dIII, *Hin*fI, *Hpa*I, *Msp*I, *Taq*I, and *Xba*I, and hybridization with β-globin gene probe produced normal-sized restriction fragments. The fragments were also of normal intensity, suggesting that the β- and δ-globin loci were intact on both chromosomes. To confirm this, we digested the DNA with five restriction enzymes known to result in polymorphic fragments. The presence of two fragments of different lengths with a given enzyme would indicate the existence of two loci. Of these five, only the *Ava*II site in the β-globin gene (21) was not informative as it yielded only a single 2.0-kb band. The other four sites—the *Hin*cII site at the $\psi\beta$-globin gene (21), the *Hin*fI site 5′ to the β-globin gene (22), and the *Bam*HI (23) and *Hin*dIII sites 3′ to the β-globin gene (24)—all revealed heterozygous patterns.

The $\psi\beta$ locus lies ~13 kb 5′ to the β-globin gene. One polymorphic *Hin*cII site occurs within the $\psi\beta$1 gene and another is 3′ to it. Digestion with *Hin*cII followed by hybridization with a $\psi\beta$1 probe yields a 3-kb band if the internal *Hin*cII site is present (Fig. 1 *a*). The subject's DNA did not contain a 3-kb fragment and thus lacked this site. However, the 6.0- and 7.6-kb bands were present, indicating the presence of a 3′ *Hin*cII site on one chromosome, and its absence on the other and confirming the presence of the $\psi\beta$-globin gene loci on both chromosomes.

Table I. Hematologic Data of Family

	Hb	MCV	MCH	HbF	HbA_2	β/α
I-1	11.0	58	18.3	0.8	2.8	0.5
I-2	14.0	91	30.1	0.9	2.7	—
II-1	11.3	56	19.0	2.7	2.9	0.37
Husband of II-1	15.8	88	29.5	0.4	3.0	
II-2	15.3	85	29.0	1.3	2.6	—
II-3	10.8	60	18.7	0.7	2.5	—
III-1 (1 yr)	8.4	78	22.8	11.2	2.5	—
(2 yr)	10.3	54	16.9	10.6	2.8	—

Abbreviations: Hb, hemoglobin; MCV, mean corpuscular volume; MCH mean corpuscular hemoglobin.

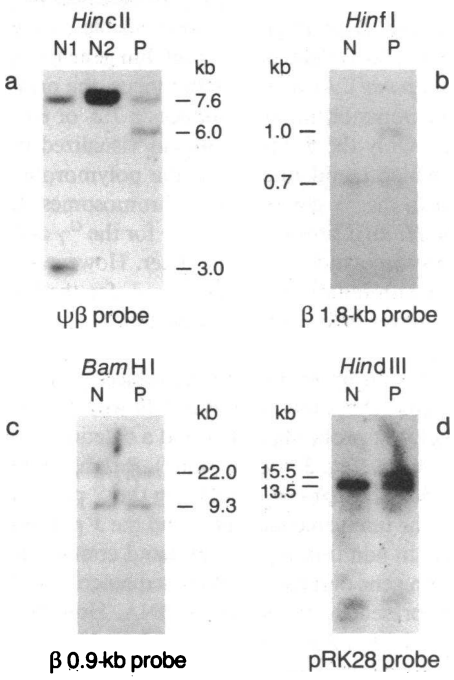

Figure 1. Autoradiogram of restriction analyses of DNA digested with (*a*) *Hin*cII and hybridized with $\psi\beta$ probe, (*b*) *Hin*fI with a 1.8-kb 5′ β probe, (*c*) *Bam*HI with 0.9-kb β probe, and (*d*) *Hin*dIII with pRK28 probe located 3′ to the β gene. The lengths of the fragments in kilobases (kb) are shown. In this and subsequent figures, N indicates normal DNA control (1 and 2 when two normals are used), and P is DNA from the subject I-1.

A polymorphic *Hin*fI site is present just 5′ to the β-globin gene. Digestion with this enzyme and hybridization with the 1.8-kb β probe results in a 0.7-kb band if the site is present, and a 1-kb band if it is absent. Both bands were present in the subject's DNA (Fig. 1 *b*) and thus two intact copies of this locus are present.

Similar results were obtained when the polymorphic *Bam*HI site 3′ to the β-globin gene was examined using the 0.9-kb β-globin gene probe (Fig. 1 *c*). Both the 22-kb band resulting from the absence of this site and the 9.3-kb band resulting from its presence are seen in the subject's DNA. Thus, two intact copies of the β-globin gene and its 3′ flanking sequence are present.

Examination of the polymorphic *Hin*dIII site 7 kb 3′ to the β-globin gene confirmed that two intact copies of this locus are present. Digestion with *Hin*dIII followed by hybridization with the pRK28 probe resulted in both a 13.5- and a 15.5-kb band in the subject's DNA (Fig. 1 *d*), indicative of the presence of the site on one chromosome and its absence on the other. Hence the normal restriction patterns obtained with many enzymes and the detection of heterozygosity for the four polymorphic restriction sites flanking the β-globin gene demonstrate that the $\psi\beta$-, δ-, and β-globin loci are intact on both chromosomes.

γ-Globin gene map. Polymorphic *Hin*dIII restriction sites are present in the second intervening sequence of the $^{G}\gamma$- and $^{A}\gamma$-globin genes (24). When DNA is digested with this enzyme and hybridized with a γ-cDNA probe, the presence or absence of the polymorphic site in the $^{A}\gamma$-globin locus yields a 2.7- or

3.5-kb band, respectively. Both fragments were visible in the subject's DNA and hence two alleles of the $^{A}\gamma$-globin gene locus are present (Fig. 2, *left panel*). At the $^{G}\gamma$-globin locus, the presence or absence of the polymorphic site produces a 7.2- or 8.0-kb band, respectively. Only the 8.0-kb band was visualized in the subject's DNA, which could imply that the polymorphic *Hin*dIII site is absent in the $^{G}\gamma$ gene on both chromosomes. If this were the case, the *Hin*dIII sites would be −− for the $^{G}\gamma$ and $^{A}\gamma$ loci on one chromosome and −+ on the other. However, a chromosome with the combination of *Hin*dIII −+ for the $^{G}\gamma$ and $^{A}\gamma$ loci *in cis* has never before been observed.

An alternate interpretation of the results is that the $^{G}\gamma$-globin gene is deleted from one chromosome, and the subject is hemizygous for the $^{G}\gamma$ locus. Digestion with *Pst*I followed by hybridization with a γ-cDNA probe suggested that a deletion had indeed affected the $^{G}\gamma$ locus (Fig. 2, *middle panel*). *Pst*I digestion normally produces a 5.1-kb fragment containing the 5′ portion of the $^{A}\gamma$-globin gene, the intergenic sequences and the 3′ portion of the $^{G}\gamma$-globin gene, in addition to a 4.0-kb band containing the rest of the $^{G}\gamma$-globin gene and its 5′ flanking sequences. Both these fragments were present in the subject's DNA. However, the intensity of the 4.0-kb band was approximately half that of the 5.1-kb band, suggesting that only one intact copy of the $^{G}\gamma$-globin gene was present.

Digestion with *Bgl*II and *Bcl*I, both of which cleave DNA outside the $^{G}\gamma$ and $^{A}\gamma$ loci, confirmed the presence of a deletion involving the γ-globin gene loci (Fig. 2, *right panel*). Normal DNA yields a 13-kb *Bgl*II fragment or a 18.0-kb *Bcl*I fragment containing both the $^{G}\gamma$- and $^{A}\gamma$-globin loci. Digestion of the subject's DNA with *Bgl*II followed by hybridization with a γ-cDNA probe produced the normal 13-kb band and a novel 11-kb band. Similarly, digestion with *Bcl*I resulted in the normal 18-kb band and a novel 12-kb band. If the deletion was confined to the short segment of the $^{G}\gamma$-globin gene, both fragments would be shortened by the same extent. The fact that the *Bgl*II fragment was shortened by 2 kb and the *Bcl*I fragment by 6 kb suggests that the deletion was extensive and not limited to a short DNA segment. The new *Bgl*II and *Bcl*I sites were probably generated by the juxtapositioning of distant DNA sequences.

Deletion of the ε-globin gene. To ascertain whether two intact copies of the ε-globin gene were present, we examined the polymorphic *Hin*cII site in that gene (21). The results were not informative in that we found no heterozygosity and observed only one 3.7-kb fragment. Hence, we determined the number of ε-globin alleles by gene dosage analysis. Equal amounts of normal and subject DNA were digested with *Eco*RI and *Xba*I, and hybridized with the ε-globin probe (Fig. 3, *left panel*). The expected 3.7-kb band was obtained in each case. However, the intensity of the bands in the subject's DNA was approximately half that of the normal DNA, suggesting the presence of only one intact copy of this locus. To ensure that equal amounts of normal and proband DNA were present, the filter was washed and rehybridized with a β-globin gene probe. The expected 5.2-kb *Eco*RI and 10.8-kb *Xba*I fragments were of equal intensity in both DNA samples (Fig. 3, *middle panel*). Hence the deletion also involves the ε-globin gene.

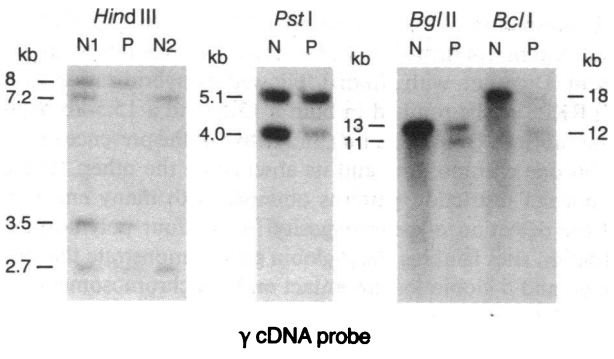

Figure 2. Autoradiogram of filters containing DNA digested with *Hin*dIII, *Pst*I, *Bgl*II, and *Bcl*I, and hybridized with the γcDNA probe JW151.

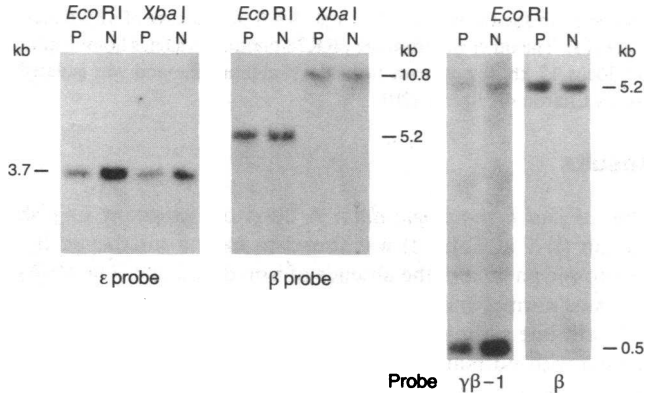

Figure 3. Comparison of intensity of hybridization of DNA from the subjects and normal. The DNA samples on the filters were digested with the enzymes indicated and were first hybridized to either the ε or γβ-1 probes. The filters were washed and rehybridized to the β-globin gene probe. Note the reduced intensity of hybridization of the subject DNA (I-1) with both the ε and γβ-1 probes, while the intensity for the β-globin probe is about equal. The faint 5.2-kb *Eco*RI fragment visualized with the γβ-1 probe is due to the presence of a short segment of this sequence in this probe.

Extent of upstream deletion. We determined the extent of the upstream deletion using the γβ-1 probe isolated from a segment of cloned DNA from the breakpoint of a large deletion that causes γδβ-thalassemia-1 (11, 12). Studies by Vanin et al. (13) have shown that this sequence is homologous to a region of DNA >90 kb upstream from the ε-globin gene. Digestion with *Eco*RI and hybridization with the γβ-1 probe produced a 0.5-kb band in both normal and subject DNA. However, the intensity of the band in the subject's DNA was less than in the normal DNA. When the filter was washed and rehybridized with a β-globin gene probe, the expected 5.2-kb band was present in equal intensity in both normal and subject DNA (Fig. 3, *right panels*). Thus, the γβ-1 locus is also deleted, and the deletion extends at least 90 kb upstream from the $^{G}\gamma$-globin locus.

Fine mapping of the deletion breakpoint. We fine-mapped the breakpoint at the 3′ end of the deletion with a 1.6-kb 3′ $^{G}\gamma$ probe extending from the *Eco*RI site in exon III to the 3′ flanking sequence. Because of sequence divergence in this region between the $^{G}\gamma$- and $^{A}\gamma$-globin genes, only the bands corresponding to the $^{G}\gamma$-globin gene were visualized. In addition to the normal fragments, we observed novel restriction fragments in the subjects' DNA after digestion with *Hpa*I (11.2 kb), *Hin*dIII (1.1 kb), *Xba*I (10.8 kb), *Bam*HI (4.7 kb), and *Apa*I (8.3 kb) (Fig. 4). The normal recognition sites for these enzymes must have been deleted from the thalassemic chromosome, and the novel fragments

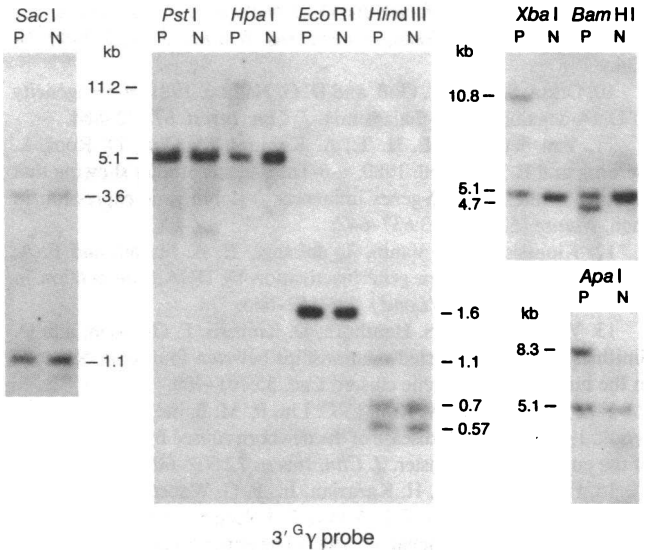

Figure 4. Autoradiography of filters hybridized with a 3′ $^{G}\gamma$ probe which extends from the *Eco*RI site at exon III to the 3′ flanking sequence. This probe does not hybridize to the $^{A}\gamma$ sequence. Note the novel fragments with the *Hpa*I, *Hin*dIII, *Xba*I, *Bam*HI, and *Apa*I digestion. Only normal fragments were obtained with *Sac*I, *Pst*I, and *Eco*RI. (The novel 11.2-kb *Hpa*I band shown here was lighter than usual; in other autoradiograms it had the same intensity as the 5.1-kb band.)

result from juxtaposition of sequences that carry these new restriction sites.

In contrast, digestion with *Eco*RI, *Sac*I, and *Pst*I produced only the expected fragments. Thus the breakpoint of the deletion must lie between the *Eco*RI site in exon III and the *Hpa*I site in the second intervening sequence. As the *Pst*I site at the 5′ region of the second intervening sequence must also have been deleted, the preservation of the normal *Pst*I fragment is probably due to the chance occurrence of a new *Pst*I site generated at approximately the same location as the normal site. The restriction map of the thalassemic chromosome is shown in Fig. 5.

The same deletion is present in the proband (II-1). To determine whether the thalassemia phenotype seen in other family members was due to the same deletion, DNA was obtained from the proband (II-1), subjected to digestion with *Bam*HI, *Bcl*I, *Blg*II, *Hpa*I, and *Xba*I and hybridized with the 3′ $^{G}\gamma$ probe (data not shown). In each case the restriction fragments obtained were

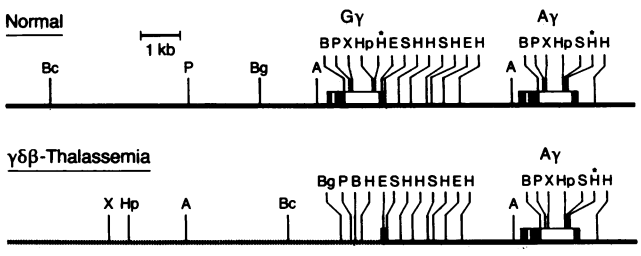

Figure 5. Fine map of the region of chromosome 11 containing the $^{G}\gamma$- and $^{A}\gamma$-globin genes. The broken line indicates DNA sequences brought in by the deletion and the asterisks denote the two polymorphic *Hin*dIII sites. Bc, *Bcl*I; P, *Pst*I; Bg, *Bgl*II; A, *Apa*I, B, *Bam*HI; X, *Xba*I; H, *Hin*dIII; Hp, *Hpa*I; E, *Eco*RI; S, *Sac*I.

identical to those of her father (I-1). Thus, the β-thalassemic phenotype segregates with the deleted chromosome.

Discussion

In this report we describe an English family in which several members manifest the hematologic picture of β-thalassemia minor. In addition to the mild microcytic hypochromic anemia characteristic of this syndrome, the typical imbalance of globin synthesis (β/α globin ~0.5) was found in two of the affected subjects in which this was studied. However, in contrast to the usual β-thalassemia heterozygote, HbA_2 levels were normal in all family members. This phenotype is inherited through three generations.

We mapped the β-globin gene cluster by restriction endonuclease analysis of DNA from one affected subject and uncovered a novel deletion on chromosome 11. Mapping data on a second affected member indicates that the same deletion is present. An extensive deletion of >90 kb extends from the $^{G}\gamma$-globin gene upstream and removes the ε-globin structural gene. The deletion breakpoint starts immediately 5′ to the *Eco*RI site in exon III of the $^{G}\gamma$-globin locus. The $^{A}\gamma$-, ψβ-, δ-, and β-globin structural genes are intact.

Although this deletion resembles the previous cases of γδβ-thalassemia in that an extensive deletion affects the β-globin gene cluster, it differs from them in several respects. First, a large portion of the β-globin gene cluster remains intact, whereas most or all of the β-globin gene cluster is deleted in the previously described cases (9–15) (Fig. 6). Secondly, the clinical presentation during the neonatal period differs from the other cases. None of the affected family members had a history of hemolytic disease of the newborn requiring transfusion, as was reported in the previous cases. The reason may be that the intact $^{A}\gamma$-globin is functional to some degree in the newborn period, thus ameliorating the severity of globin chain imbalance during the neonatal period. However, this hypothesis can only be tested by analysis of the $^{G}\gamma$- and $^{A}\gamma$-globin chains in the neonatal period. Certainly at 2 yr of age, subject III-1 with a $^{G}\gamma/^{A}\gamma$ ratio of 1:1 did not show increased $^{A}\gamma$ synthesis.

The mechanism causing the β-thalassemia phenotype in this family is not yet understood. Two explanations are possible. First, a separate mutation in the β-globin gene on the chromosome carrying the deletion could inactivate the β-globin gene. This type of double mutation has been described in the Sardinian $\delta\beta^{O}$-thalassemia, in which a nonsense mutation occurs in the β-globin structural gene on a chromosome that also harbors a high $^{A}\gamma$-producing gene (25). In order to rule out this possibility, we are in the process of cloning the β-globin gene on the mutant chromosome for sequencing and expression studies. A second possibility is that the malfunction of the β-globin gene is a result of the extensive deletion upstream from the $^{G}\gamma$ locus. This

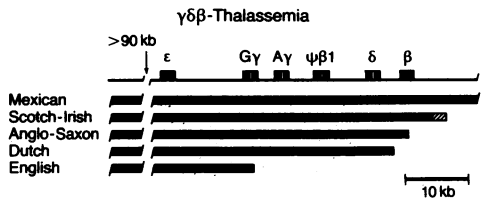

Figure 6. Summary of the deletions defined in the several γδβ-thalassemias described to date.

mechanism may be likened to the Dutch $\gamma\delta\beta$-thalassemia (11, 12) in which the deletion breakpoint occurs 2.5-kb upstream from the β-globin gene. The Dutch patient's DNA in this region was found to be hypermethylated and S1-insensitive, reflecting its inactivity. The inactivity may be due to altered chromatin structure which affects gene expression, or the juxtaposition of inhibitory sequences brought close to the β-globin gene by the deletion. In the present case, the 25 kb separating the breakpoint of the deletion and the β-globin gene makes it unlikely that the malfunction is caused by inhibitory sequences. Rather, altered chromatin structure and conformation may be responsible for the lack of β-globin gene expression.

Acknowledgments

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