$\gamma\delta\beta$ -Thalassemia due to a *de novo* mutation deleting the 5' β -globin gene activation-region hypersensitive sites

(distant deletion/nonexpressed β^S -globin gene/pulsed-field gel electrophoresis)

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ABSTRACT $\gamma\delta\beta$ -Thalassemia is a rare disorder of hemoglobin biosynthesis, characterized molecularly by partial or complete deletions of the β -globin gene complex of 100 kilobases (kb) or greater. Common to all mutants described has been the deletion of the most-5' sequences of the β -globin complex. We have used the techniques of pulsed-field gel electrophoresis and polymerase chain reaction to study a patient with a clinical $\gamma\delta\beta$ -thalassemia phenotype. This subject developed a *de novo* deletion on a maternally inherited β -globin gene chromosome involving ≈ 30 kb of sequences 5' to the ϵ gene; the deletion extends from -9.5 kb to -39 kb 5' of ϵ and includes three of the four DNase I hypersensitive sites (at -10.9 kb, -14.7 kb, and -18 kb 5' of ϵ). The remaining sequences of the β -globin complex, including the DNase I hypersensitive sites at -6.1 kb and all structural genes in cis to the deletion are physically intact, but presumably nonfunctional, as evidenced by the presence of a β^S -globin gene that is not expressed as a sickle hemoglobin. Deletion of DNase I hypersensitive sites on a previously functional β -globin gene complex confirms the significance of these sites in regulating globin gene expression.

$\gamma\delta\beta$ -Thalassemia is a rare disorder of hemoglobin biosynthesis that presents clinically as neonatal hemolytic anemia with decreased γ - and β -globin chain synthesis and postnatally develops into a benign β -thalassemia trait (1). At the molecular level the disorder is due to a heterogeneous array of large deletions of the β -globin gene complex (2–6). This complex resides within 80 kilobases (kb) on the short arm of chromosome 11 and is structurally arranged and developmentally expressed in the order of 5' ϵ - γ - δ - β . $\gamma\delta\beta$ -Thalassemia mutants described to date have involved large deletions of ≈ 100 kb or greater, which include all or part of the β -globin gene complex. The common feature of each, however, has been deletion of the most-5' sequences of the β -globin gene locus. Two of the five deletion mutants reported have retained complete β -globin genes (2, 6). DNA sequence analysis and expression of these intact genes (2, 6) determined both genes to have normal β -globin sequences that expressed normally in *in vitro* assays (7, 8).

Recent reports have identified a region important for erythroid-specific expression of the β -globin gene complex (9, 10). These include a set of four DNase I hypersensitive (HS) sites at -18 kb, -14.7 kb, -10.9 kb, and -6.1 kb 5' to the ϵ gene and a 3'-hypersensitive site 21.9 kb 3' to the β gene. The significance of these sites to globin gene expression has been demonstrated by Grosveld *et al.* (11), who achieved high levels of position-independent β -gene expression in transgenic mice by constructing a β -globin minilocus in which these most-5' and 3' HS sequences flank a β -globin gene.

These HS sites are erythroid-tissue specific, developmentally stable, and have been termed locus-activating regions to imply the essential nature of their presence to poise chromatin structure of the β -globin locus for developmentally regulated transcription of the globin genes (12).

In this report we describe an Hispanic patient with a clinical $\gamma\delta\beta$ -thalassemia syndrome, in whom a *de novo* mutation involving a partial deletion of the 5' HS sites is demonstrated. This case represents a $\gamma\delta\beta$ -thalassemia mutant in which all structural globin genes in cis linkage to the deletion are intact, but functionally inactive.

METHODS

Hematologic parameters were determined by routine methods (13). Globin-chain biosynthetic ratios were obtained by incubation of peripheral blood with [3 H]leucine, followed by chromatography on carboxymethylcellulose (14).

Pulsed-Field Gel Electrophoresis (PFGE). DNA was prepared in agarose blocks at 2×10^7 cells per ml (15, 16) from Epstein-Barr virus-transformed lymphoblast cell lines established from the patient and a normal control. DNA digested with enzymes other than *Sfi* I was isolated from cells grown in $5 \mu\text{M}$ 5-azacytidine (17). PFGE was performed in a clamped homogeneous electric field apparatus (18) with a 25-sec pulse for 21 hr. Fractionated DNA was transferred to Zetaprobe membrane after UV nicking and hybridized according to the manufacturer's recommendations (Bio-Rad).

Preparative PFGE was used to fractionate *Sfi* I-digested DNA from 1.2×10^7 cells with a 20-sec pulse for 23 hr. DNA in the 330-kb and 130-kb classes was eluted by field-inversion electrophoresis (MJ Research, Cambridge, MA) in an Elutrap apparatus (Schleicher & Schuell), according to the manufacturer's recommendations.

Conventional Restriction Endonuclease Analyses. High-molecular-weight DNA was extracted from Epstein-Barr virus-transformed cell lines or from peripheral blood leukocytes by standard methods (19). DNA was analyzed for (i) β -globin gene complex haplotypes at nine restriction endonuclease polymorphic sites (20), (ii) the presence of a β^S -globin gene with *Bsu*36I, (iii) fine mapping analysis of the deletion 3' breakpoint, and (iv) analysis of gene fragments 5' of ϵ in the maternal DNA by *Kpn* I digests. DNA was digested according to the manufacturer's recommendation (New England Biolabs) and analyzed by Southern blotting techniques as described (19).

Probes. Probes used for PFGE included a 0.55-kb *Eco*RI-*Bam*HI fragment representing sequence at -60 kb 5' of ϵ isolated from the deletion junction of $\gamma\delta\beta$ -thalassemia mutant-1 (probe 1), a normal 2.5-kb *Sph* I fragment at -55 kb 5' of ϵ representing the 5' breakpoint of $\gamma\delta\beta$ -thalassemia mu-

tant-2 (probe 2), a 0.7-kb *Bam* HI-*Pvu* II sequence at -27 kb 5' of ϵ (probe 3), a 3.3-kb *Eco* RI sequence at -22 kb 5' of ϵ (probe 4), a 0.9-kb *Bam* HI-*Eco* RI γ -IVS2 (probe 5), and a 4.4-kb *Pst* I fragment of β -globin gene (probe 6). Additional probes used for conventional Southern analyses included a 1.4-kb *Bam* HI-*Hind* III sequence at -16 kb 5' of ϵ (probe 7), a 0.55-kb sequence at -10.3 kb prepared by the polymerase chain reaction (PCR) (probe 8a), a 1.1-kb PCR probe at -10.3 kb (probe 8b), a 0.58-kb PCR probe at -8.1 kb (probe 9), a 0.45-kb *Hind* III-*Xba* I at -6 kb (probe 10), a 0.7-kb *Bam* HI ϵ gene probe (probe 11), a 1.3-kb *Bam* HI-*Eco* RI ϵ gene probe (probe 12), and a 1.7-kb *Bgl* II-*Xba* I $\psi\beta$ gene probe (probe 13). All probes were labeled with 32 P to a specific activity of 10^8 - 10^9 cpm/ μ g by either nick translation (21) or the random hexanucleotide priming technique (22).

PCR. PCR was used to prepare probes for analysis of sequences 5' of ϵ at -10,351 base pairs (bp) (5'-GACACATGCTCACATACGG-3') to -9,776 bp (5'-AGCGAAACCGAGTAGACTG-3') (probe 8a), -10,351 bp (5'-GACACATGCTCACATACGG-3') to -9,048 bp (5'-GCCTAGGCATTGTGCTGAC-3') (probe 8b), and from -8,140 bp (5'-GCTACCACTCTGGTCTAAG-3') to -7,241 bp (5'-AGTCCTCACTATAAAGTTA-3') (probe 9) (23). Nineteen base primers were synthesized on an Applied Biosystems 380B DNA synthesizer representing the above sequences. One microgram of normal DNA was amplified on a Perkin-Elmer/Cetus thermal cycler for 30 cycles according to the manufacturer's specifications (GeneAmp) (24).

RESULTS

Clinical and Hematologic Data. The proband (II-4) in Fig. 1 was born at term weighing 2730 g and developed a neonatal hemolytic anemia requiring exchange transfusion. Hematologic values for II-4 are noted in Table 1. Microcytosis and mild anemia were reported from birth and thought consistent with thalassemia trait. Family studies noted the mother and two brothers to have normal and sickle (AS) hemoglobin and the father and sister to have normal hemoglobin A. β/α -Globin-chain biosynthetic ratio of 0.35 in II-4 was also consistent with β -thalassemia trait, although neither parent had evidence of this disorder by hematologic parameters or chain synthesis (Table 1).

FIG. 1. β -Globin gene DNA haplotypes. DNA haplotypes are formulated at polymorphic restriction endonuclease sites in the β -globin locus at ϵ (*Hinc*II), γ (*Hind*III), ψ (*Hinc*II), and β (*Hinf*I, *Ava* II, *Hpa* I, and *Bam* HI) and correlated to hemoglobin phenotypes. +, Presence of cleavage site; -, absence of cleavage site. The proband (II-4) (arrow) has inherited the maternal sickle gene (S) haplotype (solid bar) but expresses only normal hemoglobin A.

Table 1. Hematologic data

	Hb, g/dl	MCV, mm ³	MCH, pg	HbEp	HbA ₂ , %	HbF, %	β/α
I-1	13.6	89	30.0	AS	2.7	0.5	0.96
I-2	17.2	92	31.0	A	2.9	0.10	0.81
II-1	16.8	88	29.0	AS	3.0	0.12	—
II-2	14.9	90	29.9	A	2.8	0.06	—
II-3	16.9	91	30.1	AS	—	—	—
II-4							
Birth	7.1	89	—	—	—	—	—
2 mo	7.9	70	—	AF	2.9	9.8	—
11 yr	11.6	59	19.8	A	3.5	0.24	0.35

Hb, hemoglobin; MCV, mean cell volume; MCH, mean corpuscular Hb; HbEp, Hb electrophoresis; HbF, fetal Hb.

DNA Haplotype Analyses. Haplotypes were formulated at nine DNA polymorphic sites in the β locus at ϵ (*Hinc*II), γ (*Hind*III), ψ (*Hinc*II), and β (*Hinf*I, *Ava* II, *Hpa* I, *Bam* HI) (20). Fig. 1 shows the segregation of these haplotypes in the pedigree. The patient II-4 and her two brothers II-1 and II-3 have each inherited the haplotype associated with the maternal sickle gene. However, the subject II-4 has no detectable sickle hemoglobin on electrophoresis or β^S -globin chains on chromatography. Analysis for the sickle mutation with the enzyme *Bsu*36I and a β -globin gene probe showed that II-4 did, indeed, have a normal β^A (1.15-kb) and a β^S globin (1.35-kb) gene at the molecular level (data not shown). These data imply that a *de novo* mutation has inactivated the β^S -globin gene inherited from her mother. The presence of the β -globin locus on both chromosomes, however, is demonstrated by heterozygosity at ϵ , γ , and β genes.

PFGE. Fig. 2 illustrates the PFGE analyses. Probes used for these analyses and their location in the β -globin locus are noted in Fig. 3 as 1-6. DNA from the patient and a normal control was analyzed with *Sal* I and *Sfi* I. A β 4.4-kb probe (probe 6) detects the normal *Sal* I fragments of 130 kb in both patient and control DNA. However, with *Sfi* I a 330-kb fragment is seen in the patient DNA in addition to the normal 130-kb fragment. Analysis with γ -IVS2 (probe 5) again demonstrates the normal 130-kb and mutant 330-kb *Sfi* I fragments in patient DNA. This probe, however, also detects normal *Sal* I fragments of 280 kb in patient and control DNA as well as a smaller 250-kb *Sal* I fragment in patient DNA. These data suggest that a deletion or rearrangement has occurred on one allele of the β -globin gene complex in this patient. The $\gamma\delta\beta$ -thalassemia DNA has a unique 5' *Sal* I fragment of 250 kb and a normal 3' fragment of 130 kb, implying that this event has occurred in 5' sequences. The abnormal *Sfi* I 330-kb mutant is also consistent with a deletion that removes both *Sfi* I sites at -17 kb and -27 kb 5' to ϵ . *Cla* I and *Kpn* I digests analyzed with a γ -IVS2 probe detected normal 25-kb *Cla* I and 40-kb *Kpn* I fragments in both patient and control DNA (data not shown), implying that sequences from -1 kb 5' to ϵ through -4 kb 3' of β are intact in this patient. These data place the deletion within the domain of the 5' HS sites.

For analysis of the deletion size, DNA digested with *Sfi* I or *Sal* I was analyzed with four probes 5' of ϵ at -60 kb (probe 1), -55 kb (probe 2), -27 kb (probe 3), and -22 kb (probe 4). In Fig. 2, PFGE analysis of *Sfi* I-cleaved DNA with the -27 kb of ϵ 0.7-kb (probe 3) probe detects a single normal 230-kb fragment in both patient and control lanes. Likewise, hybridization of a *Sal* I digest with probe 4 at -22 kb 5' of ϵ showed a single 280-kb normal fragment in both patient and control DNA (data not shown). However, analysis with the -55 kb (2.5-kb *Sph* I) probe 2 (Fig. 2) shows two *Sfi* I fragments: the mutant 330 kb and normal 230 kb. Probe 1 at -60 kb gives the same result (data not shown). The absence of the mutant

FIG. 2. PFGE. *Sfi* I and *Sal* I digests of control (N) and thalassemic (T) DNA analyzed with $\beta 4.4$ (probe 6) (A) detects a 130-kb *Sfi* I fragment in control DNA and two fragments of 130 kb and 330 kb in thalassemic DNA. A single *Sal* I fragment of 130 kb is present in both DNAs. Analysis with γ IVS2 (probe 5) (B) again detects an *Sfi* I fragment of 130 kb in control and two fragments of 130 kb and 330 kb in thalassemic DNAs. This probe also detects a 280-kb normal *Sal* I fragment in normal and two fragments of 280 kb and 250 kb in thalassemic DNAs. Analysis with 5' $\epsilon 0.7$ (probe 3) (C) detects a single 230-kb *Sfi* I normal fragment in both normal and thalassemic DNAs. Analysis with 5' $\epsilon 2.5$ (probe 2) (D) again detects two *Sfi* I fragments of 230 kb and 330 kb in thalassemic DNAs. Arrowheads, location of the 260-kb *Saccharomyces cerevisiae* mcys501 chromosome VI (Y).

330-kb *Sfi* I and 250-kb *Sal* I fragments with probes at -27 and -22 kb 5' of ϵ , respectively, and the presence of the 330-kb *Sfi* I fragment with probes at -60 and -55 kb, implies a deletion that includes the -22 -kb, but not the -55 -kb sequences. Moreover, the difference between the *Sal* I normal 280-kb and mutant 250-kb fragments also implies a deletional event of ≈ 30 kb.

Fine Restriction Mapping of the Deletion 3' End Point. Fig. 4A shows analyses with restriction endonucleases analyzed with the PCR probes 8a, 8b, and 9. Mutant fragments are detected with *Kpn* I (18 kb), *Stu* I (25 kb), *Bcl* I (3.9 kb), and *Sac* I (8.2 kb) in patient DNA with probe 9. The *Bsu*36I and *Xba* I digests, however, detect only normal fragments in the patient DNA. Analyses with the more 5' probe 8a, however, detects only normal fragments for *Kpn* I and *Stu* I in patient DNA, which appear half as intense as that of control DNA. Because the mutant fragments are not seen in the patient DNA, the deletion includes sequences represented in probe 8a at -10.3 kb to -9.8 kb. Analysis was next done with probe 8b, which includes sequences from -10.3 kb to -9.2 kb 5' of ϵ . Patient DNA shows mutant fragments with *Hind*III (1.2 kb) (Fig. 4A), *Bsu*36I (6.5 kb), and *Eco*RI (1.8 kb) fragments (data not shown). These data demonstrate that (i) the 3'-deletion breakpoint is 5' to the intact *Xba* I site at -9.5 kb 5' of ϵ , and (ii) the sequences represented in probe 8a (-10.3 to -9.8 kb), are deleted. The 3'-deletion breakpoint has, therefore, occurred within the 300-bp sequence between -9.8 and -9.5 kb 5' of ϵ . Thus, the PFGE and conventional mapping data detect an approximate 30-kb deletion from sequence 5' of ϵ extending from approximately -9.5 kb to -39 kb and including three—at -10.9 kb, -14.7 kb, and -18 kb—of the four HS sites.

De Novo Mutation. Based on haplotype analysis, the deletion is postulated to have arisen on the maternally inherited β^S chromosome. DNA from the subject's mother (I-1) and her brother (II-1) was analyzed for mutant *Kpn* I fragment with probe 9. Only the normal *Kpn* I (13.7-kb) fragment was detected in both DNAs (data not shown).

The Deletion is cis to the β^S Gene. The 330-kb and 130-kb *Sfi* I fragments were isolated from a preparative gel and analyzed for the presence of either the β^A or β^S gene by amplification of each DNA fraction by PCR and cleavage of the amplified 427-bp product with *Bsu*36I. Fig. 5 demonstrates this analysis. A single *Bsu*36I (CCTNAGG) cleavage site at codons 5–7, which generates two fragments of 338 bp and 89 bp, is present in the normal 427-bp sequence. The sickle mutation at codon 6 (GAG $\rightarrow$ GTG), however, ablates cleavage at this site and results in a single 427-bp fragment. The DNA amplified from the patient genomic DNA clearly shows the presence of the 338-bp and 87-bp (β^A) fragments as well as the 427-bp (β^S) fragment. Amplified DNA from the patient's normal 130-kb *Sfi* I fragment shows the 338-bp and 89-bp fragments representing the β^A gene. DNA from the abnormal 330-kb *Sfi* I fragment detects a single 427-bp β^S fragment. Thus, the unexpressed β^S gene is demonstrated to be in cis linkage with the deletion mutation.

DISCUSSION

In this report we describe a patient with a $\gamma\delta\beta$ -thalassemia phenotype who has a *de novo* mutation involving an ≈ 30 -kb deletion of the maternally inherited β -globin gene locus. The deletion extends from sequences at approximately 9.5 kb to 39 kb upstream to the ϵ gene, and includes three of the four upstream HS sites at -18 kb, -14.7 kb, and -10.9 kb but

FIG. 3. Long-range restriction map of β -globin locus. Probes 1–6, which are used for mapping, are represented as boxes. Normal *Sfi* I and *Sal* I cleavage sites within 200 kb surrounding the β -globin locus are illustrated in map at top; *Sfi* I and *Sal* I restriction map for thalassemia mutant DNA, generated by data in Fig. 2, is illustrated below. Thick arrows I–V represent HS sites 5' of ϵ at -18 , -14.7 , -10.9 , and -6.1 kb and the 3' β site at -21.9 kb; thin arrows represent minor hypersensitive sites. Deleted sequences are represented schematically by shaded box. C, *Cla* I; K, *Kpn* I.

FIG. 4. Fine restriction mapping of the 3'-deletion breakpoint. Bc, *Bcl* I; Bsu, *Bsu*36I; E, *Eco*RI; H, *Hinc*II; Hd, *Hind*III; K, *Kpn* I; S, *Sac* I; St, *Stu* I; and X, *Xba* I. Probes represented as 7–12 are described in text, and arrows represent the HS sites 5' of ϵ . (A) Analyses with PCR-generated probes 8a, 8b, and 9. Probe 9 sequences at -8.1 kb 5' of ϵ (Right) detect specific fragments in thalassemia DNA (T) with *Kpn* I (18 kb), *Stu* I (25 kb), *Bcl* I (3.9 kb) and *Sac* I (8.2 kb). Normal fragments are seen with *Bsu*36I (9.0 kb) and *Xba* I (2.3 kb). Probe 8a at -10.3 kb 5' of ϵ analysis (Left) detects only normal *Kpn* I and *Stu* I fragments in thalassemic DNA, which appear half as intense as the control DNA. However, probe 8b, which represents a 500-bp 3' extension of probe 8a, again detects a mutant 1.2-kb *Hind*III fragment in thalassemic DNA. (B) The normal restriction map for the above sites and corresponding probes 7–12 are illustrated in the map at top and the thalassemia mutant DNA sites below. Arrows represent upstream HS sites, and the hatched line represents juxtaposed nonglobin DNA. Slight variation in normal fragment size for *Bsu*36I, *Hind*III, and *Sac* I digests represents DNA mobility difference due to varied DNA concentrations.

leaves the remaining sequences of the β -globin locus, including the HS site at -6.1 kb, structurally intact. This deletion has presumably inactivated all the remaining intact globin genes in *cis* including a β^S gene at a distance of 50 kb from the mutation.

Clearly, the association of a structurally intact, but inactivated, β -globin locus with a *de novo* deletion of three HS sites shows the significance of these sites in the regulation of globin gene expression. This regulation has been demonstrated by Grosfeld *et al.* (11), who constructed a β -globin minilocus containing the four 5' ϵ HS sites, the β -globin gene and its flanking sequence, and the 3' HS site. High levels of position-independent human β -globin gene expression were obtained with this construct in transgenic mice, implying that this region exerts significant control over globin gene expression (11). Similarly, these sequences exert the same effect on α -globin gene expression when placed upstream to the α -globin gene and expressed in transgenic mice (25).

Other evidence for the role of these HS sequences has been demonstrated by the trans activation of these 5' and 3' HS sites in somatic-cell hybrids (12). The trans activation of the adult β -globin gene of nonerythroid cells by the murine erythroleukemia (MEL) cell environment accompanies the reformation of HS chromatin structure typical of normal erythroid tissue. Moreover, open conformation of these chromatin sites has been shown in a tissue-specific manner in all developmental stages of globin gene expression, but not in nonerythroid HL-60 cells (9). The open conformation of these HS sites in all developmental stages of erythroid expression, therefore, suggests their role in permitting access to transacting protein factors for the stage-specific expression of individual globin genes.

Further dissection of these HS sites to determine the most important sequences has been initiated by Ryan *et al.* (26), who have shown high expression in transgenic mice of a β -globin gene minilocus containing a 1.9-kb *Kpn* I–*Pvu* II fragment carrying a sequence that includes the HS II site. Their data indicate that the 1.9-kb fragment alone allows high

expression and that the 3' β -globin gene HS site is not necessary for expression. Tuan *et al.* (27) further narrowed the significant sequences to an 0.8-kb fragment with elements of an erythroid-specific enhancer, at -10.2 to -11.0 kb 5' of the ϵ globin gene. Similar erythroid tissue-specific expression of the original β -globin gene minilocus (11) was seen in the erythroid MEL and K-562 cells but not in nonerythroid L cells (28). Similar analyses by Talbot *et al.* (29) revealed slightly more efficient expression in MEL cells when a smaller 6.5-kb fragment containing HS sites I–IV was placed in both upstream and downstream orientations to a human β -globin or a *tk-neo* gene. HS sites I–IV were also seen to regenerate in uninduced MEL cells, suggesting that generation of an open chromatin configuration is a separate event from globin gene transcription (12, 28, 29). Although the individual or cooperative role of HS sites I, III, and IV has not been defined, the $\gamma\delta\beta$ -thalassemia mutant described herein provides evidence that the HS I site at -6.1 kb, present in this DNA, is not alone sufficient for β -globin locus activation. A similar observation was noted for α -globin gene expression when the HS I site alone was present upstream to the α -globin gene and expressed in transgenic mice (25). Tuan *et al.* (30) have also shown that the HS sites I, III, and IV alone do not significantly enhance expression of recombinant chloramphenicol acetyltransferase gene plasmids. Other evidence that the 3' β -globin HS site is not necessary for β -globin locus activation comes from the high persistent fetal hemoglobin deletion mutants in which this site is deleted but the β -globin locus is still activated (31).

The mechanisms generating this deletion are presently unknown. The 3'-deletion breakpoint is immediately 5' to the *Alu* 5 ϵ 3 sequence at -8.8 kb in the sequence 5' of ϵ reported by Li *et al.* (23). This region has a cluster of unusual sequences including a potential stem and loop structure, a 39-bp direct repeat, a G+C-rich sequence stretch, a 54-nucleotide tract of repeated alternating purines and pyrimidines, and a 15-nucleotide stretch of purines (23). Alternating purine-pyrimidine tracts reportedly have potential for form-

FIG. 5. Deletion is in cis linkage to the β^S gene. PCR was used for linkage analysis of the 330-kb *Sfi* I fragment to the β^S gene. The map at the top illustrates analysis for the β^S gene by PCR amplification and *Bsu*36I cleavage. Amplification primers are noted as β^{-270} (−270 bp 5' of β) (5'-ATTACGTAAATACACTTGC-3') and β^{+157} (+157 bp in β IVS1) (5'-AACCATTGATACCAACCTG-3'). Below is an ethidium bromide-stained 2% NuSieve gel representing amplified and *Bsu*36I-cleaved DNA. One microgram of normal and sickle hemoglobin (AS) control and patient total genomic DNA and 100 ng of fractionated 130 kb or 330-kb *Sfi* I (patient) DNA was amplified. Lanes: 1, pBR-*Msp* I marker; 2, control AS (total DNA, 1 μ g); 3, patient total genomic DNA (1 μ g); 4, *Sfi* I 130-kb fraction; 5, *Sfi* I 330-kb fraction.

ing left-handed structures (Z-DNA) (32). These tracts are clustered in the sequence between HS sites I and II, which might suggest some biological importance. Similar unusual sequence features have been reported in the 3'-deletion breakpoint associated with hereditary persistence of fetal hemoglobin type 3 (33).

The 5' breakpoint has been grossly mapped by PFGE to ≈ 39 kb upstream to ϵ . Sixty-four kilobases of this region 5' of ϵ has been studied by cosmid cloning and chromosome "walking" (34). Analysis for repetitive sequences by hybridization with *Alu*, *Kpn* (L1 family repeat), and total genomic DNA sequences detected ≈ 16 repetitive sequences in the upstream ϵ region. Both *Alu* and *Kpn* (L1) repeats are present at −36 kb, within the region of the predicted 5' breakpoint. Because both repeat families are reported in at least one deletion end point in β -globin gene complex deletions, their role (35, 36), if any, in generating this deletion can only be examined by sequencing the deletion junction.

This report presents the case of a $\gamma\delta\beta$ -thalassemia mutant in which all structural genes of the β -globin gene complex are found to be intact. It is also a rare mutant in which a β^0 thalassemia phenotype can be definitively assigned to the previously functional mutant β^S gene in cis to the deletion. Moreover, this deletion represents a single *de novo* mutation event that has removed three of the four upstream HS sites and has effectively inactivated a previously functional β -globin gene locus. This mutant clearly confirms the evolving

theories concerning the significance of the 5' HS sites as a locus-activating region.

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