

Heterogeneity of DNA Deletion in $\gamma\delta\beta$ -Thalassemia

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ABSTRACT By restriction endonuclease mapping, gene cloning, and DNA sequencing we have determined the region of DNA that is deleted in a family with $\gamma\delta\beta$ -thalassemia. The deletion removes the linked ϵ -, γ -, and δ -globin structural genes and terminates within the coding portion of the β -globin gene. Since the extent of DNA deletion in this family differs from that reported in another family, we conclude that $\gamma\delta\beta$ -thalassemia is heterogeneous at the molecular level.

INTRODUCTION

The β -like globin genes of man are normally arranged in a cluster spanning more than 40 kilobases (kb)¹ of DNA in the order 5'- ϵ - γ - δ - β -3', where ϵ refers to the embryonic β -like globin gene; γ and δ , the nonallelic fetal β -like globin genes; δ , the minor adult β -like gene; and β , the adult major β -globin gene (1, Fig. 1). Inherited disorders affecting the synthesis of globin chains, the thalassemia syndromes, reflect genetic lesions within or neighboring the globin genes themselves (2, 3). Among β -thalassemias, conditions in which β -globin synthesis is affected, several phenotypes are recognized. In the simplest situation only β -globin production is defective. Other globins of the β -like complex are normally produced. More complex entities involve defective synthesis of more than one β -like globin chain. For example, in two rare syndromes, $\delta\beta$ -thalassemia and hereditary persistence of fetal hemoglobin, no δ - or β -globin chains are produced from the affected chromosome. Studies using the new techniques of restriction enzyme mapping have clearly demonstrated that these latter conditions are associated with extensive deletions of DNA within the β -like complex (4-10). In $\delta\beta$ -thalassemia of the γ/δ variety (in which both γ -globin genes are active) a deletion encompassing the β and the 3'-portion of the δ -globin

genes has been described (4, 7, 10). In $\delta\beta$ -thalassemia of the γ -type (in which only γ -globin chains are synthesized) a deletion involving the entire structural genes for the γ -, δ -, and β -globins has been observed in one homozygote (4, 6). In hereditary persistence of fetal hemoglobin deletions that eliminated the δ - and β -globin genes have been documented in several homozygotes (4, 5, 7-9). The endpoint of the deletion within the γ - δ intergenic region, however, differs in two unrelated homozygotes (4, 8, 9). From these previous studies it is evident that deletions within the β -like globin gene cluster are associated with thalassemias affecting more than one β -like globin gene, and that different deletions may be observed even within phenotypically similar entities.

Kan et al. (11) described a family with a unique form of thalassemia in which γ -, δ -, and β -globin production was absent from the affected chromosome. They proposed that this form of thalassemia ($\gamma\delta\beta$ -thalassemia) might be due to a deletion involving the entire β -like globin gene complex. Recently, van der Ploeg et al. (12) studied a second family with this clinical entity and demonstrated a deletion in the DNA involving γ -, δ -, and (probably) ϵ -globin structural genes that did not extend to the β -globin gene itself. Curiously, although the deletion terminated ~2.5 kb upstream (i.e., 5'-) from the β -globin gene, this locus was not active. These data raised the interesting possibility that deletions might affect the conformation of chromosomal domains and thereby influence gene expression.

As the full spectrum of molecular defects associated with these complex forms of thalassemia is not yet known, we have examined the DNA of the first reported family with $\gamma\delta\beta$ -thalassemia to determine its genetic lesion. By restriction enzyme mapping, gene cloning, and DNA sequencing, we have determined that a deletion involving γ -, δ -, and ϵ -globin genes as well as the 5'-portion of the β -globin gene exists in this family. The deletions of DNA in the two reported families with $\gamma\delta\beta$ -thalassemia differ. Therefore, the molecular lesion in $\gamma\delta\beta$ -thalassemia is heterogeneous.

Received for publication 22 August 1980 and in revised form 27 October 1980.

¹Abbreviation used in this paper: kb, kilobase.

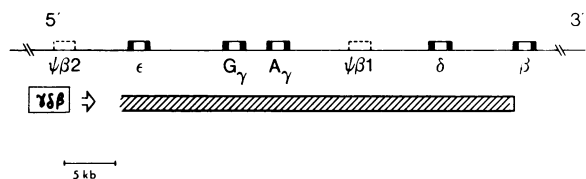


FIGURE 1 Arrangement of human β -like globin genes. The organization of $\epsilon\gamma\delta\beta$ -globin complex is depicted from the work of Fritsch et al. (1). The region of DNA deleted in the $\gamma\delta\beta$ -thalassemia family studied here is depicted below (see text).

METHODS

Restriction enzyme mapping of cellular DNA. High molecular weight DNA were prepared as described (5) from peripheral blood leukocytes of normal controls and from the affected father and daughter with $\gamma\delta\beta$ -thalassemia reported by Kan et al. (11). By history this family is of Anglo-Saxon heritage. Hematologic values and clinical histories are included in the initial studies of this family (11).

Restriction enzymes were purchased from New England Nuclear (Boston, Mass.) Boehringer Mannheim Biochemicals (Indianapolis, Ind.) and were used as suggested by the commercial supplier. Restriction mapping gel analysis was performed as previously described (5, 6, 13). Hybridization probes consisted of β - and γ -cDNA prepared as detailed in prior publications (6, 13).

Molecular cloning of a chromosomal segment of $\gamma\delta\beta$ -thalassemia DNA. DNA of the affected father with $\gamma\delta\beta$ -thalassemia was digested with the restriction enzyme Hind III and size-fractionated thereafter in a 10–40% sucrose gradient by centrifugation at 27 g for 22 h at 20°C in a Beckman SW27 rotor (Beckman Instruments, Inc., Spinco Div. Palo Alto, Calif.). Fractions including DNA of 4–6 kb in length were pooled and concentrated. 2.5 μ g of this DNA were annealed and ligated with 8.5 μ g of λ bacteriophage Charon 28 (14) vector DNA digested previously with Hind III. Charon 28 bacteriophage was kindly provided by Dr. Fred Blattner of the Laboratory of Genetics, University of Wisconsin. The DNA of this phage has a single Hind III restriction site into which foreign DNA fragments can be inserted. The ligated, recombinant DNA was subjected to in vitro packaging to generate infectious phage particles (15). Approximately 30% of the resulting phage were recombinant (i.e., contained human DNA) on the basis of phage plaque hybridization to nick-translated human DNA (16). Approximately 10^7 packaged phage (from a total of 5 μ g input human DNA) were centrifuged to equilibrium in CsCl_2 after addition of 5×10^4 Charon 4A bacteriophage (17). Fractions of this gradient were assayed on *Escherichia coli* LE392 in the presence of a galactoside indicator (17). Charon 4A DNA is 45.4 kb in length and that of Charon 28, 40.3 kb. The desired recombinant with the 5.0 kb Hind III human fragment would have an expected size of about 45 kb and band near the internal Charon 4A bacteriophage marker. Fractions of phage in this region of the CsCl_2 gradient were plated on lawns of *E. coli* LE392 and screened by hybridization with β -cDNA by the procedure of Benton and Davis (18). Among ~350,000 independent phage screened in this manner, one recombinant containing the 5.0-kb Hind III fragment was obtained. This recombinant phage was grown in liquid culture and DNA was extracted for detailed analysis.

DNA sequencing. The nucleotide sequence of the 5'-extragenic Xba-intragenic Bam HI fragment was determined by the method of Maxam and Gilbert (19, 20) after labeling of

the Bam or Xba sites at their 5'-ends. 10–15 μ g of recombinant phage DNA was digested with Xba or Bam HI, end-labeled with ^{32}P - γ ATP by the action of polynucleotide kinase, and subsequently digested with Bam HI or Xba, respectively. The labeled DNA fragments were electrophoresed in a 5% acrylamide gel and the 160 basepairs singly-labeled Xba-Bam HI fragments (labeled at either end) were isolated and sequenced (19, 20). Thin (0.38 mm) 8% acrylamide gels electrophoresed for varying times at 2,000 V were employed in the sequencing.

RESULTS

Restriction mapping of cellular DNA

Initial molecular analysis of the two affected family members with $\gamma\delta\beta$ -thalassemia involved globin gene mapping of total cellular DNA using a battery of restriction enzymes whose cleavage sites within the β -globin gene cluster have been determined (1, 4, 12). As the affected individuals in this family are heterozygous for thalassemia, we would anticipate entirely normal gene mapping patterns with both β - and γ -cDNA probes if the structural genes for γ -, δ -, and β -globins were completely deleted due to the presence of normal DNA fragments contributed from the unaffected chromosome. If, on the other hand, globin gene sequences in this complex were not completely missing, we would anticipate the appearance of novel DNA fragments with some (but not necessarily all) restriction enzymes depending on the position of the normal cleavage sites with respect to whatever deletion might be present. Lastly, if no deletion were present in the β complex, normal restriction patterns of $\gamma\delta\beta$ -thalassemia DNA would be expected.

Restriction enzyme mapping of $\gamma\delta\beta$ -thalassemia DNA was performed using nine different enzymes (Xba, Taq, Hind III, Msp, Hpa I, Bgl II, Eco RI, Pst, and Bam HI). When β -cDNA was utilized as probe, a novel β -like DNA fragment was visible with all enzymes but Bam HI. Representative hybridization patterns are shown in Fig. 2 and summarized in Table I. These data indicated that (1) the entire β -like globin gene complex is not deleted in the affected chromosome of this family and (2) this original $\gamma\delta\beta$ -thalassemia family differs from that reported by van der Ploeg et al. (12), as normal patterns were seen in their family with the enzymes Pst, Bgl II, and Hpa I. Mapping gels of DNA of our family digested with Xba, Bgl II, and Eco RI and hybridized with γ -cDNA probe were normal (not shown), as was the case with the family reported by van der Ploeg et al. (12).

The presence of novel β -like (either δ - or β -) globin DNA fragments with unaltered γ -DNA fragments initially suggested that the γ -globin genes might be deleted and that a deletion arose 5'- to the δ - and β -genes and terminated nearby or within one of these latter loci. The presence of only a single novel β -like fragment in

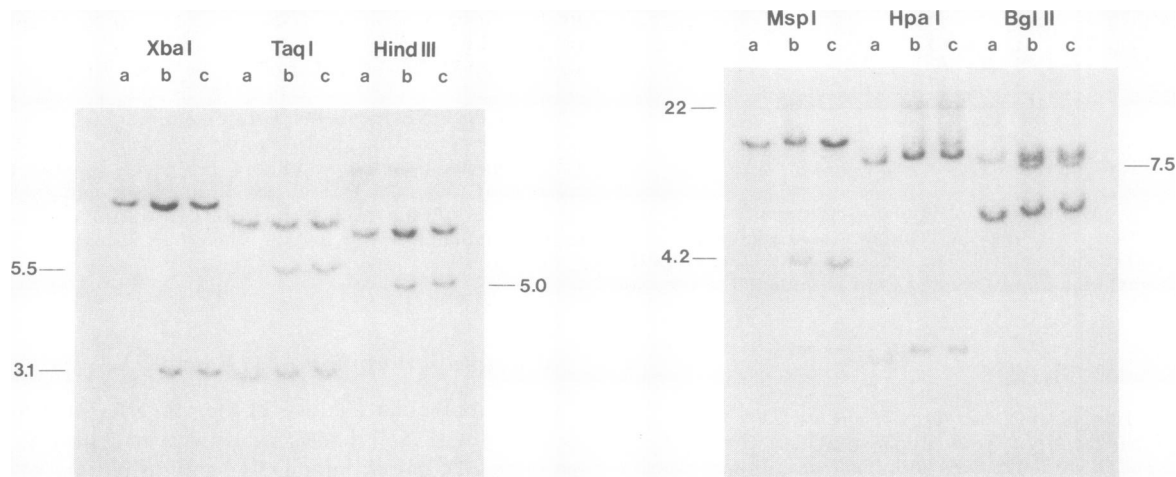


FIGURE 2 Restriction enzyme mapping gels of normal and $\gamma\delta\beta$ -thalassemia DNA. Normal (*a*) and $\gamma\delta\beta$ -thalassemia (father, *b*; daughter, *c*) DNA were digested with the indicated restriction enzymes, electrophoresed in 0.85% agarose, transferred to nitrocellular filter sheets, and hybridized with cDNA probe. The novel specific fragments detected in $\gamma\delta\beta$ -thalassemia DNA are indicated to the left and right of the gels by reference to their sizes in kilobases. In the case of Hpa I digestion a large novel band of about 22 kb was seen in the $\gamma\delta\beta$ -thalassemia DNA (*b*, and *c*); it appeared fainter than other novel bands due to incomplete transfer of the fragment during gel blotting. The faint bands visible above the normal Hpa I β -specific band in *b* and *c* were variably seen and are not thought to be significant.

each of the digests shown in Fig. 2 strongly suggested that the deletion, in fact, terminated near or within the β -globin gene and removed the δ -globin gene in the process. The cloning and sequencing data presented below prove these conclusions.

On the basis of the restriction digests shown in Fig. 2 and confirmation of the placement of restriction sites from the cloned genomic segment defined below we could construct a physical map of enzyme sites surrounding the residual β -like globin gene present on the $\gamma\delta\beta$ -thalassemia chromosome (Fig. 3). By com-

parison with the map of the normal β -globin gene region it is apparent that restriction sites 3' to the β -like sequence are normally placed for a β -globin gene, but those 5' are radically different. This physical map provided strong support for the identity of the residual β -like sequence from the $\gamma\delta\beta$ -thalassemia DNA as authentically β in origin. Of importance, the physical mapping data indicated that the deletion in the $\gamma\delta\beta$ -thalassemia DNA terminated nearer to the β -globin gene than any restriction site useful for gene mapping studies of total cellular DNA (that is, closer than the normal 5'-Hpa I site). Thus, the deletion terminated either before or within the β -globin gene.

TABLE I
 β -like DNA Fragments in $\gamma\delta\beta$ -Thalassemia DNA

Enzyme	Normal DNA		Novel fragments in $\gamma\delta\beta$ -DNA
	β -Gene	δ -Gene	
Xba I	10.8	10.8	3.1
Taq	3.1	8.6	5.5
Hind III	7.6	17.0	5.0
Msp I	10.5	10.5	4.2
Bgl II	5.0	8.4	7.5
Hpa I	7.8	1.9 + 1.4	22
Eco RI	5.6 + 3.2	2.3	4.3
Pst I	4.4	2.2	3.6
Bam HI	1.8 + 22 or 8.6	15 + 4.3	—

The size of each globin-specific DNA fragment is given in kilobase pairs of DNA. Normal β - and δ -fragments were taken from van der Ploeg et al. (12) and Fritsch et al. (4) and confirmed in our experiments.

Cloning of the residual β -like globin sequence of $\gamma\delta\beta$ -thalassemia DNA

To establish conclusively the nature of the β -like sequence present in the novel $\gamma\delta\beta$ -thalassemia fragments, the terminus of the deletion, and the physical map depicted in Fig. 3, we elected to isolate an informative DNA region by gene cloning. The 5.0-kb Hind III fragment (Fig. 2b and c) was cloned in bacteriophage Charon 28 as described in Methods. The Charon 28 vector DNA contains a single Hind III site into which this fragment was inserted. In addition, the phage DNA has no Xba site. Therefore, digestion of recombinant phage DNA with either Hind III or Xba permitted ready assessment of these cleavage sites within the cloned human segment. As shown in Fig. 4, digestion with these enzymes yielded 3.1 kb Xba and

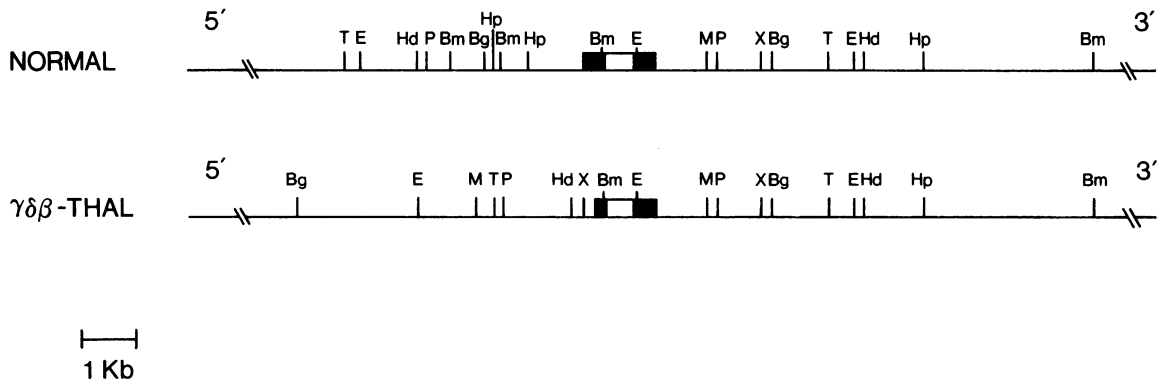


FIGURE 3 Comparison of the restriction maps of the normal β -globin gene region and the region surrounding the β -like sequence present in $\gamma\delta\beta$ -thalassemia DNA. The normal restriction map was determined by Fritsch et al. (4) and van der Ploeg et al. (12). The data of Table I, supplemented with double digestions with Eco RI + Pst and Eco RI + Msp as well as the analysis of the cloned $\gamma\delta\beta$ -thalassemia chromosomal segment, were used to construct the $\gamma\delta\beta$ -thalassemia physical map. Bg, Bgl II; E, Eco RI; M, Msp; T, Taq; P, Pst; Hd, Hind III; X, Xba; Bm, Bam HI.

5.0 kb Hind III fragments from the recombinant $\gamma\delta\beta$ -thalassemia phage DNA. The positions of the restriction sites in the cloned 5.0-kb fragment confirmed the arrangement of sites spanning the β -like sequence and the 3'-flanking region shown in Fig. 3 and, therefore, established the sequence as β -derived.

Nucleotide sequence across the 3'-terminus of the deletion in $\gamma\delta\beta$ -thalassemia DNA

Based on the restriction map (Fig. 3), we expected that the most informative segment of the cloned DNA

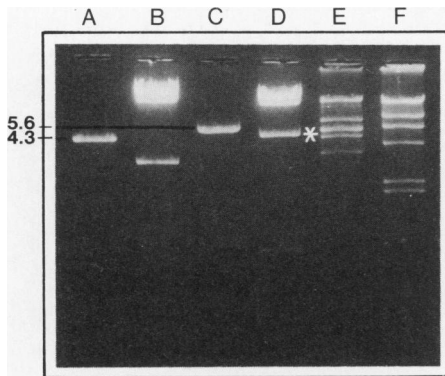


FIGURE 4 Digestion of $\gamma\delta\beta$ -thalassemia recombinant bacteriophage with Hind III or Xba. 1 μ g of recombinant phage was digested with either enzyme and electrophoresed in 0.85% agarose. DNA was visualized by staining with ethidium bromide. Size markers were electrophoresed in parallel. (A) pBR322 plasmid DNA digested with Eco RI (4.3 kb); (B) recombinant phage DNA digested with Xba; (C) pBR325 plasmid DNA digested with Eco RI (5.6 kb) and (D) recombinant phage DNA digested with Hind III. The 5.0 kb fragment (asterisk) contains the β -like sequence. Another human fragment of 0.8 kb in length was coincidentally cloned in this phage. (E and F) Wild-type lambda bacteriophage DNA digested with Eco RI and Hind III, respectively.

would reside between the 5'-extragenic Xba site and the intragenic Bam HI site (normally located at the codons for amino acids 98-100 of the β -sequence). The nucleotide sequence of this region was determined by the chemical degradation method of Maxam and Gilbert (19, 20). The sequence through the endpoint of the deletion was read in both directions beginning from the Xba or Bam HI sites (Methods) to ensure maximum sequencing accuracy. The nucleotide sequence of the majority of the Xba-Bam HI segment is displayed in Fig. 5 and compared with the normal β -globin gene sequence.

The nucleotide sequence establishes the cloned β -like sequence in the novel Hind III fragment as a β -globin sequence. The nucleotide sequence from codon 65 of the β -gene to codon 98 (the Bam HI site) is present and entirely normal. This segment cannot be that of another β -like gene. 5'-to the position of codon 65, however, the DNA sequence of the $\gamma\delta\beta$ -thalassemia fragment diverges completely from the normal β -sequence. Therefore, the novel Hind III fragment contained a β -globin gene deleted at its 5'-end to the position of codon 65 of the gene sequence. This defines the 3'-terminus of the deletion within the β -globin gene region. Absence of β -globin production from the affected chromosome is readily explained by the presence of a deletion ending within the β -globin coding region itself.

Are the δ -, γ -, and ϵ -globin genes deleted?

The restriction map of the β -globin gene region in $\gamma\delta\beta$ -thalassemia (Fig. 3) can be employed to deduce whether other structural loci of the β -like globin complex are deleted. To draw these conclusions we must propose that a single deletion event led to the formation of the $\gamma\delta\beta$ -thalassemia chromosome. Double cross-

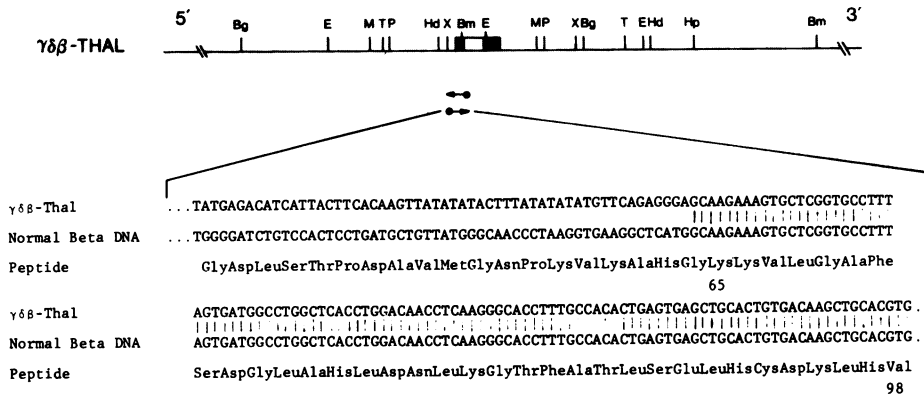


FIGURE 5 Nucleotide sequence across the 3'-terminus of the $\gamma\delta\beta$ -thalassemia deletion. The DNA sequence was determined between the 5'-extragenic Xba and intragenic Bam HI sites as described in the Materials and Methods section. The sequence in this region of the $\gamma\delta\beta$ -thalassemia. DNA is compared with normal β -globin coding sequence, previously determined by Forget et al. (21). The β -globin peptide sequence is depicted below the normal DNA sequence for comparison.

overs or two deletion events are much less likely, but cannot be entirely excluded. We shall address each structural gene of the complex in order proceeding from the δ -gene to the ϵ -globin gene in the 5'-direction. If one of these loci is not deleted, the pattern of restriction enzyme sites 5'-to the deleted β -locus on the $\gamma\delta\beta$ -thalassemia chromosome must correspond to the order of

sites in intergenic DNA stretches (the δ - β , γ - δ , ϵ - γ , and ϵ - γ regions). The restriction maps of the relevant DNA regions are shown in Fig. 6.

The δ -globin genes. Normally both the δ - and β -globin structural genes are located within a single restriction fragment after Xba and Msp digestions (4, 12, and Fig. 2). The Xba site 5' to the $\gamma\delta\beta$ -thalassemia

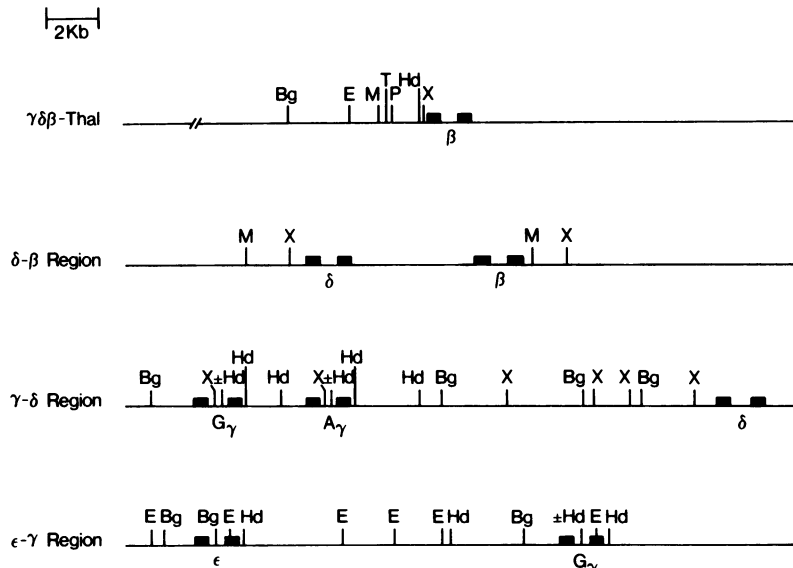


FIGURE 6 Comparison of restriction enzyme sites 5' to $\gamma\delta\beta$ -thalassemia globin gene and $\delta\beta$, $\gamma\delta$, and $\epsilon\gamma$ intergenic DNA regions. The top line depicts the placement of restriction enzyme sites flanking the $\gamma\delta\beta$ -thalassemia gene determined from blotting experiments. Below the placement of restriction sites in regions flanking the δ -, γ -, and ϵ -globin loci is shown. The restriction maps for these normal DNA regions are from references 4, 8, 9, 12, 21, and 22. The enzyme sites depicted are Bg, Bgl II; E, Eco RI; M, Msp I; T, Taq; P, Pst; Hd, Hind III; X, Xba I. Critical feature of the data summarized above is that the order of restriction sites 5' to the $\gamma\delta\beta$ -thalassemia globin gene differs from that in any of the intergene regions. Therefore, this flanking region is not contained in normal DNA from the ϵ -gene to the β -gene.

β -sequence cannot, therefore, lie in the δ - β intergenic region. The same argument applies to the Msp site. Since these cleavage sites do not normally lie in the δ - β intergenic space, the δ -globin gene must be deleted.

The γ -globin genes. Similar reasoning permits us to conclude that the γ -globin genes are deleted as well. Normally, the γ -globin genes are contained in a single restriction fragment generated by the enzyme Bgl II (4, 12). Three cleavage sites for this enzyme are also present in the γ - δ intergenic DNA (4, 12). If the γ -globin loci were present on the $\gamma\delta\beta$ -thalassemia chromosome, the Bgl II site 5' to the β -locus on this chromosome (Fig. 3), by necessity, would be one of these three intergenic Bgl II sites. The order of Eco RI, Taq, Pst, and Xba sites in this region and their positions with respect to this Bgl II site, however, do not conform to that observed in the normal γ - δ intergenic region (1, 4, 12). Therefore, both γ -globin genes must be deleted in the $\gamma\delta\beta$ -thalassemia chromosome. This deduction is consistent with the restriction mapping of $\gamma\delta\beta$ -thalassemia DNA with γ -cDNA as probe that revealed no novel γ -specific fragments. The normal-appearing γ fragments arose from the unaffected chromosome of the $\gamma\delta\beta$ -thalassemia patients.

The ϵ -globin gene. The embryonic ϵ -globin gene normally lies about 13 kb upstream from the γ -globin gene (1, 22). Again, the pattern of restriction sites in the region 5' to the β -globin sequences of the $\gamma\delta\beta$ -thalassemia chromosome does not resemble that found in cloned genomic DNA of the ϵ - γ -intergenic region or from the immediate ϵ -globin gene region itself (1, 22, 23). Therefore, this locus is also deleted from the affected chromosome.

In summary, restriction mapping data revealed the presence of novel β -like DNA fragments in $\gamma\delta\beta$ -thalassemia. By gene cloning we demonstrated that these sequences are from an authentic β -globin gene that was deleted at its 5'-end to the position of codon number 65. Comparison of restriction sites 5' to this partially deleted gene with restriction maps throughout the $\gamma\delta\beta$ -globin gene cluster revealed that the ϵ -, γ -, and δ -globin loci are all missing on the affected chromosome. The endpoint of the deletion upstream from these loci has not been determined more precisely. These findings are depicted in Fig. 1.

DISCUSSION

Thalassemias that affect the synthesis of more than one member of the $\gamma\delta\beta$ -globin gene complex ($\delta\beta$ -thalassemia, hereditary persistence of fetal hemoglobin, and $\gamma\delta\beta$ -thalassemia) are rare entities that appear to result from deletions within the gene cluster on the affected chromosome. We have concentrated here on perhaps the rarest member of this class of disorders, $\gamma\delta\beta$ -thalassemia, identified to date in only two families,

one of Dutch and the other of Anglo-Saxon ancestry. Originally Kan et al. (11) suggested that the condition might be due to a genetic lesion, most likely a deletion, that inactivated the γ -, δ -, and β -globin loci.

Van der Ploeg et al. (12) showed that the Dutch family had an extensive deletion including the γ -, δ -, and probably ϵ -globin genes but no deletion closer than 2.5 kb to the β -globin gene. If there were not a coincident presence of a β -thalassemia gene on the affected chromosome, these findings indicated that gene expression might be adversely affected by distant deletion of DNA. This is a novel possibility with wide-reaching implications for the control of eucaryotic gene transcription.

The molecular lesion in the first reported $\gamma\delta\beta$ -thalassemia family studied here, however, is more easily reconciled with the clinical phenotype. The deletion on the affected chromosome renders production of γ -, δ -, and β -globins impossible by virtue of removal of either the entire structural genes (γ , δ) or merely a portion (β). In this respect this family is uninformative with regard to the interesting speculations raised by van der Ploeg et al. (12). Nevertheless, comparison of the deletions in these two $\gamma\delta\beta$ -thalassemia families permits us to conclude that the precise deletions in this entity are heterogeneous. This molecular heterogeneity is a recurring theme among thalassemia syndromes. In both deletion and nondeletion varieties of α - and β -thalassemias similar phenotypes may result from different anatomical molecular defects (3). Since the deletion of the ϵ -globin gene has apparently occurred in both $\gamma\delta\beta$ -thalassemia families, it might be more appropriate to rename this rare syndrome " $\epsilon\gamma\delta\beta$ -thalassemia" to reflect the molecular pathology more specifically.

An important, unresolved question regarding deletions associated with thalassemias is what determines the endpoints of the observed deletions? On the one hand, the endpoints might represent positions of accidental breakage of DNA within the globin complexes. Alternatively, specific sequences within the DNA may facilitate misalignment of homologous segments and result in unequal crossing-over events (3). A candidate for regions of DNA capable of promoting such events could be the highly repeated DNA sequences that are interspersed throughout the human genome and the globin complexes (1, 24). Such sequences are present in the region immediately 5' to the β -sequence of the $\gamma\delta\beta$ -thalassemia chromosome studied here (unpublished observations), in the region 5' to a partially deleted α -globin gene found in α -thalassemia (25) (unpublished observations), and in the approximate position of the 5'-termini of at least one variety of hereditary persistence of fetal hemoglobin deletion (1, 4). Further study of the endpoints of various thalassemia deletions should focus on the role, if any, of

repeated DNA sequences in the evolution of specific deletions. If such sequences are regularly found near the deletion boundaries in other thalassemias, more precise mechanisms for their facilitation of the deletions themselves will have to be entertained.

ACKNOWLEDGMENTS

We thank Catherine Lewis for preparation of this manuscript. This work was supported by grants from the National Institutes of Health and the National Foundation-March of Dimes and by a Research Career Development Award of the National Heart, Lung, and Blood Institute to Dr. Orkin.

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