Rapid Detection of Deletions Causing $\delta$ Thalassemia and Hereditary Persistence of Fetal Hemoglobin by Enzymatic Amplification

By J.E. Craig, R.A. Barnetson, J. Prior, J.L. Raven, and S.L. Thein

A considerable number of deletions of variable size and position that involve the $\beta$-globin gene complex on chromosome 11 are associated with the clinical entities of hereditary persistence of fetal hemoglobin (HPFH) and $\delta$ thalassemia. Specific deletions appear to be associated with consistent phenotypes and some are known to be recurrent. To facilitate the molecular diagnosis of uncharacterized patients with HPFH and $\delta$ thalassemia, oligonucleotide primers have been designed to enzymatically amplify deletion-specific products for nine known deletions, which include those responsible for HPFH-1, HPFH-2, HPFH-3, Spanish ($\delta$)\textsuperscript{0} thalassemia, hemoglobin (Hb) Lepore, Sicilian ($\delta$)\textsuperscript{0} thalassemia, Asian-Indian inversion-deletion ($\delta$)\textsuperscript{0} thalassemia, and Turkish inversion-deletion ($\delta$)\textsuperscript{0} thalassemia. Using this approach, we have successfully characterized the molecular basis for $\delta$ thalassemia in 23 individuals from 16 families of diverse ethnic origins. Thirteen individuals from this group were shown to be heterozygous for the 13.4-kb Sicilian deletion, two were heterozygous for the 100-kb Chinese $\alpha^6$$\gamma$(+$\gamma$)\textsuperscript{0} deletion, four were heterozygous for the Turkish form of inversion-deletion $\delta$ thalassemia, and three were heterozygous for the Asian-Indian form of inversion-deletion $\alpha^6$$\gamma$(+$\gamma$)\textsuperscript{0} thalassemia. One Vietnamese subject was heterozygous for a 12.6-kb deletion, which we have fully characterized at the molecular level. Sequence analysis of the breakpoint regions of the Chinese deletion and the Turkish rearrangement indicates that, in each case, the mutation is likely to have arisen from a single origin. This hypothesis is supported by the evident geographical clustering of the various deletions described here.

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**MATERIALS AND METHODS**

**Patient samples.** Whole-blood samples from the patients described in this study were collected using EDTA as anticoagulant. An automated cell counter was used to obtain hematological data from freshly collected blood samples. Hb electrophoresis was performed at pH 8.9 on cellulose acetate. The percentage of Hb A\textsubscript{2} was measured by elution and spectrophotometry, and Hb F by alkaline denaturation.

**Oligonucleotide primers.** Each deletion, three oligonucleotide primers have been designed for use in the same amplification reaction that led to the production of a unique deletion-specific product in the presence of the deletion and a normal control band of a different size in the presence of the normal allele.

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normal DNA, and consequently do not lead to any detectable amplification product with normal DNA as the template. However, in the presence of the deletion in question, the primer sites are brought close to proximity and lead to the amplification of a deletion-specific band. A third PCR primer, designed to work under similar conditions to the deletion-specific primers, was synthesized complementary to sequence removed by the deletion in question, and leads to the amplification of a normal control band in the presence of the primer flanking the 5' deletion breakpoint. Consequently, two bands are amplified with DNA from patients heterozygous for the deletion, whereas only the normal control band is amplified with DNA from individuals not carrying the deletion. Furthermore, since the deletion-specific band alone is amplified when the patient is homozygous for the deletion concerned, heterozygotes and homozygotes are distinguishable by this technique. Inversion-deletion rearrangements are characterized by the presence of two deletions, with inversion of the intervening region of DNA. In this report, the 5' deletion and its breakpoints are referred to as A, and the 3' deletion and its breakpoints are referred to as B (Fig 1 and Table 2). The two deletions flanking the intervening DNA in each of these rearrangements are considered independently for the application of the screening strategy.

Figure 1 summarizes the extent of the various deletions and the positions of the amplification primers, and Table 1 summarizes the sequence of the primers with their corresponding GenBank (HUMHBB) coordinates where available.

DNA analysis. DNA was extracted from peripheral blood leukocytes as previously described. The PCR protocols were developed using DNA from patients known to be heterozygous, and in some cases homozygous, for the deletion concerned as a positive control, and DNA from normal individuals as a negative control. The positive-control DNA had been previously characterized by extensive restriction endonuclease mapping and Southern blot hybridization. PCR amplification was performed using a DNA thermal cycler (Perkin Elmer-Cetus) in a 25-μL reaction volume containing 100 ng of genomic DNA, 0.2 mmol/L each of dGTP, dATP, dTTP, and dCTP, 0.5 U of Taq DNA polymerase (Advanced Biotechnologies) and the three oligonucleotide primers, in a buffer containing 10 mmol/L Tris-HCl, pH 8.3, at 20°C and 1.25 to 2.5 mmol/L MgCl₂. Ten picomoles of each of the three primers was used per reaction in all cases, with the exception of the HPFH-2 deletion. In this instance, it was found that the two bands produced in heterozygotes were of equal intensity only if the amount of the normal control primer (B2) was reduced to 2 pmol. The thermal
to the production of both the deletion-specific band and the control band in heterozygotes, the deletion-specific product alone in homozygotes (when available), and the normal control band alone in DNA samples known not to carry the deletion in question. In the case of the two inversion-deletion rearrangements, the amplification reaction was performed independently for each of the deletion breakpoints (designated breakpoint A and B in Table 2, in which A is the 5' deletion and B is the 3' deletion). Reactions were performed on more than one occasion and no false-positive or false-negative results were obtained. When the reaction conditions and cycling profiles were optimized, we observed no reaction failures.

To test the efficacy of this method as a screening strategy, ß-thalassemia segregating in 16 unrelated families from various worldwide geographical regions was studied. The 23 individuals from these families ranged in age from 10 to 78 years, and the molecular basis for their phenotypes had not been previously characterized. The relevant hematological parameters for this group are summarized in Table 3. All patients had hypochromic microcytic red blood cell indices and normal Hb A2 levels, and the majority were mildly anemic. Hb F levels were elevated in all cases, and ranged from 4.2% to 23%. It is noteworthy that the degree to which Hb F was elevated showed considerable variability among individuals subsequently shown to be heterozygous for the same deletion. In some cases, affected members of the same family showed significant differences in Hb F levels.

DNA from the individuals was sequentially screened for the different ß-thalassemia deletions. A known heterozygote for the particular deletion and a normal DNA were amplified concurrently to act as positive and negative controls, respectively. The primers for the 13.4-kb Sicilian deletion were used initially, and 13 of the 23 individuals were shown to be heterozygous for this mutation (data not shown). The 13 affected individuals included four of the apparently unrelated Italian individuals, one Greek individual, and all eight of the British subjects. Only the 1,585-bp normal control band was amplified in the remaining subjects, with the exception of the Vietnamese individual (see below).

The remaining nine individuals in which the Sicilian deletion had not been detected were screened sequentially for the Chinese G(γ(3)-ß) thalassemia deletion, the Asian-Indian inversion-deletion, and the Turkish inversion-deletion thalassemia using the amplification primers described. The two Chinese subjects were found to be heterozygous for the approximately 100-kb Chinese deletion. The three Iranian siblings were all heterozygous for the Asian-Indian form of inversion-deletion G(γ(3)-ß) thalassemia. The remaining four individuals (two Italian and two Greek; the latter mother and son) were heterozygous for the Turkish form of inversion deletion (ß) thalassemia. This recently described complex rearrangement is probably the basis for the Macedonian form of ß-thalassemia, which was previously thought to be due to a simple 18- to 23-kb deletion, but in retrospect generated similar-sized abnormal bands on restriction analysis to the complex Turkish rearrangement. Vietnamese (ß) thalassemia is due to a 12.6-kb deletion. Amplification of DNA from the Vietnamese individual with

**Table 1. Details of Oligonucleotide Primers**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5'→3')</th>
<th>GenBank Coordinates</th>
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<tbody>
<tr>
<td>A1</td>
<td>AGAATGTCACTTATAAGAAGT</td>
<td>50767-50808</td>
</tr>
<tr>
<td>A2</td>
<td>CACTTTAATTCGTCCTCAGGA</td>
<td>52403-52380</td>
</tr>
<tr>
<td>A3</td>
<td>ACTGATATTTGAGAAATTGGAC</td>
<td>N/A</td>
</tr>
<tr>
<td>B1</td>
<td>GATGTCATTTAGTCTTAAGAAG</td>
<td>44610-44631</td>
</tr>
<tr>
<td>B2</td>
<td>TGCTGATACTACTTCTTCTTAC</td>
<td>46816-46797</td>
</tr>
<tr>
<td>B3</td>
<td>CTTTCTGTTCAAGGCTTTAATTC</td>
<td>45451-45430</td>
</tr>
<tr>
<td>C1</td>
<td>GCCAGACACGTAGTGTCACTTGAAG</td>
<td>N/A</td>
</tr>
<tr>
<td>D1</td>
<td>AGCTGCCCACCTGCTCTTTT</td>
<td>N/A</td>
</tr>
<tr>
<td>E1</td>
<td>GACACACATGAGCAAGCAACCAAT</td>
<td>54586-54610</td>
</tr>
<tr>
<td>E2</td>
<td>CGATCCTTCAATATGCTACCAAG</td>
<td>61848-61870</td>
</tr>
<tr>
<td>E3</td>
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<td>62763-62742</td>
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<td>54792-54992</td>
</tr>
<tr>
<td>F2</td>
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<td>56566-56533</td>
</tr>
<tr>
<td>F3</td>
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<td>69500-69481</td>
</tr>
<tr>
<td>G1</td>
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</tr>
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<td>G2</td>
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<td>J5</td>
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Abbreviation: N/A, not available.

Cycling conditions and the concentration of MgCl₂ were optimized for each reaction, as summarized in Table 2. In all cases, the Taq polymerase was diluted in 1 μL of PCR buffer and added to the other reagents only when the denaturation temperature of 94°C had been reached, in order to increase the specificity of the reactions. Reaction samples were overlaid with 25 μL of liquid paraffin. After amplification, a 5-μL aliquot of the product was subjected to electrophoresis in a 1.2% agarose gel and stained with ethidium bromide (0.5 μg/μL).

Restriction endonuclease analysis and DNA sequencing. Certain deletion-specific PCR products were subjected to further analysis by restriction endonuclease digestion and sequencing. Restriction endonuclease digestions were performed under the directions of the manufacturer (Boehringer Mannheim, Mannheim, Germany) and the digested PCR products were subjected to electrophoresis in 1.2% agarose gels. For sequencing, one of the amplification primers was biotinylated to allow preparation of a single-stranded template using magnetic beads (Dynabeads M-280 Steptavidin) and a magnetic particle concentrator (MPC-E; Dynal, Warrll, UK). The single-stranded product was directly sequenced by the dideoxy termination method using Sequenase and reagents supplied by USB Corp. (Cleveland, OH).

**RESULTS**

The results of using the oligonucleotide primers shown in Fig 1 and Table 1 with normal and positive-control DNA are summarized in Fig 2. For each simple deletion, amplification using the three primers as described consistently led...
primers F1, F2, and F3, designed to detect the Sicilian 13.4-kb deletion, unexpectedly produced amplification of a band of approximately 2 kb in addition to the 1,585-bp normal control band (Fig 3). The normal control band of 1,585 bp was amplified by primers F1 and F2, indicating the presence of one normal allele. However, the origin of the band of approximately 2 kb was uncertain. The same result was obtained consistently on at least different occasions. Primers F1 (GenBank HUMHBB coordinates 54972 to 54992) and F3 (69500 to 69481) are 14,528 bp apart in normal DNA and consequently lead to no amplification when DNA from normal individuals is used as the template. However, in the presence of the 13.4-kb Sicilian deletion, the priming positions for F1 and F3 are brought into close proximity and permit the amplification of the Sicilian deletion-specific fragment of 1,150 bp. It was postulated that the 2-kb band amplified from the DNA of the Vietnamese individual resulted from a deletion smaller than the Sicilian deletion, but still lying between primers F1 and F3. Since primers F1 and F3 are normally 14.5 kb apart, the presence of the 2-kb band would thus predict a deletion of approximately 12.5 kb.

Amplification of the mutant 2-kb band alone was achieved using primers F1 and F3. The 2-kb PCR product was then subjected to restriction endonuclease analysis with enzymes that cut in the region between primers F1 and F3. The results (not shown) confirmed that the 2-kb product was the result of amplification from a mutant allele carrying a deletion of approximately 12.5 kb. The 5’ breakpoint was shown to lie between the intact Rsal site at position 55919 (GenBank HUMHBB) and the deleted HindIII site at position 56111. The 3’ breakpoint was similarly localized to between the intact PstI site at position 68649 and the deleted AvaII site at position 68377. To localize precisely the breakpoint of this Vietnamese (βδ) thalassemia deletion, primer S1 (Fig 4) was synthesized to enable sequencing of single-stranded DNA isolated by amplification of the 2-kb deletion-specific product with biotinylated F1 and nonbiotinylated F3 (data not shown). The breakpoints of the deletion were determined by comparing the nucleotide sequence of the amplified DNA with the published sequence of the β-globin cluster in this region (GenBank file HUMHBB) (Fig 4). The deletion is 12,584 bp in length and extends from IVS-II of the β-globin gene to the 6.4-kb L1 repeat element located 3’ of the β-globin gene, thus removing exon III of the β-globin gene and the entire β-globin gene. Two breakpoints are possible, 56,007 → 68,591, or 56,008 → 68,592, due to the presence of a single-base (T) homology common to both ends. The Vietnamese 5’ breakpoint lies just 47 to 48 bp down-
Fig 2. Ethidium bromide-stained agarose gels illustrating the products obtained after amplification using primers specific for the nine respective deletions as detailed in Table 2. In each case, lane 1 represents normal control DNA, lane B is a water blank, and lane M represents marker DNA. (A) HPFH-1 using primers A1, A2, and A3. Lanes 2 and 3 represent homozygotes for HPFH-1. The sizes of the fragments in the marker DNA (lane M) are 8.0, 7.1, 6.0, 4.8, 3.5, 2.7, 1.9, 1.85, 1.5, 1.4, 1.15, 1.0, 0.68, 0.49, and 0.37 kb (Boehringer Mannheim DNA Molecular Weight marker VII). (B) HPFH-2 using primers B1, B2, and B3. Lanes 2 and 3 are heterozygotes for HPFH-2. See (A) for sizes of the marker DNA. (C) HPFH-3 using primers B1, C1, and C2. Lane 2 represents a heterozygote and lane 3 represents a homozygote for HPFH-3. Sizes of the fragments in the marker DNA (BglII) are 1,358, 1,098, 872, 603, 310, 281/271, 234, and 194 bp, respectively. (D) Spanish (δβ)° thalassemia using primers A1, A2, and D1. Lanes 2 and 3 represent heterozygotes and lane 4 represents a homozygote for the deletion. See (A) for sizes of the marker DNA. (E) Hb Lepore using primers E1, E2, and E3. Lanes 2 and 3 represent heterozygotes for the Hb Lepore deletion. See (C) for sizes of the marker DNA fragments. (F) Sicilian (δβ)° thalassemia amplified with primers F1, F2, and F3.
stream from that of the Sicilian 13.4-kb deletion, and the 3' breakpoint is 747 or 748 bp upstream from the Sicilian 3' breakpoint. The extent of the deletion and its similarity to the Sicilian deletion are illustrated in Fig 4.

Restriction endonuclease and sequence analysis of the deletion-specific PCR products for the Sicilian, Chinese, and Turkish mutations. Thirteen of the 23 individuals were heterozygous for the Sicilian 13.4-kb deletion. The breakpoint region of this deletion, as determined by Henthorn et al from cloned DNA, is characterized by two orphan nucleotides at the recombination site, and two single-base variations immediately 3' of the breakpoint. The first of the latter variations (G → A at 69,353) abolishes a cleavage site for TaqI, and the second (T → G at position 69,373) creates a cleavage site for the enzyme BglII. The deletion-specific PCR products of the 13 individuals with the Sicilian deletion were subjected to restriction analyses with TaqI and BglII as previously described, and in all 13 cases the TaqI site was absent and the BglII site was present (data not shown). Furthermore, we have shown recently that these sequence variations are absent in a series of normal individuals. This suggests that the sequence variations are unique to the Sicilian deletion and that the deletion is of a single origin.

For the two cases of Chinese γ(γδβ) thalassemia, the deletion-specific PCR products were directly sequenced us-
ing one of the amplification primers as a sequencing primer. In both cases, the deletion breakpoint was found to be identical to that initially described by Mager et al., including the presence of deletion specific PCR products containing deletion breakpoints A and B, respectively, were sequenced in three individuals (patients no. 17, 18, and 19; Table 3), and compared with the sequence obtained from cloned DNA of the Turkish patient reported by Kulozik et al. In all three cases, the breakpoint sequence for both deletions flanking the inverted region of DNA was identical to that initially described, including the presence of a single orphan nucleotide (+ C) at the deletion junction of deletion A.

**DISCUSSION**

The conventional approach to characterizing deletions that occur within the β-globin cluster has been to undertake extensive restriction endonuclease mapping and Southern blot hybridization using radioactively labeled probes. Such an approach may take considerable time and still require eventual cloning of an abnormal restriction fragment and subsequent sequence analysis to define the breakpoints precisely. The difficulty of applying this approach has been a limiting factor in the accumulation of large numbers of patients with the same mutation to allow phenotype/genotype correlations to be drawn. Furthermore, the time spent characterizing deletions that are eventually shown to be identical to other known cases slows the analysis of novel deletions.

The PCR-based strategy described in this report enables the screening of uncharacterized individuals with a pheno-
type of deletional HPFH or \( \delta \beta \) thalassemia for nine previously described deletions. The choice of these mutations for the screening protocol was based on the fact that each had either been described in more than one family in the literature, or on the basis that we had characterized more than one case in the past by conventional methods. Many of the \( \beta \) cluster deletions not included in this screening method have been described in only one family, and as such are likely to represent isolated mutations. The method is rapid, uses no radioactivity, and has reproducible results. A further advantage of this method for rapidly diagnosing deletions is that once a deletion-specific PCR product is amplified, it can be readily sequenced directly to enable a precise comparison of the breakpoint with those cases previously reported. We have successfully applied this strategy to characterize several deletions in the \( \beta \)-globin cluster and have now characterized the molecular basis for \( \delta \beta \) thalassemia in all of the 16 unrelated families presented here, thus establishing the efficacy of the PCR-based approach. Cases of \( \delta \beta \) thalassemia that remain uncharacterized despite screening for all the mutations outlined in this report are likely to have (1) a novel deletion; (2) a nondeletional form of \( \delta \beta \) thalassemia, such as the Sardinian variant, which is due to a point mutation inactivating the \( \beta \) gene,\(^27\) in cis to a point mutation in the \( \gamma \) gene promoter upregulating Hb F production\(^{28,29}\); or (3) another previously described deletion not included in our screening strategy.

In the study of \( \beta \)-globin cluster deletions, the degree to which upregulation of the \( \gamma \) genes occurs with different deletions is of particular interest and may lead to the identification of putative enhancer elements 3' to the \( \beta \)-globin gene.\(^3,30\) The comparison between the phenotypes associated with different deletions has been of prime importance in the delineation of such elements, but in some cases has been based on a relatively small number of affected individuals. Phenotypic analysis of the individuals accumulated in this study clearly demonstrates that while hypochromic microcytic red blood cell indices and normal Hb A2 levels are consistently present in all the mutations studied, the degree to which Hb F is elevated may vary widely for a given mutation (even between members of the same family). This finding underscores the complexity of the regulation of Hb F production. Although some of this variation may be technical (interlaboratory variability), factors linked to the \( \beta \)-globin cluster known to affect \( \gamma \) gene expression, such as the \( XmnI-G \) polymorphism,\(^31,32\) or unlinked factors known to segregate independently of the \( \beta \)-globin cluster could also account for some of the observed variability.\(^33,34\) The finding of considerable variation in Hb F expression among heterozygotes for the same deletion (Table 3) should lead to caution in the interpretation of phenotype/genotype comparisons for deletions that have been based on small numbers of affected individuals.

It is noteworthy that the Vietnamese deletion has been detected and characterized by the screening strategy using amplification primers that were designed to identify the 13.4-kb Sicilian deletion. No regions of close homology exist flanking the breakpoints of the Vietnamese deletion, and
thus it is likely to have arisen by a nonhomologous recombination event. However, the regions of the 5' and 3' ends of the deletion are also the site of other recombination events. The 5' breakpoint of this 12.6-kb deletion lies in IVS-II of the δβ globin gene and is just 47 to 48 bp downstream of the Sicilian 5' breakpoint. This region is AT-rich and is also the site of a TGTGTGTGTG motif, which is repeated in exon 3 of the δ gene where breakpoint B of the Asian-Indian inversion-deletion is situated (84 bp downstream from the Vietnamese 5' breakpoint). A Laotian 12.5-kb deletional variant of δβ thalassemia has also been described in this region.35 The breakpoints of this deletion were localized by restriction endonuclease mapping; the 5' breakpoint also lies in IVS-II of the δ gene, but lies 18 to 24 bp downstream of the Vietnamese deletion breakpoint.35 The Laotian deletion ends approximately 0.7 kb upstream from the Sicilian 3' breakpoint in the L1 repeat sequence, which makes it very similar to the deletion causing Vietnamese δβ thalassemia. Recently, Trent al have described a 12.5-kb deletion in a Thai individual37 with δβ thalassemia, which by restriction mapping has similar breakpoints to the Laotian deletion. The possibility that the Vietnamese, Laotian, and Thai deletions are identical cannot be excluded, because breakpoint sequence information is not available in the latter two cases. An alternative explanation would be that two or possibly three different deletions have arisen independently due to the incompletely understood recombinogenic nature of both the δ IVS-II region and the L1 repeat element 3' of the δ-globin gene, where the 3' breakpoints of at least six deletions are clustered.4,35-38

Of relevance to the discussion regarding the origin of mutations such as those presented here is the finding that all cases of the Sicilian and Chinese deletions and the Turkish inversion-deletion rearrangement appear to have identical breakpoint sequences. The deletion junction includes identical orphan nucleotides of unknown origin in all three cases. This finding strongly suggests that these mutations are derived from single mutational events. The hypothesis of a common origin has been suggested previously for the Sicilian form of δβ thalassemia24 and would explain the observation that various deletions appear to show a tendency for geographical/racial clustering. In view of the geographical clustering of the deletions, it is suggested that the ethnic origin of the individual concerned should be the primary guiding factor in the initial choice of amplification primers. The ability to diagnose δβ thalassemia rapidly has recently proved to be useful for prenatal diagnosis in a situation in which one partner was heterozygous for a δ thalassemia allele and the other partner was found to be heterozygous for one of the deletions described in this report.

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REFERENCES


Rapid detection of deletions causing delta beta thalassemia and hereditary persistence of fetal hemoglobin by enzymatic amplification

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