Molecular and Hematologic Characterization of Scottish-Irish Type (εγδβ)± Thalassemia

By R.J. Trent, B.G. Williams, A. Kearney, T. Wilkinson, and P.C. Harris

The DNA deletion associated with an example of (εγδβ)± thalassemia (Scottish-Irish type) was characterized. The deletion is approximately 205 kb in length and involves the ε, Gγ, Aγ, δ, and β globin genes. The breakpoint is located 263 bp 3' to exon 3 of the β globin gene. An LI (KpnI) repeat element approximately 320 bp in size is found at the 3' end of the novel DNA sequence. Different clinical phenotypes for three heterozygous neonates suggest that the deletion alone does not predict severity of (εγδβ)± thalassemia at this age.

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GENES FOR THE HUMAN β globin cluster that code for embryonic (ε), fetal (γ), and adult (δ, β) globin chains are closely linked on chromosome 11p15 in the order 5'-ε-Gγ-Aγ-ψβ-δ-β-3'. The α globin gene cluster comprises one embryonic (ζ) and two fetal/adult genes (α2, α1) on chromosome 16p13.3 in the order 5'-ζ-ψα2-ψα1-α2-α1-θ1-3'. A number of nonfunctioning pseudogenes (ψ) are found in each cluster. Embryonic hemoglobins (Hb) comprise Hb Gower 1 (ζγζ), Hb Gower 2 (εζζ), and Hb Portland (ζγζ). Fetal Hb (αζζ) is produced from approximately 6 weeks of intrauterine development. During postnatal development, a switch from fetal to adult Hb (Hb A, αβ) occurs.

Thalassemias are genetic disorders that result from reduced output of α globin chains (α thalassemias) or β-like globin chains (δβ thalassemias). As a result, microcytic hypochromic red blood cells and anemia to a variable extent are produced. (εγδβ)± Thalassemia is a rare disorder first described in 1972. Because fetal genes are nonfunctional the newborn with heterozygous (εγδβ)± thalassemia has a microcytic hemolytic anemia of variable severity. The hematologic picture subsequently changes to that of a β-thalassemia-like disorder after 6 months of age, when switch to the adult globin genes has been completed. DNA mapping studies have identified six forms of this thalassemia that have in common extensive deletions involving the β globin gene cluster. They include Dutch type (deletion of ε, Gγ, Aγ, ψβ, and δ genes), Anglo-Saxon type (deletion of ε, Gγ, Aγ, ψβ, δ, and 5'β genes), Scottish-Irish and Mexican-American types (deletions of ε, Gγ, Aγ, ψβ, δ, and β genes), English type (deletion of ε and most of the Gγ gene), and Spanish type (a deletion extending upstream 5' of the ε gene). The present study characterizes the DNA breakpoint in the Scottish-Irish type of (εγδβ)± thalassemia. Contrasting clinical phenotypes were observed in three affected neonates.

MATERIALS AND METHODS

Patients

Four generations of a family (B) comprising a total of 14 individuals were studied. All were born in Australia. Grandparents of the propositus were English, although the grandmother's maiden name suggested an Irish origin.

Hematological Parameters

Except where indicated, blood counts were obtained with a Coulter Model S Plus Cell counter (Coulter Electronics, Hialeah, FL). HbA1c levels were quantitated by cellulose acetate electrophoresis. HbF was modified by the Bette method. Ferritin was measured by radioimmunoassay. α/β Ratios were determined by a standard method.

DNA Analysis

DNA was isolated from peripheral blood buffy coats by phenol chloroform extraction. Gene mapping followed standard techniques. DNA probes included p1L1.3 (λ), phd 3.3 (γ), Pp3.9 (ψβ), PstI β 4.4 (β), 2.5 SphI, and pRK29.14 High molecular weight DNA was digested partially with the restriction endonuclease Sau3A and size-fractionated to ~20 kb on a sucrose gradient. Fragments were cloned into the λ phage vector EMBL3 according to standard protocol. Plaques were screened with the β globin gene probe. From a positive clone a 3.5-kb KpnI/SalI fragment was subcloned into pUC19. DNA sequences were compared with sequences in the NBRF and GenBank data bases using the Wilbur-Lipman (Match) and the Lipman-Pearson (MatchFast) algorithms, respectively, through the Molecular Biology Information Service of the C.S.I.R.O. DNA from an affected individual as well as a cytogenetically normal lymphoblastoid cell line was isolated in agarose blocks and digested with restriction enzymes SfiI and SalI as described previously. PFGE was performed in a field inversion apparatus controlled by the DNASTAR Pulse System (DNASTAR, Madison, WI). DNA was run for 86 hours at 130 V with a ratio of forward to reverse pulses of 3. The initial forward pulse was for 3 seconds, increased to a final forward pulse of 100 seconds. PFGE for (εγδβ)± thalassemia DNA was performed in a Biorad CHEF-DRII system (Biorad, Richmond, CA). DNA was run for 24 hours at 200 V with pulse times ramping from 20 to 50 seconds. An additional probe for pulsed field gel electrophoresis (PFGE) (pBWEG) was prepared by taking a 2.8-kb KpnI/EcoRV fragment from the 3.5-kb subclone described above.

RESULTS

Adult Hematological Phenotypes

Pedigree for family B is given in Fig 1. This family has been followed by one hematologist (T. Wilkinson) for over 20

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SCOTTISH IRISH (βγδ)n THALASSEMIA

Fig 1. Pedigree for family

B. Propositus is II1.

years. Eight affected individuals were studied, three (III1, IV1, IV4) as neonates. The propositus is II1. Blood counts show mild microcytic hypochromic anemias in adults with (βγδ)n thalassemia (Table 1). Basophilic stippling and target cells are frequently prominent in the peripheral blood. Affected adults have HbA2 levels that are at the upper limit of normal. Only one individual (II1, a 21 year old woman) had a moderate elevation in HbF. An α/β biosynthesis ratio of 2.3 in the propositus was consistent with a β thalassemia-like disorder. A number of individuals, particularly II3, had been investigated and treated for iron deficiency over many years.

Neonate Phenotypes

III1 was pale and jaundiced at birth. He had palpable splenomegaly and a cord blood Hb of 13.2 g/dL. Target cells and basophilic stippling were prominent in the peripheral blood. III1's birth weight was 8.5 pounds and he was not

Table 1. Hematological Data for Family B

<table>
<thead>
<tr>
<th>Family Member</th>
<th>Sex/Age (yr)*</th>
<th>Hb (g/dL)</th>
<th>MCV (fl)</th>
<th>MCH (pg)</th>
<th>Reticulocyte (%)</th>
<th>Ferritin (μg/L)</th>
<th>HbA2 (%)</th>
<th>HbF (%)</th>
<th>α/β Ratio</th>
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</thead>
<tbody>
<tr>
<td>I1</td>
<td>F/73</td>
<td>11.9</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>II1</td>
<td>M/58</td>
<td>12.7</td>
<td>61</td>
<td>20</td>
<td>CBT</td>
<td>—</td>
<td>85</td>
<td>3.8</td>
<td>&lt;1.0</td>
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<tr>
<td>II2</td>
<td>F/53</td>
<td>14.0</td>
<td>92</td>
<td>32</td>
<td>N</td>
<td>1.3</td>
<td>2.4</td>
<td>&lt;1.0</td>
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<tr>
<td>II3</td>
<td>F/41</td>
<td>10.1</td>
<td>—</td>
<td>—</td>
<td>CB</td>
<td>—</td>
<td>&lt;3.7</td>
<td>&lt;1.0</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>III1</td>
<td>F/21</td>
<td>11.6</td>
<td>62</td>
<td>20</td>
<td>CBT</td>
<td>—</td>
<td>20</td>
<td>3.4</td>
<td>2.5</td>
</tr>
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<td>III2</td>
<td>M/24</td>
<td>16.3</td>
<td>84</td>
<td>31</td>
<td>N</td>
<td>—</td>
<td>3.6</td>
<td>&lt;1.0</td>
<td>1.2</td>
</tr>
<tr>
<td>III3</td>
<td>F/40</td>
<td>11.0</td>
<td>65</td>
<td>20</td>
<td>CT</td>
<td>1.0</td>
<td>63</td>
<td>3.8</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>III4</td>
<td>M/28</td>
<td>13.6</td>
<td>60</td>
<td>20</td>
<td>CB</td>
<td>1.3</td>
<td>34</td>
<td>3.7</td>
<td>&lt;1.0</td>
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<tr>
<td>III5</td>
<td>F/37</td>
<td>14.5</td>
<td>95</td>
<td>31</td>
<td>N</td>
<td>2.1</td>
<td>14</td>
<td>3.6</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>IV1</td>
<td>F/17</td>
<td>11.0</td>
<td>65</td>
<td>20</td>
<td>CT</td>
<td>3.3</td>
<td>24</td>
<td>3.6</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>IV2</td>
<td>F/20</td>
<td>12.2</td>
<td>83</td>
<td>25</td>
<td>CT</td>
<td>—</td>
<td>—</td>
<td>70.0</td>
<td>—</td>
</tr>
</tbody>
</table>

Normal adult values: Hb, 16 ± 2 (M), 14 ± 2 (F); Mean corpuscular volume (MCV), 86 ± 10; mean corpuscular hemoglobin (MCH), 29 ± 3; reticulocyte 0.2–2.0; ferritin, 20–300 (M), 15–200 (F); HbA2, 1.5–3.7; HbF, <1.0; α/β, <1.0. Affected individuals are I1, II1, III1, III2, III3, IV1, and IV4. Blood counts in I1 and II1 were obtained with a Coulter model ZB counter. Blood films in III1 and IV1 (see Fig 2) were normal. IV2 and IV4 had no clinical problems as neonates.

Abbreviations: C, central pallor; B, basophilic stippling; T, target cells; N, normal blood film; —, not done.

*Age at time of testing.
†Blood sample taken from umbilical cord.
delivered prematurely. There was no evidence of immune hemolysis on blood film and his Coomb's test was negative. III₁ required repeated blood transfusions and was subsequently discharged from hospital 18 days after delivery with a Hb of 10.7 g/dL. Twenty-eight days postnatally, his Hb had fallen to 8.5 g/dL. In contrast, two other affected neonates (IV₁ and IV₄) had uneventful postnatal periods and did not require blood transfusions or prolonged hospitalization. Cord blood Hbs and MCVs for the latter were 12.2 g/dl and 92 fl (IV₁) and 12.2 g/dL and 83 fl (IV₄) (Table 1).

DNA Mapping

Deletion breakpoint. Normal restriction fragments were obtained with e, y, and ψβ probes. In affected individuals, intensities of bands measured with a densitometer were reduced (data not given). Total deletion of the e, y, and ψβ regions was confirmed by DNA polymorphisms, which showed affected individuals to be hemizygous (Table 2). The restriction fragment length polymorphism (RFLP) patterns for III₁ were initially read as −−/−− and −−/−− respectively; those for III₁ were +−/+− and +−/+−. However, RFLPs for the latter are incorrect unless there is nonpaternity or this individual and her father are hemizygous. The former explanation is unlikely given the rare occurrence of this disorder. Abnormal restriction fragments were detected with the β globin gene probe (Table 3, Fig 2). The latter were not detectable if a 5′ fragment or an intervening sequence (IVS) sequence fragment of the β probe was used for hybridization. This indicated that it was the 3′ component of the PstI 4.4-kb β probe that was hybridizing to the novel DNA fragment. A tentative restriction map that involved deletion of the e, y, ψβ, and β globin genes was constructed and subsequently confirmed by DNA sequencing (Fig 3).

Deletion size. The 5′ extent of the deletion was sought with DNA probe 2.5 SphI, which is located ≈105 kb upstream of the β globin gene.13 No abnormal bands were detected with this probe. Double-hybridizations using 2.5 SphI and pRK29 probes (19 kb 3′ to the β globin gene) demonstrated a reduced hybridization signal with 2.5 SphI consistent with deletion of this segment. The minimum size of the deletion is thus 105 kb (Fig 4). PFGE of normal DNA digested with SalI and SfiI and hybridized consecutively with probes 2.5 SphI and pBWEG identified the same

<table>
<thead>
<tr>
<th>Subject</th>
<th>Polymorphism (probe/restriction enzyme)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>II₁ (father)†</td>
<td>−−/−−</td>
</tr>
<tr>
<td>II₂ (mother)</td>
<td>+−/+−</td>
</tr>
<tr>
<td>III₁ (daughter)†</td>
<td>−−/−−</td>
</tr>
<tr>
<td>III₂ (son)</td>
<td>+−/+−</td>
</tr>
</tbody>
</table>

*+, RFLP present; −−, absent; 0, deletion.
†Affected individuals.

**Table 2. DNA Polymorphisms for Family B**

<table>
<thead>
<tr>
<th>Restriction Endonuclease</th>
<th>Normal Size (kb)*</th>
<th>Family B Pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>PstI</td>
<td>4.4</td>
<td>N + 3.5</td>
</tr>
<tr>
<td>BglII</td>
<td>5.0</td>
<td>N + 4.4</td>
</tr>
<tr>
<td>HindIII</td>
<td>7.8</td>
<td>N + 6.0</td>
</tr>
<tr>
<td>EcoRV</td>
<td>15.5</td>
<td>N + 1.8†</td>
</tr>
<tr>
<td>PvuII</td>
<td>12.8</td>
<td>N + 7.5</td>
</tr>
<tr>
<td>HincII</td>
<td>5.4</td>
<td>N + 4.4</td>
</tr>
<tr>
<td>Kpnl</td>
<td>39.9</td>
<td>N + 6.5</td>
</tr>
<tr>
<td>SaeI</td>
<td>16.4</td>
<td>N + 6.1</td>
</tr>
<tr>
<td>XbaI</td>
<td>11.1</td>
<td>N</td>
</tr>
<tr>
<td>BamHI</td>
<td>1.9, 9.4</td>
<td>N</td>
</tr>
<tr>
<td>EcoRI</td>
<td>5.6, 3.8</td>
<td>N + 4.0†</td>
</tr>
</tbody>
</table>

Abbreviation: N, normal.

*Restriction fragments derived from GenBank sequence data.
†The 1.8-kb EcoRV site was confirmed on DNA sequence and a 4.0-kb EcoRI fragment identified from DNA sequence.
SCOTTISH IRISH (εγβ)° THALASSEMA

Fig 3. Restriction map for (A) the normal β globin gene and (B) the deletion in family B. The novel DNA fragment in family B is shown by a broken line, the breakpoint by a vertical arrow. Horizontal arrows define the L1 repeat and triangles the Alu repeats located downstream of the β globin gene. Restriction sites are E, EcoRI; H, HindIII; P, PstI; G, BglII; B, BamHI; N, HindII; X, XbaI; R, EcoRV; K, KpnI; V, PvuII; and S, Sall. The extent of the PstI 4.4-kb β gene probe is shown.

Fig 4. PFGE map for normal DNA (A and B) derived from reference 8 and confirmed in this study. Locations of probes SphI and pRK29 are shown. The ~150-kb SfiI fragment comprises 70 kb upstream of the β gene and 80 kb downstream. Bands obtained after hybridization of normal DNA with probes 2.5 SphI and pBWEG are shown in C and D. A novel ~205-kb fragment was found following digestion of (εγβ)° thalassemia DNA with SfiI and hybridization to probes pRK29 and pBWEG (E).

normal ~290- and ~250-kb fragments, respectively. Therefore, the 5′ breakpoint for Scottish-Irish (εγβ)° thalassemia is located within these two fragments (Fig 4). DNA from (εγβ)° thalassemia, when digested with SfiI and hybridized to the pRK29 probe, gave both the normal ~150-kb band and an additional ~205-kb band (Fig 4). 125 kb of the latter is novel sequence because ~80 kb are derived from the normal segment 3′ to the β gene. Additional proof that the ~205 kb represented novel sequence was obtained by identifying the same band on rehybridization of the above filter with the pBWEG probe. Therefore, the 5′ breakpoint is 125 kb from the β gene and so the total deletion, based on the SfiI map, is (250 + 10 + 70) – (125) or ~205 kb.

DNA Sequencing

Five hundred eighty-six base pairs (bp) of DNA sequenced are shown in Figs 5 and 6. The 3′ 106 bp are identical to the sequence found 3′ to the normal β globin gene and place the breakpoint at 263 bp 3′ from the terminator codon on exon 3. The remaining 480 bp of sequence represent novel DNA of
which approximately 320 bp show homology to a number of sequences. On the Match program the latter included human HLA-DP-β1 and α exon genes (85% match 272/321 bp); human leukocyte interferon i, 1, and m genes (83% match 110/133); human monomeric alphoid satellite DNA (83% match 272/328); and the L1 (KpnI) repeat found between the human ε and Gγ globin genes (88% match 282/320 bp). DNA sequence data confirmed the deletion map in Fig 4 and identified an EcoRI restriction site immediately 5′ to the EcoRV site. The novel EcoRI fragment generated measured approximately 6.1 kb in size and is 3.4 kb downstream from the β globin gene (Fig 3). The significance of the L1 repeat at the breakpoint in Scottish-Irish α′ thalassemia has been established data for the 3′ flanking region by an interrupted line. An EcoRI recognition site (GAATTG) is found at position 15 and one for EcoRV (GATATC) at 89. DNA from both strands was sequenced. The only difference from published data for the 3′ flanking region was an additional C at position 526.

**DISCUSSION**

The Scottish-Irish type of (γδβ)α′ was first described in 1983. The upstream end of this deletion was estimated to extend at least 100 kb 5′ to the β gene cluster. The 3′ end was placed between the β globin gene and a polymorphic HindIII restriction site 7 kb downstream from the β gene. Abnormal restriction fragments were not detected because of the β globin gene probes used. The present example of (γδβ)α′ thalassemia has a deletion length of ~205 kb, which involves the ε, Gγ, Aγ, Bγ, δ, and β globin genes. DNA mapping, cloning, and sequencing identified the downstream breakpoint at 263 bp 3′ to the third exon of the β globin gene (Fig 3). The similarity of the breakpoint region and the ethnic background of the propositus indicates that the (γδβ)α′ thalassemia characterized is an example of the Scottish-Irish type.

DNA database searches identified a sequence of approximately 320 bp near the 3′ breakpoint, which had homology to a number of human genes/DNA fragments. The latter included HLA-DP, leukocyte interferon, and alphoid satellites. These DNA sequences have structural features consistent with L1 (KpnI) repeats. Confirmation that the above 320-bp sequence is an L1 repeat was obtained when homology searches identified an 88% match with L1 Heg, an 8.6-kb KpnI repeat element located between the human ε and Gγ globin genes (Fig 3). An example of a hemophilia defect that resulted from insertion of an L1 repeat element has been reported. It was proposed in this instance that dispersal of an L1 repeat via an RNA intermediate produced hemophilia by an insertional mutation. It is intriguing that the 320-bp L1 sequence found in the novel (γδβ)α′ thalassemia fragment is located near a second L1 repeat. The latter is approximately 6.1 kb in size and is 3.4 kb downstream from the β globin gene (Fig 3). The significance of the L1 repeat at the breakpoint in Scottish-Irish (γδβ)α′ thalassemia is unclear. Taramelli and colleagues failed to detect repetitive elements in the breakpoint regions of Anglo-Saxon and Dutch (γδβ)α′ thalassemias. L1 repeats constitute approximately 5% of the human genome. There are seven such elements located within the 100 kb upstream of the β globin gene. It is likely that the L1 repeat in Scottish-Irish (γδβ)α′ thalassemia and its proximity to the normal L1 repeat 3′ to the β globin gene is fortuitous. An illegitimate recombination event, such as proposed in the great majority of α and β globin deletions, is more likely responsible for the deletion.

Hematologic and clinical phenotypes described in neonates with (γδβ)α′ thalassemia are variable. They range from a severe life-threatening microcytic hemolytic anemia requiring blood transfusion to an asymptomatic condition. The two Scottish-Irish neonates described in 1983 both required blood transfusion and prolonged hospitalization. Three of nine infants with the Dutch variety died. Five of the living six required blood transfusions, whereas the remaining one improved with no treatment. A blood transfusion was required for the infant with the Anglo-Saxon deletion. Variability in severity was also seen in the three affected family B with the L1 repeat underscored by a solid line and the normal β gene 3′ flanking region by an interrupted line. An EcoRI recognition site (GAATTG) is found at position 15 and one for EcoRV (GATATC) at 89. DNA from both strands was sequenced. The only difference from published data for the 3′ flanking region was an additional C at position 526.
neonates in the present study. One required blood transfusion and prolonged hospitalization; the remaining two were asymptomatic. Exacerbation in the thalassemias may occur following infections or other hematological conditions predisposing to hemolysis. These were not present in the severely affected infant described above. However, an unusual feature of the \((\epsilon\gamma\delta\beta)\) thalassemia is improvement of the thalassemic phenotype as the switch from fetal to adult hemoglobin occurs. Thus, it is possible that the timing of the HbF switch may also influence severity. If the latter is a component in modulating the severity of \((\epsilon\gamma\delta\beta)\) thalassemia, the preterm infant with high levels of HbF\(^{29}\) may be particularly at risk for a more severe disorder.

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