Inclusion Body β-Thalassemia Trait in a Swiss Family Is Caused by an Abnormal Hemoglobin (Geneva) With an Altered and Extended β Chain Carboxy-Terminus due to a Modification in Codon β114


We have analyzed the sequence of the β globin gene of a chromosome that is linked to the occurrence of an inclusion body β-thalassemia characterized in the heterozygote by moderate anemia, severe red cell abnormalities, splenomegaly, inclusion body formation, elevated Hb A2 levels, and an increased in vitro α/β chain synthetic ratio. The data indicate a change in codon 114 from CTG (Leu) to -GG that resulted in a frameshift and the presumed modification of an abnormal β chain that is 156 residues long with a completely different C-terminal amino acid sequence. The change in codon 114 gives a -GGGCC- sequence that creates a new ApaI site; the resulting 2.6-kilobase fragment has been observed in all subjects with this thalassemia condition. Protein structural analyses failed to demonstrate any trace of the abnormal β chain, even in reticulocytes and nucleated red cells that were isolated by density gradient centrifugation. The inclusion bodies appear to contain mainly normal α chains. It is assumed that the structure of the β-Geneva chain prevents it from combining with normal α chains; this results in a rapid breakdown of the abnormal protein during the early stages of red cell maturation and an accumulation of free α chains.

β-Globin gene cloning and sequence analysis. DNA from subject IV-2 (Fig I) was digested with BclI, and the 10.5-kilobase (kb) fragment was ligated into BamHI-EcoRI double-digested EMBL-3 phage DNA. Recombinant phages were packaged in vitro (“Gigapack plus” packaging extracts, Stratagene, La Jolla, CA) and plated on P2392 host strain. The β globin gene from the β-thal chromosome in the positive clones was identified by the presence of the AavII restriction site in βIVS-II (see later) by using Southern blotting and the βIVS-II probe. The β globin gene was subcloned as a 3.7-kb BglII-PstI fragment into the BamH1-PstI double-digested Blue-script plasmid (Stratagene) by using DH 5α as competent cells (BRL, Bethesda, MD). DNA sequence analysis was performed by the dyeoX chain termination method of Sanger and Coulson with a modification of the sequenc analysis protocol (Stratagene). The three synthetic 19-20-mer oligonucleotides used as primers were located at nucleotides 105 to 96 5' to the Cap site, at 529 to 548 in IVS-II (reverse primer), and at 1233 to 1251 in IVS-II. The region of the cloned gene was sequenced extended from 80 bp upstream from the Cap site to 50 bp downstream from the termination site and excluded some 750 bp in the middle of IVS-II.

Protein structural analyses. These involved analyses of tryptic peptides of the AE:β chain that were isolated from a red cell lysate by carboxymethyl (CM)-cellulose chromatography of precipitates.

MATERIALS AND METHODS

Blood samples. These were collected in vacutainers with EDTA as an anticoagulant and studied at the institution in Geneva; samples from six family members were shipped by air to Augusta, GA, for further analyses. Informed consent was obtained.

Hematology. Routine hematologic studies were done with automated cell counters and with standard procedures. Methodology to analyze blood samples for the presence of inclusion bodies and unstable Hb and for the relative synthesis of α and β chains has been presented before.1 The presence of a possible abnormal Hb in red cell lysates was evaluated by electrophoretic and chromatographic procedures routinely in use in our laboratories.13,14 Hb A2 was quantitated by microcolumn chromatography13 and Hb F by an alkali denaturation procedure.14 Hb F was isolated by diethylaminomethyl–cellulose chromatography,11 and its γ chain composition was determined by reverse-phase high-performance liquid chromatography (HPLC).11

DNA analysis. DNA was isolated from white cells by the procedure described by Poncz et al.11 Haplotypeing was done for six members of the family and included the following restriction sites: HindIII S' to ε, XmnI S' to γ, HindIII at εγ and γ, PvuII S' to β, HincII at βδ and 3' to it, TaqI S' to β, AavII at β, and HpaII and BamHI 3' to β. Methodology and probes have been listed before.13,14

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isolated from red cells of the propositus, which were separated by density gradient centrifugation using phosphate-buffered dextran solutions of 24 to 34 g/100 mL, and of washed whole red cells of the propositus. The methodology included the separation of the peptides by HPLC, rechromatography of several peptides by additional HPLC procedures, amino acid analyses using fully automated HPLC, rechromatography and chromatographic methods or by heat and isopropanol stability tests.

**DNA analyses.** Initial data indicated that none of the four carriers had an anomaly in the arrangements of the \( \alpha \) globin genes (all were \( \alpha a/\alpha a \)), and no deletion was detectable in the \( \beta \) globin gene cluster. The haplotype of the affected chromosome was \([+\ldots++\ldots++]\); because the \( XmnI \) site \( 5' \) to \( \delta \gamma \) was absent, a low \( \delta \gamma \) value was expected.\(^8\)\(^9\) This was indeed observed for subjects 111-2, 111-6, and IV-2 but not for IV-1, who had a \( \delta \gamma \) value of 55.4% and an \( XmnI \) site on the normal chromosome that he inherited from his father, subject III-1, who had a homozygosity at that site and a high \( \delta \gamma \) value of 68.7% (see Table 1). Three of the four affected members had the \( Avall \) site at both \( \beta \) globin genes, while the normal chromosome of subject IV-2 did not have this restriction site. Thus, DNA from this

### Table 1. Hematologic and Hb Composition Data for Six Members of Family D

<table>
<thead>
<tr>
<th>Pedigree No.</th>
<th>III-1</th>
<th>III-2</th>
<th>III-4</th>
<th>III-6</th>
<th>IV-1</th>
<th>IV-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex-age</td>
<td>M-44</td>
<td>F-44</td>
<td>F-38</td>
<td>M-38</td>
<td>M-22</td>
<td>F-22</td>
</tr>
<tr>
<td>Hb (g/dL)</td>
<td>14.9</td>
<td>9.5</td>
<td>14.5</td>
<td>11.6</td>
<td>10.6</td>
<td>10.5</td>
</tr>
<tr>
<td>PCV (L/L)</td>
<td>0.433</td>
<td>0.290</td>
<td>0.420</td>
<td>0.340</td>
<td>0.336</td>
<td>0.340</td>
</tr>
<tr>
<td>RBC ( \times 10^{12}/L )</td>
<td>4.93</td>
<td>3.55</td>
<td>4.61</td>
<td>4.41</td>
<td>4.81</td>
<td>4.95</td>
</tr>
<tr>
<td>MCV (fl)</td>
<td>88</td>
<td>81</td>
<td>90</td>
<td>77</td>
<td>70</td>
<td>68</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>30.0</td>
<td>26.8</td>
<td>31.5</td>
<td>26.0</td>
<td>22.0</td>
<td>21.0</td>
</tr>
<tr>
<td>MCHC (g/dL)</td>
<td>34.0</td>
<td>33.0</td>
<td>35.0</td>
<td>34.0</td>
<td>31.5</td>
<td>31.0</td>
</tr>
<tr>
<td>Reticulocytes (%)</td>
<td>0.9</td>
<td>36.0*</td>
<td>0.7</td>
<td>42.5*</td>
<td>4.6</td>
<td>5.8</td>
</tr>
<tr>
<td>WBC ( \times 10^9/L )</td>
<td>5.1</td>
<td>16.7</td>
<td>5.4</td>
<td>14.1</td>
<td>7.2</td>
<td>7.1</td>
</tr>
<tr>
<td>Platelets ( \times 10^9/L )</td>
<td>294</td>
<td>900*</td>
<td>185</td>
<td>570*</td>
<td>293</td>
<td>427</td>
</tr>
<tr>
<td>Morphology</td>
<td>Normal</td>
<td>Abnormal†</td>
<td>Normal</td>
<td>Abnormal†</td>
<td>Abnormal†</td>
<td>Abnormal†</td>
</tr>
<tr>
<td>Heinz bodies</td>
<td>—</td>
<td>+ + +</td>
<td>—</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
</tr>
<tr>
<td>Hb ( A_2 ) (%)</td>
<td>1.8</td>
<td>3.6</td>
<td>2.4</td>
<td>ND</td>
<td>4.0</td>
<td>3.3</td>
</tr>
<tr>
<td>Hb ( F ) (%)</td>
<td>0.8</td>
<td>2.4</td>
<td>0.4</td>
<td>1.6</td>
<td>3.6</td>
<td>1.6</td>
</tr>
<tr>
<td>( \delta \gamma ) in Hb F (%)</td>
<td>68.7</td>
<td>26.3</td>
<td>—</td>
<td>25.1</td>
<td>55.4</td>
<td>21.4</td>
</tr>
</tbody>
</table>

Abbreviations: PVC, packed cell volume; MCV, mean corpuscular volume; MCHC, mean corpuscular Hb concentration; ND, not determined.

*By alkali denaturation.\(^8\)
†Extreme anisocytosis and poikilocytosis; basophilic stippling, erythroblasts.
‡By reverse-phase HPLC.\(^7\)\(^8\)

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**RESULTS**

**The family.** As many as 44 members of this French-Swiss family were evaluated; at least 14 suffered from congenital anemia that was diagnosed in 1973 in Zurich as a "severe form of \( \beta \)-thalassemia trait with inclusion bodies and splenomegaly." Figure 1 illustrates an abbreviated pedigree and identifies the six family members participating in this latest study. Hematologic data are given in Table 1. Four subjects, including the propositus III-2, had a significant hemolytic anemia characterized by an extreme anisocytosis and poikilocytosis, basophilic stippling, and reticulocytosis (Fig 2). The Hb \( A_2 \) level was elevated at 3.3% to 4.0%, and the Hb F level was slightly increased (1.6% to 3.6%). The propositus, a 44-year-old female who was splenectomized at the age of 23 years, was studied in great detail. Relevant information includes numerous erythroblasts with large inclusion bodies in the peripheral blood upon staining with brilliant cresyl blue (Fig 2), decreased red cell survival (\( ^{31} \)Cr, autologous) at 20 days (half-life), ineffective erythropoiesis (\( ^{3} \)Fe) with increased plasma iron turnover (2.71 mg/100 mL/d), increased erythrocyte iron turnover (2.28 mg/100 mL/d) and a decreased incorporation into mature red cells (53%), \( \alpha/\beta \) in vitro synthesis ratio for reticulocytes of 2.06 and 1.67 for bone marrow, bone marrow morphology consisting of erythroid hyperplasia, large cytoplasmic inclusions in erythroblasts after staining with methyl violet, transferrin saturation of 56%, serum ferritin at 416 ng/mL, and no abnormal Hb detectable in red cell lysate by electrophoretic and chromatographic methods or by heat and isopropanol stability tests.

Fig 1. Partial pedigree of family D. Family members III-1, III-2, III-4, III-6, IV-1, and IV-2 participated in the present study. Ages are listed in the right upper corner; patients III-2 and III-6 are splenectomized.

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![Pedigree Diagram](image-url)
INCLUSION BODY β-THALASSEmia TRAIT

Fig 2. (Top) Peripheral blood film of subject IV-1 showing marked anisopoikilocytosis (Wright stain; original magnification x2,000). (Bottom) Peripheral blood film of propositus 111-2 who was splenectomized in 1965. Some cells containing large inclusion bodies are indicated with arrows. Incubation was with brilliant cresyl blue (original magnification x200).

A

B

Fig 3. Sequence analyses of part of the β globin gene of the AvaII-positive chromosome of subject IV-2, labeled Geneva. (Top) Autoradiograph of a DNA sequencing gel showing the sequence of codons 111 through 117. (Bottom) Sequence data for part of exon 3 of the abnormal β globin gene. The deletion of the dinucleotide CT and the insertion of the nucleotide G in codon 114 results in an out-of-frame reading through codon 156 and the suggested synthesis of an abnormal β chain with 156 amino acid residues.

Protein structural analyses. Initial analyses of the AE-β chain and isolated inclusion bodies failed to identify a β globin chain abnormal variant; the digests of the inclusion bodies contained peptides from both α and β chains, with the α peptides being present in excess (data not shown). Digests from the washed whole red cells also contained mainly peptides from α and β chains, as did the digests of Hb isolated from red cell fractions separated by density gradient centrifugation and of Hb from precipitates considered to be mainly inclusion bodies (data not shown). Calculation of the α/β ratios in these digests by the molar concentrations of selected tryptic peptides showed excess α chain in the Hb isolated from (precipitates of) the red cells with the highest density.

DISCUSSION

The results of the analyses described here provide strong evidence that the severe β-thal-like condition in this family is caused by the presence of an abnormal and probably most unstable β chain variant. The modification observed in codon 114 results in a frameshift, and the β chain to be synthesized is not only ten residues longer but also has unusual structural features inasmuch as the new structure starting from residues 114 through 156 will have no acidic residues, five basic residues (three Lys and two Arg or three Lys in the C-terminus, 114 through 146, of β), four Trp residues, one...
Our data confirm earlier observations that red cell precipitates contain mainly α globin chains.13

The formation of the β chain of Hb Geneva, like those of some other unstable β chain abnormal Hbs such as Hb Indianapolis11 and Hb Showa-Yakushiji,12 is associated with a β-thal phenotype that is clinically more severe than that observed in heterozygotes for a βα-thal. Unlike other abnormal α and β chains with elongated carboxy-termini, the β-Geneva chain is, because of its peculiar C-terminal sequence, not expected to combine with itself or any other globin chain to form a dimer or even a tetramer and will be rapidly hydrolyzed. This continuous enzymatic breakdown of the β-Geneva chain, which is likely synthesized at a rate similar to that of βα, may exhaust the proteolytic defense mechanism of the developing red cell to the extent that proteolysis of free α chain is considerably decreased. Thus, free α chains will accumulate and subsequently precipitate in the red cell precursors to a greater extent than observed in the erythrocytes of subjects with a βα-thal heterozygosity. This will result in damage to the red cell membrane and in an ineffective erythropoiesis with clinical hemolysis and splenomegaly. Milder β-thal phenotypes are seen in patients with other unstable β chain variants. These conditions are likely caused by a similar, but perhaps quantitatively different mechanism.

REFERENCES


Inclusion body beta-thalassemia trait in a Swiss family is caused by an abnormal hemoglobin (Geneva) with an altered and extended beta chain carboxy-terminus due to a modification in codon beta 114

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