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 \(\beta 114\)


We have analyzed the sequence of the \(\beta\) globin gene of a chromosome that is linked to the occurrence of an inclusion body \(\beta\)-thalassemia characterized in the heterozygote by moderate anemia, severe red cell abnormalities, splenomegaly, inclusion body formation, elevated \(Hb A_2\) levels, and an increased in vitro \(\alpha/\beta\) chain synthetic ratio. The data indicate a change in codon 114 from CTG (Leu) to \(-GG\) that resulted in a frameshift and the presumed morphology of an abnormal \(\beta\) 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 \(Apel\) 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 \(\beta\) chain, even in reticulocytes and nucleated red cells that were isolated by density gradient centrifugation. The inclusion bodies appear to contain mainly normal \(\alpha\) chains. It is assumed that the structure of the \(\beta\)-Geneva chain prevents it from combining with normal \(\alpha\) chains; this results in a rapid breakdown of the abnormal protein during the early stages of red cell maturation and an accumulation of free \(\alpha\) chains.

\(\beta\)-Globin gene cloning and sequence analysis. DNA from subject IV-2 (Fig 1) 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 \(\beta\) globin gene from the \(\beta\)-thal chromosome in the positive clones was identified by the presence of the \(\Avell\) restriction site in \(\beta\)IVS-II (see later) by using Southern blotting and the \(\beta\)IVS-II probe. The \(\beta\) globin gene was subcloned as a 3.7-kb \(BglII-PstI\) fragment into the \(BamHI-PstI\) double-digested Blue-script plasmid (Stratagen) by using DH 5a as competent cells (BRL, Bethesda, MD). DNA sequence analysis was performed by the dyeoxy chain termination method of Sanger and Coulson with a modification of the sequencing protocol (Stratagen). 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 that 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-\(\beta\) 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 \(\alpha\) and \(\beta\) chains has been presented before. The presence of a possible abnormal Hb in red cell lysates was evaluated by electrophoretic and chromatographic procedures routinely in use in our laboratories. \(Hb A_2\) was quantitated by microcolumn chromatography and \(Hb F\) by an alkali denaturation procedure. \(Hb F\) was isolated by diethylaminoethyl-cellulose chromatography, and its \(\gamma\) chain composition was determined by reverse-phase high-performance liquid chromatography (HPLC).

DNA analysis. DNA was isolated from white cells by the procedure described by Poncz et al. Haplotyping was done for six members of the family and included the following restriction sites: \(HinfI\) S’ to \(\epsilon\), \(XmnI\) S’ to \(\gamma\), \(HindIII\) at \(\gamma\) and \(\epsilon\), \(PvuII\) S’ to \(\psi\), \(HinfI\) at \(\phi\) and 3’ to it, \(TaqI\) S’ to \(\beta\), \(AvaiI\) at \(\beta\), and \(HpaII\) and \(BamHI\) 3’ to \(\beta\). Methodology and probes have been listed before.

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isolated from red cells of the propositus, which were separated by
density gradient centrifugation using phosphate-buffered dextran
dilutions 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 procedures, amino acid analyses using fully automated
HPLC, rechromatography.

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 A2 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 brillian cresyl blue (Fig 2), decreased red cell survival ($^{51}$Cr, autologous) at 20 days (half-life), ineffective erythropoiesis ($^{59}$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 consist-
ing of erythroid hyperplasia, large cytoplasmic inclusions in
erthroblasts 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.

DNA analyses. Initial data indicated that none of the
four carriers had an anomaly in the arrangements of the $\alpha$
globin genes (all were $aa/aa$), and no deletion was detectable
in the $\beta$ globin gene cluster. The haplotype of the
affected chromosome was $[+]_{+}++_{+}++_{+}++;$ be-
cause the $XmnI$ site $5'_{+}$ was absent, a low $G_{+}$ value was
expected.\(^9\) This was indeed observed for subjects 111-2,
III-6, and IV-2 but not for IV-1, who had a $G_{+}$ value of 55.4%
and an $XmnI$ site on the normal chromosome that he
inherited from his father, subject III-1, who had a homozy-
gosity at that site and a high $G_{+}$ value of 68.7% (see Table 1).
Three of the four affected members had the $Avai$ 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>Pedigee 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>
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<tbody>
<tr>
<td>Sex-age</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M-44</td>
<td>F-44</td>
<td>F-38</td>
<td>M-38</td>
<td>M-22</td>
<td>F-22</td>
<td></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></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></td>
</tr>
<tr>
<td>RBC (10$^9$/L)</td>
<td>4.93</td>
<td>3.55</td>
<td>4.61</td>
<td>4.41</td>
<td>4.81</td>
<td></td>
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<tr>
<td>MCV (fl)</td>
<td>88</td>
<td>81</td>
<td>90</td>
<td>77</td>
<td>70</td>
<td></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></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></td>
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<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></td>
</tr>
<tr>
<td>WBC (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></td>
</tr>
<tr>
<td>Platelets (10$^9$/L)</td>
<td>294</td>
<td>900*</td>
<td>185</td>
<td>570*</td>
<td>293</td>
<td></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>Hb A2 (%)‡</td>
<td>1.8</td>
<td>3.6</td>
<td>2.4</td>
<td>ND</td>
<td>4.0</td>
<td></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></td>
</tr>
<tr>
<td>$G_{+}$ in Hb F (%)‖</td>
<td>68.7</td>
<td>26.3</td>
<td>—</td>
<td>25.1</td>
<td>55.4</td>
<td></td>
</tr>
</tbody>
</table>

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

*By alkali denaturation.\(^9\)
†By microcolumn chromatography.\(^9\)
‡By reverse-phase HPLC.\(^7\,8\)
INCLUSION BODY β-THALASSEmia TRAIT

Fig 2. (Top) Peripheral blood film of subject IV-1 showing marked anisopoikilocytosis (Wright stain; original magnification x 2,000). (Bottom) Peripheral blood film of propositus III-2 who was splenectomized in 1965. Some cells containing large inclusion bodies are indicated with arrows. Incubation was with brilliant cresyl blue (original magnification x 200).

Fig 3. Sequence analyses of part of the β globin gene of the Avell-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.

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 β114 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 in three Lys in the C-terminus, 114 through 146, off β'), four Trp residues, one 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 β114 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 in three Lys in the C-terminus, 114 through 146, of β'), four Trp residues, one
Of major interest is the question of the composition of the inclusion bodies. One can expect that aggregates of abnormal β chains together with α chains, which should be present in excess as a result of loss of β chains, will both contribute to the formation of inclusions in nucleated red cells. Our structural analyses gave no indication for the presence of an abnormal β chain, not even in reticulocytes or nucleated red cells, which supports the assumption that this unstable protein is rapidly eliminated by proteolysis. 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 Indianapolis21 and Hb Showa-Yakushiji,22 is associated with a β-thal phenotype that is clinically more severe than that observed in heterozygotes for a ββ-thal. Unlike other abnormal α and β chains with elongated carboxy-termini,23 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 β, 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 ββ-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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