Observations on the Levels of Hb A₂ in Patients With Different β-Thalassemia Mutations and a δ Chain Variant

By J.F. Codrington, H-W. Li, F. Kutlar, L-H. Gu, M. Ramachandran, and T.H.J. Huisman

Hb A₂ and its variant B₂ (α₂δ₁₆(A13)Gly → Arg) were quantitated in the blood of subjects with three different types of β-thalassemia and with the δ-B₂ anomaly in cis or in trans to the β-thalassemia determinant. In one family, the δ-B₂ mutation was in cis to a newly discovered codon 47 (+A) frameshift. The levels of Hb A₂ and B₂ were nearly the same and approximately 70% higher than those in simple Hb B₂ heterozygotes. In two additional families, the δ-B₂ variant was in trans to either a deletional β-thalassemia (1,393 bp) involving part of the β-globin gene and part of the β-globin gene promoter, or to the − 88 C → T promoter mutation. In both instances, the Hb B₂ level was increased by approximately 80%, but the Hb A₂ level was increased by approximately 270% and 200%, respectively. These data indicate two mechanisms that will cause an increase in δ chain production. One is consistent with a general mechanism concerning the relative excess of α chains in β chain deficiencies which will combine with δ chains to form variable levels of Hb A₂ dependent on the severity of the β chain deficiency. The second concerns the loss of β-globin gene promoter activity, perhaps by an absence of (or decreased) binding of specific protein(s) to this segment of DNA and a concomitant increase in δ-globin gene promoter activity in cis.

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The increase in the level of Hb A₂ in β-thalassemia (thal) heterozygotes was discovered in 1957 when the percentage of Hb A₂ was found to be approximately twice that in normal adults. More recent studies have indicated that the relative increase depends on the type of β-thal allele that is present. For instance, carriers of some mild types of β-thal, such as those caused by the C → T mutation at IVS-1-6' or by A → G mutations in the polyadenylation site, have only slight increases in their Hb A₂ percentages, while heterozygotes for a silent type of β-thal due to the C → T mutation at nucleotide (nt) − 101 of the β-globin gene promoter have nearly normal Hb A₂ levels. These observations are consistent with the concept that α chains combine much more readily with the β chain of Hb A than with the δ chain of Hb A₂, and that in conditions with variable β chain deficiencies, the increase in Hb A₂ level should vary accordingly. However, there are exceptions to this rule; for instance, persons with a heterozygosity for either one of two mild promoter mutations (namely, C → T at position − 88 or A → G at position − 29, which are common among blacks) have Hb A₂ values at least as high as those observed among heterozygotes for the more severe β-thal alleles, like the C → T mutation at codon 39 or the G → A mutation at IVS-1-110 in Mediterranean patients. Furthermore, δ-thal heterozygotes with a δ chain variant like δ-B₂ or δ16(A13)Gly → Arg in cis or in trans to the thalassemia mutation have similar amounts of Hb A₂ and the Hb A₁ variant, indicating that the increased Hb A₂ synthesis in β-thal is derived from δ genes both in cis and in trans to the β-thal mutation.

Unusually high Hb A₂ levels of 7% to 9% have been found in β-thal heterozygotes with a deletional defect that involves the 5' segment of the β-globin gene and part of the β-globin gene promoter. The deletions concern a 292-bp deletion observed in a Turkish family, a 1,393-bp deletion observed among blacks, and the much larger 4,237-bp deletion seen in members of a Czechoslovakian family, all types share the loss of the 5'β promoter. We recently detected a β-thal heterozygote from Surinam who had the 1,393-bp deletion and a Hb B₂ heterozygosity and who exhibited disproportionate levels of Hb A₂ and Hb B₂. This observation prompted us to reevaluate some members of two families with both β-thal and Hb B₂ heterozygositities who were described over 25 years ago.

MATERIALS AND METHODS

Blood samples. Samples from three members of family K and from four members of a second black family (family M) were collected in The Netherlands (two samples) and Surinam (five samples) and shipped by air to Augusta, GA. Blood samples from one black male of family K, his 6-year-old twin daughters, and from a fourth, unrelated adult black female were obtained locally. Collection was in vacutainers with ethylenediamine tetraacetate as anticoagulant. Informed consent was obtained.

Methods. Hematologic data were collected with an automated cell counter. Hb F was quantitated by cation exchange high performance liquid chromatography (HPLC) using the PolyCAT (Columbia, MD) column system. Hb A₂ and Hb B₂ were quantitated by the same PolyCAT HPLC procedure and by the analytic diethylylaminoethoxy (DEAE)-cellulose procedure with Tris-KCN-HCl developers and a pH gradient; a similar method was used in the earlier publications describing some of these individuals.

DNA was isolated from white blood cells with the method described by Poncz et al. Identification of the B₂ variant (δ16(A13)Gly → Arg) was by hybridization of amplified DNA with probes specific for a G → C mutation in codon 16 of the β-globin gene. A similar procedure was used to identify one of the β-thal mutations; this methodology has been described in previous publications. The approximately 1.4-kb deletion present in the third family was detected by gene mapping as shown earlier. The mutation in the third family was new and was identified through sequencing of amplified DNA. It was confirmed by hybridization of amplified DNA with a specific, radiolabeled oligonucleotide probe.
RESULTS

The three families and their \( \beta \)-thal types. Family \( K \) is of Dutch-Surinam origin and was first described in 1961.\(^7\) Fourteen members with a \( \beta \)-thal heterozygosity also had a heterozygosity for Hb B\(_2\) or \( \alpha_{2}\beta_{16}(A13)\)Gly \( \rightarrow \) Arg; no normal members carried the Hb B\(_2\) variant and no \( \beta \)-thal heterozygote was without it. This observation was explained by assuming that the two abnormalities were linked and occurred on the same chromosome. The average values for HPLC procedure may be slightly too high because of contamination with a minute quantity of a modified Hb S.

The average Hb A\(_2\) value (2.2\%) for 12 normal family members (measured by carboxyl-methyl-cellulose chromatography\(^6\)). Nearly 30 years later, it was possible to obtain blood samples from three \( \beta \)-thal heterozygotes (listed in Table 1). The \( \beta \)-thal mutation in this family had not been observed before, and was identified through sequencing as a frameshift in codon 47 (+A), which was confirmed by hybridization of amplified DNA with appropriate probes (Fig 1).

Family \( R \) is a black family from central Georgia who were described in 1963\(^4\) because Hb C, \( \beta \)-thal, and Hb B\(_2\) occurred in different combinations. One subject, E.R., had the \( \beta \)-thal and Hb B\(_2\) heterozygositites; family studies showed that the two anomalies were not linked and occurred on opposite chromosomes. Subject E.R. had 1.85\% Hb B\(_2\) and a rather high Hb A\(_2\) level of 2.7\%, while his mother, with a simple Hb B\(_2\) heterozygosity, had 1.15\% Hb B\(_2\) and 1.25\% Hb A\(_2\). He was reevaluated 26 years later; his twin daughters, 6 years of age, were found to have a simple Hb B\(_2\) heterozygosity. The \( \beta \)-thal mutation in this family was the second most common frameshift in codon 47 (+A), which was confirmed by hybridization of amplified DNA with appropriate probes (Fig 1).

Family \( M \), from Surinam, consisted of parents and two children. The father had an Hb B\(_2\) trait and a \( \beta \)-thal heterozygosity, the mother an Hb S trait, one child Hb S-\( \beta^A \)-thal, and the other child Hb S trait combined with a Hb B\(_2\) heterozygosity. The \( \beta \)-thal was identified as the 1,393-bp deletion with a 5’ end point approximately 600 bp 5’ to the Cap site and a 3’ end point approximately 500 bp from the 5’ splice junction of IVS-II.\(^1,3\) Details of the study of family \( M \) will not be presented.

The mutation of G \( \rightarrow \) C in codon 16 of the \( \delta \)-globin gene leading to the synthesis of the \( \delta \)-chain of Hb B\(_2\) (\( \delta 16(A13)\)Gly \( \rightarrow \) Arg) was confirmed for all three families through hybridization of amplified DNA with \( ^3 \)P-labeled oligonucleotide probes.

The percentages of Hb A\(_2\) and Hb B\(_2\) Quantitation was with two procedures. The DEAE-cellulose chromatographic method resembled that used in the older experiments\(^5\); it had the disadvantage of a decreased recovery of Hb B\(_2\), particularly in somewhat older red blood cell lysates. The data obtained with the PolyCAT HPLC method were highly reproducible, even after storage of the samples at 4°C for an extended period of time. The percentages determined with both procedures are listed in Table 1; although the absolute values given for some samples in the two groups differ considerably, the relative quantities for Hb B\(_2\) and Hb A\(_2\) (listed as 100B\(_2\)/total) were similar.

The percentages of Hb A\(_2\) and Hb B\(_2\) in the three members of family \( K \) with the codon 47 (+A) frameshift were nearly the same, confirming previously published results.\(^1\) The average PolyCAT values of 2.41\% and 2.49\% were approximately 70\% higher than the average values of 1.46\% and 1.49\% observed for two Hb B\(_2\) heterozygotes. Subject E.R., with the \( -88 \) (C \( \rightarrow \) T) mutation, averaged 2.55\% for Hb B\(_2\) and 4.48\% for Hb A\(_2\); this corresponds to approximately 80\% and 200\% increases over the levels in the Hb B\(_2\) heterozygotes. The total value of 7.03\% for \( \delta \) chain containing hemoglobin types was high; the average value for Hb A\(_2\) in 10...

Table 1. The Levels of Hb A\(_2\) and Hb B\(_2\) in Hb B\(_2\) Heterozygotes With and Without an Additional \( \beta \)-Thal Heterozygosity

<table>
<thead>
<tr>
<th>Subject*</th>
<th>Sex/Age (y)</th>
<th>DEAE-Cellulose Chromatography</th>
<th>PolyCAT HPLC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>B(_2) (%), A(_2) (%), Total (%)</td>
<td></td>
</tr>
<tr>
<td>Family K (Hb B(_2) in cis, ( \beta )-thal mutation, codon 47 (+A))</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S.K. (IV-1)</td>
<td>F/36</td>
<td>1.98</td>
<td>2.51</td>
</tr>
<tr>
<td>H.R. (III-4)</td>
<td>M/64</td>
<td>2.52</td>
<td>2.72</td>
</tr>
<tr>
<td>E.C.R. (III-2)</td>
<td>F/60</td>
<td>1.71</td>
<td>2.36</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td>2.07</td>
<td>2.53</td>
</tr>
<tr>
<td>Family R (Hb B(_2) in trans, ( \beta )-thal mutation, ( -88 ) (C ( \rightarrow ) T))</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E.R. (II-2)</td>
<td>M/39</td>
<td>2.17</td>
<td>4.39</td>
</tr>
<tr>
<td>Second collection</td>
<td></td>
<td>2.46</td>
<td>4.75</td>
</tr>
<tr>
<td>Family M (Hb B(_2) in trans, ( \beta )-thal mutation, 1,393-bp deletion)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L.M.</td>
<td>M/54</td>
<td>1.77</td>
<td>4.75</td>
</tr>
<tr>
<td>Controls (( \beta )-thal absent)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S.B.</td>
<td>F/6</td>
<td>1.36</td>
<td>1.60</td>
</tr>
<tr>
<td>L.B.</td>
<td>F/6</td>
<td>1.29</td>
<td>1.50</td>
</tr>
<tr>
<td>D.C.</td>
<td>F/37</td>
<td>1.24</td>
<td>1.49</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td>1.30</td>
<td>1.53</td>
</tr>
<tr>
<td>D.M.</td>
<td>F/13</td>
<td>1.51</td>
<td>1.68</td>
</tr>
</tbody>
</table>

Abbreviation: ND, not determined.

*Numbers between parentheses identify the numbers in the pedigrees as published in 1961\(^7\) and 1963.\(^3\) The controls S.B. and L.B. are the twin daughters of E.R. of family R. The control D.M., who is the daughter of L.M., has an additional Hb S heterozygosity; the value for Hb A\(_2\) with the polyCAT HPLC procedure may be slightly too high because of contamination with a minute quantity of a modified Hb S. From www.bloodjournal.org by guest on September 24, 2017. For personal use only.
\( \beta \)-thal heterozygotes with the same mutation was 5.4% ± 0.4%, with a range of 4.5% to 6.6% (unpublished data). The level of Hb B2 in L.M. of family M, with the 1,392-bp deletion, was 2.51% and that of Hb A2 was 5.47%, an increase of approximately 80% and of 270% over the values for Hb B2 and Hb A2, respectively, in the Hb B2 heterozygotes. The level of 7.98% for \( \delta \) chain containing Hb types is similar to values observed for other patients with the same deletion.6,10,13 The values for Hb A2 and Hb B2 in the one nonthalassemic member of this family who also has an Hb S heterozygosity were nearly the same.

**DISCUSSION**

The results obtained in this study, summarized in Table 1, suggest the following. First, simple Hb B2 heterozygotes have nearly equal levels of Hb A2 and Hb B2, indicating that the 
\( G \rightarrow C \) substitution at codon 16 of the \( \delta \) gene (Gly \( \rightarrow \) Arg in the \( \delta \) chain) does not affect the relative synthesis of this variant chain and, thus, the presence of this \( \delta \) gene marker offers an opportunity to study the relative formation of Hb A2 derived from \( \delta \) chains in cis or in trans to a \( \beta \)-thal mutation. The one child of family M with Hb B2 and Hb S heterozygosities also had closely similar Hb A2 and Hb B2 values. Second, the nearly equal values for Hb A2 and Hb B2 in family K with the frameshift at codon 47 (\( +A \)) confirm earlier data1 and suggest that mutations within the \( \beta \)-globin gene do not influence the relative synthesis of \( \delta \) chains derived from \( \delta \) genes in cis or in trans to the \( \beta \)-thal gene but only the total Hb A2 formation. Third, the high level of Hb A2 of nearly 5.5% in a subject with the deletional \( \beta \)-thal who has a \( \delta \)-B mutation in trans and a Hb B2 level of 2.5% indicates that the excess Hb A2 is derived from \( \delta \) chains in cis to the \( \beta \)-thal deletion. Finally, the high level of Hb A2 (nearly 4.5%) in a patient who had the –88 C \( \rightarrow \) T mutation and the Hb B2 mutation in trans (the Hb B2 value was 2.5%) showed that this mutation in the CACC box of the 5'\( \beta \) promoter increases Hb A2 formation from \( \delta \) chains in cis to this \( \beta \)-thal mutation. There are considerable differences between the Hb A2 and Hb B2 percentages found for this person; in 1963, at the age of 11 years, the values were Hb A2 2.70% and Hb B2 1.80% and, in 1990, at the age of 39 years, the average Hb A2 and Hb B2 values by DEAE-cellulose chromatography were 4.57% and 2.32%, respectively. The considerably lower values found in the earlier study might be due to a coexisting iron deficiency, although this possibility was not evaluated at that time.

These data suggest that at least two mechanisms can be responsible for higher levels of Hb A2 in \( \beta \)-thal heterozygotes. One general mechanism concerns the relative excess of \( \alpha \) chains in \( \beta \) chain deficiencies that is present in all types of \( \beta \)-thal, albeit at different levels dependent on the type of \( \beta \)-thal. The second mechanism appears to be loss of \( \beta \)-globin gene promoter activity. Popovich et al12 were the first to suggest that the transcription of the \( \delta \)-globin gene is affected by loss of the 5'/\( \beta \) promoter, perhaps because the 5'\( \beta \) and 5'\( \delta \) promoters are influenced by the same 3'\( \beta \) enhancer. The data presented here support this hypothesis and suggest that a mutation in the proximal CACC has a nearly similar effect as the loss of the entire 5'\( \beta \) promoter. It may be that loss of (or decreased) binding of a specific protein or a complex of proteins to this segment of DNA results in a decrease in or absence of \( \beta mRNA \) formation and, indirectly, in an increase in \( \delta mRNA \) formation because protein binding at the CACC box of the 5'\( \delta \) promoter is not affected. Such a difference would not exist when the \( \beta \)-thal mutation is outside this promoter region and the formation of \( \beta mRNA \) (although ineffective) is not influenced by this mutation. Another mechanism explaining the high \( \delta \) chain production is that the deletion of the \( \beta \)-globin gene promoter or the mutations in the TATA (–29, A \( \rightarrow \) G) or CACC (–88, C \( \rightarrow \) T) boxes

![Fig 1. Identification of the frameshift at codon 47 (+A) of the \( \beta \) chain leading to a \( \beta \)-thal. (Left) Part of a sequencing gel locating the frameshift at codon 47. (Right) Confirmation of the codon 47 (+A) frameshift by hybridization of the amplified DNA with \( 32P \)-labeled probes.](image)
THE LEVELS OF Hb A₂ IN DIFFERENT β-THAL TYPES

partially removes competition for limiting transcription factors, which makes these more readily available to the δ-globin gene promoter. However, this would affect equally the promoters of both δ-globin genes and not only the δ-globin gene in cis to the β-thal allele.

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