Molecular Analysis of Japanese δβ-Thalassemia

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A DNA fragment containing the deletion junction region from a Japanese individual with homozygous δβ-thalassemia has been cloned. A clone containing the normal DNA surrounding the 3' breakpoint of this deletion and a clone carrying the Gγ- and Aγ-globin genes of this patient were also isolated. Sequences of the deletion junction and both γ-globin genes were determined. A comparison of these sequences with previously determined sequences of the normal counterparts revealed that the 5' breakpoint is located between 2,134 and 2,137 base pairs (bp) 3' to the polyA site of the Aγ-globin gene, the 5' breakpoint is located just downstream of the 3' border of the fetal γ-globin duplication unit, and no molecular defects are evident within the γ-globin gene region. A comparison between the sequences of the normal DNA surrounding the 3' breakpoint and the normal DNA surrounding the 5' breakpoint shows that deletion is the result of a nonhomologous recombination event. There are A + T-rich stretches near the 5' and 3' breakpoints in the normal DNA, and a portion of an Alu repeat is located in the region 3' to the 3' breakpoint. Southern blot analysis using probes 3' to the β-globin gene showed that the deletion extends in the 3' direction further than any other deletions associated with δβ-thalassemia and hereditary persistence of fetal hemoglobin (HPFH) heretofore reported. These results are discussed in terms of the mechanism generating large deletions in mammalian cells and three models for the regulation of γ-globin and β-globin gene expression in humans.

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probe. All DNA sequences were determined by the dideoxy chain termination method. DNA sequences were analyzed using Hitachi HIBIO DNASIS and GENAS software.

RESULTS

Hematologic data. The proband is a 30-year-old Japanese woman with moderate microcytic and hypochromic anemia (Table 1). She also had slight icterus and splenomegaly. The blood film showed moderate anisocytosis and poikilocytosis of the RBCs. Studies of liver function showed the following: GOT 24 U, GPT 16 U, LDH 275 U, and total bilirubin 4.3 mg/dL, all findings consistent with a diagnosis of thalassemia intermedia. Electrophoretic studies of the RBC lysates showed only HbF and a complete absence of Hbs A and A2. The absence of δ and β chains was confirmed by chromatographic analysis of globins on CM cellulose; only α and γ chains were demonstrated. After incubation of the peripheral blood cells of the proband for two hours with [3H]-leucine, the cells were lysed and the consistent globin chains were separated by CM cellulose chromatography. The total radioactivity incorporated into the α and γ chains and the specific activities of the chains were determined. There was a marked imbalance of globin chain production, as reflected in a γ/α chain production rate of 0.44 (Table 1).

The proband, a product of a consanguineous marriage and her 8-year-old son were examined. Hematologic and hemoglobin analytic data are shown in Table 1. The son had a slight anemia and morphologic changes in the blood cells compatible with a mild form of thalassemia. According to the results of the acid elution test, the HbF was distributed unevenly among the son's RBCs. These results indicate that the proband is homozygous and the son is heterozygous for β-thalassemia. Usually, the hematologic abnormalities of heterozygous for β-thalassemia has a more severe microcytosis and hypochromia than the proband. To explain this discrepancy, the proband is homozygous and the son is heterozygous for δβ-thalassemia (Jp-thal) deletion was located between the EcoRI site 2.7 kb 3' to the Ay-γ-globin gene and the BglII site 0.5 kb 5' to the ββ-globin gene, and extends in the 3' direction deleting the β-β, β- and ββ-globin genes. With a γ-globin gene probe, genomic mapping data identified a novel 8.5-kb BamHI fragment present in DNA from the proband, indicating that this BamHI fragment spans the Jp-thal deletion. To isolate a clone containing this fragment, recombinant phages containing size-fractionated BamHI fragments from the proband were screened with the Hind probe (Fig 1a). Two hybridizing phages were isolated; both contained the 8.5-kb BamHI fragment identified by genomic mappings. A restriction map of one of two phages, JpT-1, is shown in Fig 1b. JpT-1 contains the fragment expected for a clone spanning the deletion junction. A comparison of the map of this clone with the map of the corresponding region of the normal individuals (Fig 1a) demonstrated that the EcoRI site normally present 2.7 kb downstream of the Ay-γ-globin gene was absent, but that a novel EcoRI site appeared 2.6 kb 3' to the Ay-γ-globin gene in JpT-1, indicating that the 5' deletion point was located in the region between the novel EcoRI site and the HindIII site located 1.1 kb downstream of the Ay-γ-globin gene in both the normal clone and the JpT-1. Our previous study on the proband indicated that the 2.3-kb EcoRI fragment normally located in the 3' flanking region of the Ay-γ-globin gene was present in the DNA. One possible explanation for this discrepancy is that there is only a 0.1 kb difference in the size of the EcoRI fragment located 3' to the Ay-γ-globin gene between the DNA's of a normal individual and the proband. The 2.2-kb, 0.6-kb, and 1.4-kb EcoRI fragments were subcloned and a region of ~1.4 kb including the deletion junction (Fig 1b) was sequenced.

Isolation of a normal clone containing the 3' breakpoint. To clone normal DNA surrounding the Jp-thal 3' breakpoint, the 1.4-kb EcoRI fragment containing single copy sequences (Fig 1b) was isolated from phase JpT-1. This fragment was used as a probe. When a Charon 4A phage library of partially digested HaeIII/A1uI fragments was screened with this EcoRI fragment, one clone, N3', containing a 14.6-kb insert was isolated. Figure 1c is a restriction enzyme map of this clone, which also includes the 3' breakpoint of the Jp-thal deletion. The 3.0-kb EcoRI fragment located near the 5' end of this insert was subcloned, and the DNA sequence of the 742-base pair (bp) region (Fig 1c), including the 3' breakpoint, was determined.

Comparison of the sequences involved in deletion formation. We compared the DNA sequences of the Jp-thal deletion junction region with those of the regions containing the 5' and 3' breakpoints in the normal DNA. The normal 5'

Table 1. Hematologic Data and Hemoglobin Analysis for Proband (A) and Her Son (B)

<table>
<thead>
<tr>
<th>Individuals</th>
<th>A</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBCs (x 10^12/L)</td>
<td>4.85</td>
<td>6.04</td>
</tr>
<tr>
<td>Hb (g/dL)</td>
<td>12.0</td>
<td>12.8</td>
</tr>
<tr>
<td>MCV (fL)</td>
<td>76.0</td>
<td>67.0</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>24.7</td>
<td>21.2</td>
</tr>
<tr>
<td>MCHC (g/dL)</td>
<td>32.6</td>
<td>31.7</td>
</tr>
<tr>
<td>Reticulocytes (%)</td>
<td>2.4</td>
<td>1.0</td>
</tr>
<tr>
<td>Aγ:γ-A1:γ</td>
<td>38.9:61.1:0.0</td>
<td>50.7:49.3:0.0</td>
</tr>
<tr>
<td>HbA2 (%)</td>
<td>0.0</td>
<td>2.9</td>
</tr>
<tr>
<td>Hbf (%)</td>
<td>100</td>
<td>7-8</td>
</tr>
<tr>
<td>non α/α ratio</td>
<td>γ/α - 0.44</td>
<td>β/α - 0.73</td>
</tr>
</tbody>
</table>
DNA sequence is from the intergenic region of the A\(\gamma\)- and \(\psi\)\(\beta\)-globin genes\(^{27}\) and the normal 3' DNA sequence is from phage N3\(^{3}\). When the sequences were aligned, as in Fig 2, and analyzed using software, no homology between the normal 5' and 3' DNAs was found. A comparison of the normal 5' and deletion spanning sequences showed that the 5' breakpoint was 2,134 or 2,137 bp 3' of the polyA site of the A\(\gamma\)-globin gene.

The normal DNAs in the vicinity of the 5' and 3' breakpoints were examined for distinctive features that might have facilitated the deletion event. For convenience, we designated the nucleotide at the 5' end of the 3' normal sequence analyzed at the nucleotide level as position 1 (Fig 2). Many stretches of A+T-rich sequences are found around the junction of the 3' normal DNA and in the region of the 5' normal sequence around the deletion breakpoint. Positions 396 through 609 of the 3' normal sequence are part of the Alu family repeat\(^{18}\) and position \(-\) 120 through \(-\) 152 of the 5' normal sequence is homologous to the 3' end of the L1 family sequence.\(^{19}\) No significant direct repeats and inverted repeats could be detected in the 5' and 3' normal DNAs and there were no LTR-like sequences.\(^{20}\) When 500-bp sequences surrounding each breakpoint were analyzed for possible secondary structures, we found no structures significantly more stable than those in several other regions.

Isolation of a clone containing the Gy- and A\(\gamma\)-globin genes of Japanese \(\delta\beta\)-thalassemia. To eliminate the possibility of a molecular defect within the \(\gamma\)-globin gene, which reduces synthesis of the \(\gamma\)-globin chain, a Charon 4A partial EcoRI library constructed from the proband was screened, using the Hin \(\gamma\) probe. One clone containing both Gy and A\(\gamma\) genes was obtained. We determined the nucleotide sequence that extends from 200 bp from 5' of the cap site to 150 bp 3' of the polyA site of both \(\gamma\)-globin genes of this phage clone. Comparison of the nucleotide sequence with that of the known sequence of the Gy\(\gamma\)-globin and A\(\gamma\)-globin genes\(^{17}\) revealed no molecular defects, including deletion, as well as point mutations that cause abnormalities in RNA transcription, RNA processing, or premature termination of translation (data not shown).

Relation of the 3' breakpoints of the Japanese \(\delta\beta\)-thalassemia and other types of HFPFH and \(\delta\beta\)-thalassemia. The Jp-thal deletion extends an unknown distance 3' to the \(\beta\)-globin gene into a region not previously characterized. Several deletions which extend far beyond the \(\beta\)-globin gene cluster are shown in Fig 3. To localize the 3' endpoint of the Jp-thal, we mapped the normal DNA encompassing the 3' breakpoint, using probes HFPFH-3D and 1.3 derived from the normal 3' flanking DNA located \(-\) 90 kb and \(-\) 100 kb downstream from the \(\beta\)-globin gene, respectively.\(^{9,10,21}\) Genomic DNAs from the proband and two normal subjects (N-1 and N-2) were digested with PvuII, PstI, and BclI/PstI and blot hybridized with either HFPFH-3D or 1.3 probes. In each blot analysis, the DNA fragment (probe 5'\(\gamma\)) specific to the human \(\gamma\)-globin sequence was used as a probe to eliminate the possibility of an unsatisfactory DNA preparation (Fig 4).

When the DNA from normal individuals was used, the probe HFPFH-3D hybridized to a 7-kb PvuII fragment and the probe 1.3 hybridized to 20-kb PstI and 6-kb BclI/PstI fragments. In the DNA from N-2, intensity of the 6-kb BclI/PstI band is less than that of N-1 and a 20-kb band is visible. Only the 20-kb fragment is visible in the DNA from both N-1 and N-2, digested with PstI, thereby indicating that the BclI site on the 20-kb PstI fragment is polymorphic. On the other hand, no specific fragments hybridized to either probe HFPFH-3D or 1.3 (7 kb for PvuII digests, 20 kb for PstI digests, and 20 or 6 kb for PstI/BclI digests) when the DNA of the proband was used. The 5'\(\gamma\) probe hybridized to the fragment of the expected size in the three subjects. We also analyzed the 3' breakpoint of the Jp-thal using probe pRK29 located \(-\) 18 kb downstream from the \(\beta\)-globin gene. No specific bands were observed when the DNA from Jp-thal was used (data not shown). The 3' endpoints of the deletion associated with HFPFH-1 and Spanish \(\delta\beta\)-thalassemia are located just 5' to the probes HFPFH-3D and 1.3, respectively.\(^{9,10}\) These results indicate that the Jp-thal deletion extends through the 3' breakpoint of the Spanish \(\delta\beta\)-thalassemia deletion as well as the HFPFH-1 deletion. Thus, the 3' breakpoint of Jp-thal deletion is more distant from the
\[ \text{\textbeta-globin gene cluster than that of any other deletion associated with HPFH and \textdelta-thalassemia.} \]

\textbf{DISCUSSION}.

The proband we studied had a slight anemia, RBC indices and morphologic changes typical of thalassemia, 100% HbF in the peripheral blood, and an imbalance in the \(\alpha\)-globin and \(\gamma\)-globin chain synthesis. Her son is a heterozygote with a small deletion in the \(\gamma\)-globin gene cluster. Based on these results, the proband appears to be homozygous for \(\delta\)-thalassemia. This condition results from a large deletion starting within the \(\beta\)-globin gene cluster and extending to the 3' flanking region of the \(\beta\)-globin gene. To elucidate the mechanisms involved in chromosomal rearrangements in mammalian cells and to identify regions important in the control of \(\gamma\)-globin gene expression, we performed further molecular analyses of the Jp-thal. We found that the Jp-thal deletion was generated by a nonhomologous breakage and reunion event. A + T-rich stretches were found near the 5' and 3' breakpoints in the normal DNA. Wavy lines show A + T-rich stretches in the 5' normal DNA.

\[ \text{There are A + T-rich stretches around the junction of the 3' normal DNA: a segment 21 - 160 (78\% A + T), a segment 610 - 724 (70\% A + T), and a segment 1,019 - 1,150 (79\% A + T). Many stretches of A + T-rich sequences are also found in the region of the 5' normal sequence around the deletion breakpoint: a segment 490 - 530 (79\% A + T), a segment 300 - 331 (74\% A + T), and a segment 231 - 260 (73\% A + T). Broken arrow shows a portion of an Alu family repeat and its direction. Solid arrow indicates the short L1 family homologous region. Positions 396 through 609 of the 3' normal sequence are part of the Alu family repeat and position 120 through 152 of the 5' normal sequence is homologous to the 3' end of the L1 family sequence.} \]
stream of the 3' breakpoint in the normal DNA. A 33-bp region homologous to the 3' end of the L1 family is found ~330 bp upstream of the 5' breakpoint in the normal DNA. In HPFH-1,25 HPFH-3,26 and Spanish δβ-thalassemia,27 the recombinational events occurred within the Alu sequences. The short L1 family homologous region mentioned above is known to demarcate the ends of the fetal globin gene duplication units.1 The similar region is also found downstream of the Gγ-globin gene. The 5' breakpoint of Jp-thal was located in the region just downstream of the 3' end of this duplication unit. Shen et al28 suggested that the region near the 3' end of this duplication unit would be preferred substrates of chromosomal rearrangement. However, the breakpoint of Jp-thal is not precisely located within Alu or L1 sequences, and all of these sequence features are very common in most intergenic DNA. Therefore, their location near deletion breakpoints could be coincidental.

Vanin et al29 proposed that the deletion of similarly sized loops of DNA anchored to the nuclear matrix may account for some chromosomal rearrangements associated with some cases of HPFH and of δβ-thalassemias. Recently, matrix association regions (MARs) were identified in several instances and were found to be A + T-rich DNA segments associated with topoisomerase II and control elements of the gene expression.30,31 Cockerill and Garrard proposed that MARs play fundamental roles in the functional organization of chromatin loop domains.31 In the Jp-thal deletion, the regions around the 5' and 3' breakpoints in normal DNA are A + T-rich, and the 5' breakpoint could be located near the border of two functional domains: γ-domain and δβ-domain.32,33 Moreover, the 5' breakpoint is located ~1 kb downstream of an enhancer-like sequence of the Aγ-globin gene35 (Fig 3). These findings suggest that MARs exist near the regions in which the 5' and the 3' breakpoints of Jp-thal are located.

Studies of the extent of deletions associated with HPFH or thalassemia have led to three main hypotheses to explain the different expressions of the fetal globin genes in these two conditions. In Fig 3, the extent of deletions of HPFH-1,9,21,25 HPFH-2,29 Spanish δβ-thalassemia,10,27 and Chinese δβ-thalassemia36 are shown in comparison with that of the Jp-thal deletion. The first hypothesis suggests that a region between the ψβ-globin and δ-globin genes includes important control sequences that normally cause the shutdown of the fetal γ-globin genes in adults.37 The thalassemia phenotype observed in the Jp-thal patient is not consistent with this hypothesis because this putative regulatory area is deleted and no apparent molecular defects exist within the γ-globin gene region in Jp-thal. The second hypothesis suggests that DNA sequences located downstream of the normal β-globin cluster, but brought near the γ-globin gene by the deletion, are important in regulating the expression of the gene.38 The 3' breakpoint of the Jp-thal deletion in normal DNA is further distant from the β-globin gene cluster than that of the Spanish δβ-thalassemia deletion. The region considered to contain the enhancer-like element13,39 is brought close to the fetal globin gene in HPFH-1 and HPFH-239 but is deleted in the Jp-thal patient as well as in Spanish δβ-thalassemia. The absence of the region that enhances the γ-globin gene expression may explain the thalassemia phenotype of this patient. The third hypothesis proposes that fetal and adult globin gene chromatin domains have distinct 5' and 3' boundaries and that the persistent expression of HbF occurs in the Jp-thal patient as well as in Spanish δβ-thalassemia.32,33,34 The Jp-thal deletion is compatible with this hypothesis because it may remove the domain containing the adult δ-globin and β-globin genes and one of the two boundaries of the fetal globin domain.

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