A patient with homozygous β thalassemia of German/Italian descent was found to be doubly heterozygous for the common IVS1-110 G → A mutation of the β globin gene and for a novel C → T mutation of the proximal CACCC-box of the β globin gene promoter at position −87 relative to the transcription start site (cap). Transcription analysis in an HeLa cell transfection assay indicated a 45% to 51% residual activity of the gene with the −87 C → T mutation relative to normal, further underlining the physiologic role of the affected promoter element. The finding of an only moderately reduced transcriptional activity of the β globin gene with the −87 C → T mutation corresponds well with the clinical phenotype of the reported patient, which is characterized by a late onset of symptoms, moderate anemia, and normal physical development. The ethnically German mother of the propositus has minimal anemia with only slightly changed red blood cell indices, which can also be explained by the relatively high residual activity of the gene with the −87 C → T mutation.

© 1991 by The American Society of Hematology.
a viral infection of the upper respiratory tract and an anemia of 7.3 g/dL, requiring blood transfusion. For the following 10 years the transfusion frequency was about twice per year. On these occasions his Hb decreased from a steady state of between 8.5 and 9.5 g/dL to between 5.5 and 6.5 g/dL, usually at the time of a viral infection. The patient’s physical and sexual development proceeded normally. However, he did develop marked skeletal deformities of his face (Fig 1) and massive hepatosplenomegaly. During the past 2 years the transfusion frequency has been increasing to about six per year; this increase is probably related to hypersplenism.

An analysis of the ethnically German mother showed minimal anemia (Hb 12.4 g/dL, Hk 38%) with slightly hypochromic and microcytic red blood cell indices (MCV 84 fL, MCH 26%) and increased HbA2 (5.5%) and HbF (3.4%). The Italian father was not available for study.

RESULTS

α Globin genotype. α globin gene mapping demonstrated a normal complement of four α globin genes (αα/αα) in the propositus and his mother.

β Globin haplotype. The patient and his mother are both homozygous for the common Caucasian haplotype I.11 A detailed mapping of the β globin gene cluster did not show any abnormal restriction fragments. This normal gene map, together with the finding of two different β globin gene alleles (see below), excludes the presence of large deletions within the β globin gene complex.

β Globin genotypes. An initial analysis of PCR-amplified β globin gene fragments with oligonucleotide probes directed at the common Mediterranean β thalassemia mutations detected the IVS1 position 110 G → A mutation on one allele of the propositus, but was not found in the mother’s DNA (not shown). Therefore, this mutation is likely to be of paternal origin.

Direct sequencing of the patient’s PCR-amplified DNA showed the heterozygous inheritance of a thus far undescribed C → T mutation at position −87 relative to the transcription start site (cap) affecting the proximal CACCC promoter element (Fig 2). A pair of oligonucleotides containing the wild type or the mutated sequence at this position was synthesized and hybridized to independently amplified samples of the propositus and his mother. This analysis confirmed/demonstrated the presence of the −87 C → T mutation in both samples (Fig 3). The patient is thus doubly heterozygous for this novel mutation of the β globin gene promoter and one of the common Mediterranean β thalassemia mutations (−87 C → T/IVS1-110 G → A). Sequencing of the entire β globin gene contained in the vector used for transcription analysis showed the normal sequence except for the −87 C → T mutation.

The functional effect of the −87 C → T mutation was investigated by comparing the transcription of the mutated with the wildtype gene in transfected HeLa cells (Fig 4).
Normal β globin mRNA protects complementary sequences of 197 of the 767 nucleotides of the EcoRI/PstI β globin gene riboprobe. Fragments of normal length were detected by analysis of HeLa cells transfected with the wildtype and the mutated β globin gene. The accumulation of β globin and HSV1-TK RNA was quantified by scintillation counting of standardized slices excised from the polyacrylamide gel in four independent experiments. The relative intensities of the HSV1-TK signals were used to correct for different transfection efficiencies. The ratios of the thus corrected amounts of the β globin -87 C → T/wildtype RNA ranged from 0.45 to 0.51 in the four experiments (mean 0.48). The mutated β globin gene was thus estimated to be about 45% to 51% as active as the wildtype gene.

**DISCUSSION**

The promoter of the human β globin gene contains sequence motifs that have been highly conserved during evolution and are designated ATAAA-, CAAT-, and CACCC-boxes according to their conserved nucleotide sequence. The ATAAA-box is located 26 to 31 bp and the distal element from 101 to 105 bp 5' of the cap site. These promoter elements act in cis to bind transacting factors that are important for a regulated transcription of the β globin gene. Including the change reported here there are 10 known β thalassemia mutations located in the promoter area. Six affect the ATAAA-box and result in a moderate reduction of transcriptional activity in transfected cells (Table 1). However, the phenotype of affected patients appears variable. The -29 A → G, the -30 T → A, and the -31 A → G mutations are associated with a relatively mild clinical picture in homozygous patients, whereas the -28 A → C mutation was found in a patient with transfusio-dependent thalassemia major (Table 1). The reason for the discrepancy between the phenotypic effect and the behavior of the -28 A → C gene in transfected cells is not clear. A mutation at position -101 relative to cap, ie, in the distal CACCC-box, results in a particularly mild form of β thalassemia that does not produce any hematologic abnormalities in the heterozygote (silent β thalassemia), and in compound with another more severe β thalassemia mutation produces only moderate anemia.14,29

Mutations of the proximal CACCC-box have been described at position -87 (C → G)11 and at position -88 (C → T).13 The -88 mutation and the -87 C → G mutations are associated with a mild clinical phenotype of homozygous β thalassemia.30,31

Transcription analysis of these mutated genes indicated a residual activity of between 20% and 30% relative to normal.13 The -87 C → T mutation of the proximal CACCC-box described here results in a residual activity of about 45% to 51% relative to a normal gene in an HeLa cell transcription assay. This finding correlates with the remarkably mild clinical phenotype in the patient with homozygous β thalassemia and with the only slight hematologic changes in his heterozygous mother. However, it was surprising to find an HbA2 level of 5.5% in the mother, as the increased HbA2 in heterozygous β thalassemia is thought to be due to the lack of β chains, thus promoting the low-affinity binding of excess α with δ globin chains. Therefore, one might have expected a lower HbA2 level in a heterozygote for the -87 C → T mutation.

The differences in the behavior between genes carrying the -87 C → T or C → G mutations is a further example for the variable effect of different mutations at the same site. It is conceivable that the transversion of a purine base for a

**Table 1. Effect of β Globin Gene Promoter Mutations on the Clinical Phenotype and the Transcriptional Activity in a Transfection Assay**

<table>
<thead>
<tr>
<th>Promoter Mutation (ref.)</th>
<th>Transcriptional Activity in a Transfection Assay</th>
<th>Clinical Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>-28 A → C (8,9)</td>
<td>≈ 30%</td>
<td>Thalassemia major</td>
</tr>
<tr>
<td>-28 A → G (12)</td>
<td>20–30%</td>
<td>Not reported</td>
</tr>
<tr>
<td>-29 A → G (10)</td>
<td>25%</td>
<td>Thalassemia intermedia</td>
</tr>
<tr>
<td>-30 T → A (15)</td>
<td>Not reported</td>
<td>Thalassemia intermedia</td>
</tr>
<tr>
<td>-30 T → C (33)</td>
<td>Not reported</td>
<td>Not reported</td>
</tr>
<tr>
<td>-31 A → G (14)</td>
<td>45%</td>
<td>Thalassemia intermedia</td>
</tr>
<tr>
<td>-87 C → G (11,12,30)</td>
<td>20–30%</td>
<td>Thalassemia intermedia</td>
</tr>
<tr>
<td>-87 C → T (this report)</td>
<td>45–51%</td>
<td>Thalassemia intermedia</td>
</tr>
<tr>
<td>-88 C → T (13,31)</td>
<td>20–30%</td>
<td>Thalassemia intermedia</td>
</tr>
<tr>
<td>-101 C → T (16,29)</td>
<td>30% in a Friend cell CAT promoter assay</td>
<td>Silent β thalassemia</td>
</tr>
</tbody>
</table>
pyrimidine in the case of the C → G mutation has a more profound effect on the tertiary structure of the DNA than the C → T transition. A similar example among the β thalassemia mutations can be found at position 5 of the first intron where transversions of the normal G by a C or a T almost completely inactivate the gene, whereas a G → A transition results in only moderate reduction of splicing efficiency and a mild clinical phenotype.

The cluster of four CACCC-box mutations underlines the likely physiologic significance of that sequence motif in β globin gene expression. It is notable in this context that the promoter of the poorly expressed β globin gene does not contain this element. It is also remarkable that all the known mutations of the proximal CACCC-box, including that described here, have a more marked effect on β globin gene activity than the −101 mutation of the distal CACCC-box, probably reflecting a more important functional role of the proximal element.

ACKNOWLEDGMENT

We gratefully acknowledge the continuous support by Professors C.R. Bartram, B. Kubanek, and H. Seliger during our work. We also thank Anja Fröhlich for synthesizing the oligonucleotides, and H. Barro and A. Jacobs for editing the manuscript.

REFERENCES

and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. Nucleic Acids Res 12:7035, 1984


Thalassemia intermedia: moderate reduction of beta globin gene transcriptional activity by a novel mutation of the proximal CACCC promoter element

AE Kulozik, A Bellan-Koch, S Bail, E Kohne and E Kleihauer