A New Mutation in IVS-1 of the Human β Globin Gene Causing β Thalassemia Due to Abnormal Splicing

By George F. Atweh, Corinne Wong, Robin Reed, Stylianos E. Antonarakis, Ding-er Zhu, Prabhakat K. Ghosh, Tom Maniatis, Bernard G. Forget, and Haig H. Kazazian, Jr

A G to T transversion at the fifth nucleotide of the first intervening sequence (IVS-1) of the β-globin gene has been identified in cloned β-thalassemia genes of two unrelated individuals, one of Mediterranean and the other of Anglo Saxon ancestry. In each patient the mutation was present in a different β globin gene framework, defined by intragenic restriction site polymorphisms, thereby suggesting the occurrence of independent mutations. The study of the RNA products of one of these cloned genes, after transfer and transient expression in HeLa cells, showed partial inactivation of the normal donor splice site of IVS-1 and activation of two major and one minor cryptic splice sites. Only one of the two major cryptic sites was utilized in a cell-free splicing extract. The effects of this mutation on messenger RNA (mRNA) splicing are similar to that of another β thalassemia gene with a G to C transition at the same positions.

RESULTS

Cloning and sequence analysis of β-thalassemic genes. Haplotype analysis of the Mediterranean patient revealed heterozygosity for haplotypes Va and Vb, which differ only at the polymorphic Hind III site S′ to the β gene2 (Fig 1). One of the two β-thalassemia genes from this patient was studied earlier by Treisman et al15 and was found to contain a G to A transversion at IVS 2 position 1. This mutation abolishes the Hph I restriction endonuclease site normally present at the exon 2/IVS-2 junction and is commonly associated with haplotype Vb.5 Haplotype Va, on the other hand, is commonly associated with a G to T substitution at IVS-1 position 1. Hybridization studies using synthetic oligonucleotides ruled out the presence of this mutation in the patient’s DNA (data not shown). The β-thalassemia gene from the haplotype Va chromosome was cloned and identified by the retention of the Hph I recognition site normally present at the IVS-2 donor splice site. Sequence

From the Hematology Section, Department of Medicine, Yale University School of Medicine, New Haven, CT; the Pediatric Genetics Unit, The Johns Hopkins University School of Medicine, Baltimore; and the Department of Biochemistry and Molecular Biology, Harvard University, Cambridge, MA.

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Address reprint requests to Bernard G. Forget, Hematology Section, Department of Medicine, Yale University School of Medicine, New Haven, CT 06510.

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HAPLOTYPe ANALYSIS

| Patient 1 | Haplotype Va | - | - | - | + | + | - |
| Patient 2 | Haplotype Vb | - | - | - | - | - | - |
| Patient 2 | Haplotype I  | - | - | - | - | + | + |

Fig 1. Results of haplotype analysis in the two patients. Haplotype Va is commonly associated with a G → T substitution at IVS1 position 1; haplotype Vb is commonly associated with a G A substitution at IVS-2 position 1; and haplotype I is often associated with either a G → A transition at IVS-1 position 110 or β-nonsense 39.

analysis of the promoter region, the three exons, and all intron-exon junctions of this gene revealed, as the only abnormality, a G to T transversion at IVS 1 position 5 (Fig 2).

Haplotype analysis was also performed on a nuclear family of Northern European ancestry. The thalassemia allele was of haplotype I (Fig 1) and was inherited from both parents, who were first cousins, facilitating cloning of the β-globin gene from the homozygous patient. The two most frequent mutations associated with this commonest Mediterranean chromosome background are the G to A transition at IVS-1 position 110 and the β-39 nonsense mutation. Both mutations were shown, by oligonucleotide hybridization, to be absent in this patient (data not shown), thereby making this gene a candidate for a new mutation. Nucleotide sequence analysis using synthetic oligonucleotide primers revealed a G to T substitution at IVS-1 nucleotide 5, the same mutation identified in the first patient described above.

Gene transfer and gene expression studies in HeLa cells. Processing of RNA transcribed from the Mediterranean β-thalassemic gene was studied by transfecting in parallel a normal β-globin gene and the thalassemic gene, both cloned in the expression vector pSVplac, into cultured HeLa cells. The cytoplasmic RNA was isolated and subjected to S1 mapping studies using a uniformly labeled single-stranded probe that extends from the promoter of the β-globin gene to the Eco RI site in exon 3 (Fig 3A). RNA transcribed from the normal β-globin gene protects a 223-nt fragment corresponding to exon 2 and a 143-nt fragment corresponding to exon 1. The 49-nt fragment corresponding to exon 3 can only be seen on high percentage gels (data not shown). RNA transcribed from the β-thalassemic gene with the G to T mutation at IVS-1 position 5 (IVS1-5*) protects normal amounts of the 223-nt fragment corresponding to exon 2, decreased amounts of the 143-nt fragment corresponding to exon 1, and two additional fragments, 128 and 105 nt in size. Transfection of HeLa cells with a different cloned β-thalassemic gene containing a G to C mutation at IVS-1 position 5* gave a qualitatively similar pattern of S1 nuclease protection (data not shown). A similar pattern was also obtained using reticulocyte RNA from the Mediterranean patient (data not shown), thus demonstrating that the same splicing defects occur in vivo; an additional band of 270 nt was also detected corresponding to the alternatively spliced β-globin mRNA from the patient's other β-thalassemic gene carrying a point mutation at IVS-2 position 1.16

Splicing at the IVS-1 donor splice site was also evaluated using a 3' end-labeled Hgi Al fragment (Fig 3B) as a probe.

β-GLOBIN GENE

Normal CAGTTGGT
Thalassemia CAGTTGGT

Fig 2. Side-by-side comparison of the DNA sequence of the Mediterranean β-thalassemic gene from haplotype Va and a normal β-globin gene in the region of the IVS-1 donor splice site. The mutated nucleotide (G) is denoted by (*). The schematic diagram of the β-globin gene shows the position of this mutation at the fifth position of IVS-1. The GT invariant dinucleotides of the donor splice site are underlined.
Fig 3. Analysis of RNA resulting from transient expression of β-globin genes after transfer into HeLa cells. (A) S1 nuclease mapping study of RNA transcribed from the IVS1-5T β-thalassemic gene and a normal β-globin gene using a uniformly labeled single-stranded probe represented on the schematic map below the autoradiograph. The 223-nt fragment (exon 2) and the 143-nt fragment (exon 1) are seen in the normal (NI) lane. Additional fragments (128 nt and 105 nt) are seen in the IVS1-5T lane. (B) S1 nuclease mapping of the same RNAs and RNA from the IVS 1-5C gene using a 3' end-labeled probe represented on the schematic map below the autoradiograph. A 90-nt fragment is seen in the NI lane that corresponds to the part of exon 1 complementary to the probe. Both IVS1-5T and IVS1-5C lanes show decreased amounts of the 90-nt fragment in addition to three other fragments (102, 74, and 52 nt); a fourth 114nt fragment of unknown significance is present in all lanes. (C) cDNA products of a primer extension study using RNA from the IVS1-5T gene or a normal gene (NI) and a primer from exon 2 represented on the map below the autoradiograph. The NI lane shows the expected 226-nt cDNA product, while the IVS1-5T lane shows decreased amounts of the 226-nt product in addition to two abnormal products (210 and 188 nt).

In another S1 nuclease mapping experiment. The autoradiograph in Fig 3B shows the expected 90-nt fragment when RNA from the normal gene was used and four additional fragments when RNA from the IVS1-5T gene was used. The 52- and 74-nt fragments correspond to the aberrantly spliced RNAs that generated the fragments of 105 and 127 nt respectively in Fig 3A. These protected fragments result from the utilization of cryptic splice sites at codons 18 and 25 in exon 1. The 102-nt fragment is consistent with the utilization of a cryptic splice site at position 12 of IVS-1. This donor splice site is utilized much less efficiently than the cryptic splice sites in exon 1. The fragment of 114 nt was seen with the normal β gene as well as the β-thalassemic gene. The significance of this fragment is not clear; however, its size is consistent with a splicing event at a cryptic donor splice site at position 24 of IVS-2. A qualitatively similar pattern of splicing was obtained with the β-thalassemic gene containing a G to C mutation at IVS-I position 5 (IVS1-5C) (Fig 3B, lane 3). The difference in intensity of the bands between RNA from IVS1-5T (lane 2) and that of IVS1-5C (lane 3) is probably due to differences in transfection efficiency of the two genes in the particular experiment shown.

RNA processing was further evaluated by a primer extension study using a 5' end-labeled synthetic oligonucleotide, complementary to the mRNA sense-strand of exon 2, as a primer in the reverse transcription reaction. The cDNA product of the RNA transcribed from the normal gene is 226 nt in size (Fig 3C). RNA from the IVS1-5T gene directed the synthesis of cDNA products of 226, 210, and 188 nt in size. The abnormal cDNA products (210 and 188 nt) have sizes consistent with mRNA abnormally spliced at the two cryptic splice sites within exon 1. The minor species thought to result from splicing at the cryptic splice sites within IVS-1 are not visualized in this study.

In vitro splicing in the cell-free extract. To determine the effects of the IVS1-5T mutation on the in vitro splicing of human β-globin pre-mRNA, we examined both normal and thalassemic SP6 precursors containing exon 1, IVS-1, and exon 2 in the HeLa cell nuclear extract. The results of this analysis are shown in Fig 4. Splicing products and intermediates were identified by comparison with the known species generated from the normal precursor. In addition, splicing intermediates were identified by examining the products generated when the in vitro reaction was carried out in the absence of KCl. Under these conditions only products from the first step of the reaction, exon 1 and the IVS-1/exon 2 lariat, are observed. These analyses revealed that the predominant splicing product generated from the IVS1-5T precursor is derived from utilization of the cryptic 5' splice site in codon 25 of exon 1. In addition, some normally spliced RNA is observed. However, by contrast with the in vivo...
at an adenine residue 37 nucleotides upstream from site, is also utilized when the cryptic 5' splice site is splice sites. Competitive interactions between the normal and mutant 5' splicing efficiency of the thalassemic precursor is less than 5' cryptic that of the normal precursor, probably as a consequence of activated, we localized the branchpoint in the mutant IVS-1. 

Using the previously developed branchpoint mapping technique, we found that the site of lariat formation occurs at the same position in both the mutant and normal introns (data not shown).

**DISCUSSION**

The Mediterranean patient studied here has severe transfusion-dependent β-thalassemia major resulting from a profound decrease in β-chain synthesis. One of the two β-globin genes from this patient was studied earlier by Treisman et al and was found to have a mutation that converts the GT invariant dinucleotide of the IVS-2 donor splice site to an AT and thus abolishes normal splicing completely at that site. Haplotype analysis of polymorphic restriction sites in the β-globin gene cluster in this patient revealed heterozygosity for haplotypes Va and Vb. Haplotype Va is the one commonly associated with the above GT to AT base substitution. Haplotype Va, on the other hand, is commonly associated with a G to T base substitution at IVS-1 position 1. Hybridization studies using synthetic oligonucleotides ruled out the presence of the latter mutation in this patient's DNA. DNA sequence analysis of the haplotype Va gene in our patient revealed a G to T transversion at IVS 1 position 5. This mutation is within the consensus sequence of the donor splice site, a region that is believed to be critical for accurate mRNA splicing.

The second patient in this study differs from the first patient in terms of geographic origins (Northern vs Southern Europe) as well as in haplotypes (I v Va; Fig 1) and β-globin gene frameworks (1 v 2). This patient also differs in that he is homozygous for the mutation, as his parents are first cousins. His clinical phenotype is also typical of transfusion-dependent β-thalassemia major. Despite the difference in chromosome backgrounds, however, both patients have the same mutation of RNA splicing. Both cases represent instances in which the expected mutation/haplotype correspondence was absent. As shown here and emphasized in another survey of seven thalassemia genes, close association of a specific mutation and a particular haplotype is not invariant. Several examples of mutation spread to different ethnic groups, and disparate chromosome backgrounds have been observed and have provided strong evidence for independent origins of the same mutation.

For the β' mutation to migrate from haplotype I to Va and framework 1 to 2, a crossing-over event between the mutation and IVS-2 position 74 is required. Occurrence of a recombination event within a short stretch of DNA is less likely than two independent origins of the same mutation in the two ethnic groups. It is of interest that of a relatively small number of β-thalassemia mutations (7 of 37 known mutations) in which recurrences have occurred, two are mutations at the same nucleotide, IVS-1 nt 5.

When we introduced the cloned Mediterranean thalassemic gene into HeLa cells using a transient expression vector and analyzed its mRNA products in S1 nuclease mapping experiments, we demonstrated decreased utilization of the usual IVS-1 donor splice site and utilization of at least three surrounding cryptic splice sites. Two of these cryptic splice sites are in exon 1 of the gene and one is in

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**Fig 4.** In vitro splicing of normal and IVS1-5' human β-globin precursors. 32P-labeled SP6 precursors were synthesized in vitro, spliced in the HeLa cell nuclear extract, and then fractionated on a 5% denaturing polyacrylamide gel. The structures of each of the RNA species are indicated adjacent to the autoradiogram. The boxes refer to exons, and the line indicates the intron. The closed loop indicates the lariat form of the intron. The mutant products and intermediates are designated by an asterisk. The unlabeled faint band seen in lane 2 (also barely visible in lane 1) results from exonuclease degradation of the small amount of uncapped mRNA in the reaction mixture. The sizes of the bands are as follows: unspliced precursor: 497 nt; exon 1/exon 2: 367 nt; and exon 1: 155 nt; the corresponding mutant products are shorter by 16 nt. Lane 1: Normal precursor; Lane 2: Precursor carrying IVS1-5' mutation, KCl in the splicing reaction; Lane 3: Precursor carrying IVS1-5' mutation, KCl absent from the splicing reaction.

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Results, no evidence was obtained for utilization in vitro of the cryptic 5' splice site at codon 18. Figure 4 also shows that the splicing efficiency of the thalassemic precursor is less than that of the normal precursor, probably as a consequence of competitive interactions between the normal and mutant 5' splice sites.

To determine whether the normal site of lariat formation, at an adenine residue 37 nucleotides upstream from the 3' splice site, is also utilized when the cryptic 5' splice site is activated, we localized the branchpoint in the mutant IVS-1.
IVS-1 mutation causing $\beta$ thalassemia

IVS-1. As illustrated in Fig 5, the majority of the RNA products that accumulated in HeLa cells were spliced at the two cryptic splice sites within exon I. These cryptic splice sites are also commonly utilized in other $\beta$-thalassemic globin genes that carry different mutations within the consensus sequence of the IVS-1 5' splice site, including a $\beta$-thalassemia gene isolated earlier from an Asian Indian patient that was shown to have a G to C transition at IVS-1 position 5, the same position that is mutated in the genes from our patients. Splicing of the mutant precursor mRNA in a cell-free extract also revealed decreased utilization of the normal splice site but, in contrast to the results in intact cells, only one of the cryptic sites in exon 1 was utilized (Fig 5). Similar differences in the relative amounts of spliced RNAs obtained from cryptic sites have been observed with most of the other thalassemia mutants that have been analyzed both in vivo and in vitro. This may be due to differences in the concentrations of the various factors in intact cells compared to that in the in vitro extracts.

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A new mutation in IVS-1 of the human beta globin gene causing beta thalassemia due to abnormal splicing

GF Atweh, C Wong, R Reed, SE Antonarakis, D Zhu, PK Ghosh, T Maniatis, BG Forget and HH Jr Kazazian