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The mRNA transcripts from a mutant β-globin gene derived from splicing at preferential cryptic sites

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We have analyzed mRNA transcripts from β-globin genes carrying a homozygous point mutation at the 5′ splicing site of the first intron, using a method allowing in vivo analysis of mRNA transcripts. As expected, this mutation decreases normal splicing of mRNA when cryptic splicing sites are utilized. We have observed that, in reticulocytes, most mature mRNA transcribed from β-globin genes derives from specific sites of abnormal splicing. Our results differ from those previously obtained using mutant β-globin genes introduced in cultured cells and indicate a preferential processing of the abnormal globin mRNA species in red cell precursors.

Thalassemia, β-; mRNA splicing

1. INTRODUCTION

The human β-globin gene provides a good model for understanding the mechanism of mRNA transcription and processing. Several point mutations in β-globin genes have been observed at the junction of exons and introns and particularly in the GT dinucleotide at the 5′ splicing site. Those mutations are responsible for β-thalassemia (reviewed in [1]). It is now well-established that the GT dinucleotide is necessary for correct splicing of mRNA precursors [2].

The most commonly used procedure to investigate the effect of mutation at the splicing sites has been the analysis of the expression of cloned mutant genes after introduction in cultured cells. This procedure has made it possible to show that mutation in the 5′ or 3′ splicing site consensus sequences results in abnormal splicing due to the activation of cryptic splicing sites (reviewed in [3]). This approach, however, does not take into consideration differences in tissue-specific factors between red blood cell precursors and transformed cells, as previously suggested [4,5].

In the present report, we studied the consequences of a point mutation observed on both alleles of a patient affected by β-thalassemia. This mutation lies in the GT dinucleotide of the splice junction of the first intron (IVS 1) of the β-globin gene (IVS 1-2 T ⟶ C). β-Globin cDNAs were prepared from reticulocytes and further amplified by polymerase chain reaction (PCR) [6] in order to determine the in vivo effect of the mutation on splicing and maturation of mRNA occurring in the red cell precursors of the patient.

2. MATERIALS AND METHODS

2.1. *Subject*

The patient was diagnosed, using conventional laboratory investigation, as having β-thalassemia. He was affected by a mild form of β-thalassemia, and was not regularly transfused. He had not been splenectomized. DNA analysis was performed. We determined that the molecular defect was a homozygous mutation (T⟶C) at position IVS 1-2.

2.2. *Oligonucleotide used*

For cDN synthesis and PCR amplification, two oligonucleotides (palm 4 = 5′-GCCCATAACAGCATCAGGAGT-3′ and palm C = 5′-GCTTCTGACACAACTGTGTT-

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CAC-3′) were synthesized on a Pharmacia gene assembler (Pharmacia LKB, Sweden). Palm 4 is complementary to the β-globin mRNA sequence of the second exon of the β-globin gene. The palm C sequence is localized in the first exon of the β-globin gene. Detection of β-globin mRNA transcripts was performed with an oligonucleotide commonly used to detect a normal coding region of the β-globin gene [8] that is located between the sites used for PCR amplification.

2.3. *Amplification and sequencing*

RNA of blood cells was prepared following lysis in guanidinium thiocyanate and cesium chloride purification [7]. cDNA was synthesized and further amplified according to the method described in [9] with the following modifications: cDNA was synthesized from 0.5 μg RNA using palm 4 as 3′ primers. After addition of 50 pmol each of palm 4 and palm C, PCR amplification was performed. Amplified products were separated on 5% acrylamide gels and excised from the gel. Gel pieces were scratched into 0.2–0.5 ml of 10 mM Tris/1 mM EDTA. After heating for 1–2 h at 50°C, 1 μl of elution mixture was used to generate single strand DNA after PCR amplification using unequal molar amounts of primers [10]: the mixtures containing 0.1 pmol of 5′ primer and 50 pmol of 3′ primer (or the reciprocal primer ratio) were subjected to 15–20 cycles of polymerisation-denaturation. The nucleotide sequences were determined using modified T7 DNA polymerase (sequenase USB) according to the manufacturer's recommendations.

2.4. *Analysis of PCR amplified products*

5–10 μl of mixture from cDNA amplification were run on 2% agarose gel and transferred to Biodyne B nylon membranes (Pall Industry, NY) in 0.2 N NaOH. Membranes were hybridized with $1–5 \times 10^6$ cpm of 5′ ^{32}P-labelled oligonucleotide and washed with 3 M tetramethylammonium chloride following the procedure described in [11].

3. RESULTS AND DISCUSSION

We have analyzed transcripts from the β-globin gene in reticulocytes of a subject affected by β-thalassemia. This β-thalassemia was related to a homozygous T⟶C transition in the GT dinucleotide of the first splice junction of the β-globin gene.

cDNA was prepared from RNA extracted from circulating reticulocytes. A primer was used to generate DNA fragments complementary to the 5′ end of the β-globin mRNA. Double-stranded cDNA was further synthesized and PCR amplified using this primer and another located in the 5′ non-coding end of the mRNA. In the normally spliced mRNA this results in the amplification of a 216 base-pairs long DNA fragment. This region is interrupted in the β-globin gene by a non-coding sequence (IVS 1) 130 base-pairs long. Amplification products were analyzed by gel electrophoresis (fig.1A). Five main bands were detected exhibiting an apparent migration of about 72–180–215–230 and 240 base pairs.

In order to identify products that contain β-globin mRNA sequences, amplified cDNA were transferred onto a nylon membrane and hybridized to an oligonucleotide of complementary sequences of the second exon of the β-globin gene [8] (fig.1B).

Under stringent washing conditions, only DNA of an apparent length of 180–215–230 and 240 base pairs remained hybridized to the probe. We verified that the use of an oligonucleotide differing only by a single central mismatch does not elicit any autoradiographic signal (not illustrated). The 215 base pairs fragment, whose size corresponded to that expected from a normal splicing event, was indeed observed in the β-globin cDNA of a normal subject (fig.1, lane 1). The other fragments can be related to the expressions of cryptic splicing sites. In order to locate the splicing sites, amplified β-globin cDNA were separated in acrylamide gel; the four β-globin-related DNA fragments were extracted from the gel and further PCR amplified using an unequal molar ratio of primer to allow accurate nucleotide sequence determination. Two cryptic splicing sites were identified at position −38 upstream of the normal splicing site and position +13 downstream (fig.2). These two sites and another −16 upstream were previously located using different approaches [12,13].

Using the primers described above, we failed to obtain readable patterns concerning the nucleotide sequence of the cDNA which migrated at an apparent size of 240 base pairs. This may indicate that this cDNA consists of a mosaic of different cDNA [6]. To verify this hypothesis we amplified this cDNA using the 3′ primer (palm 4) and another primer located only 30 bases upstream of the normal 5′ splicing junction. Nucleotide sequences of the product thus obtained are illustrated in fig.3. The main component of the cDNA was a product that can be related to the use of the cryptic site at position +13 (see fig.2B). The presence of other components in the studied DNA is derived from the appearance of minor bands in the background of the autoradiograms. This result indicates that this 240 base pairs DNA fragment is indeed a mosaic of the 230 base-long cDNA, and other β-globin or non-β-globin cDNA. The

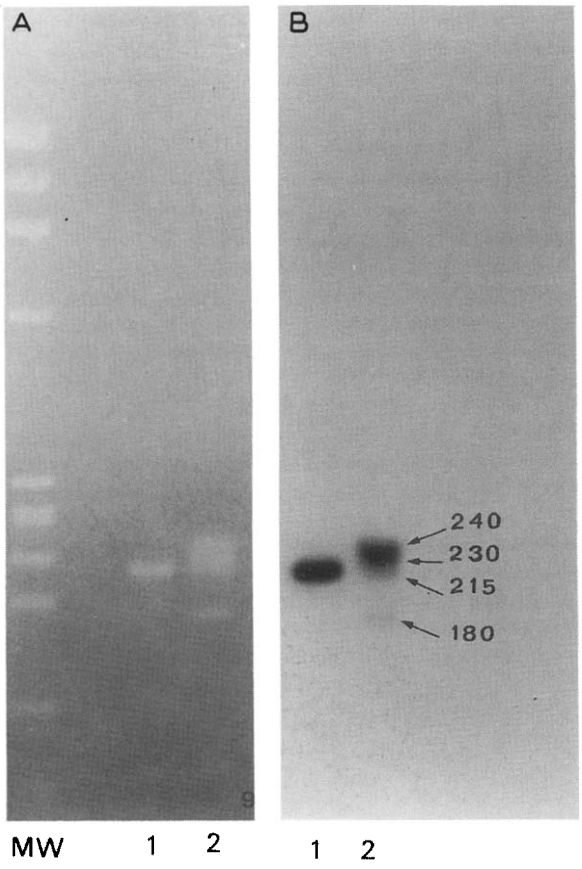

Fig.1. Analysis by agarose gel and Southern blot of the β-globin-related cDNA. Transcripts from the thalassemia affected patient (2) and those from a normal subject (1) are compared. Molecular weight markers (MW) are fragments of *Hae*III digested x174 RF DNA. Transcripts were resolved by agarose gel electrophoresis (A), transferred onto a nylon membrane and hybridized to a β-globin-specific oligonucleotide probe (B). The apparent size of the fragments hybridizing to the probe is indicated. The autoradiogram was voluntarily overexposed in order to visualize bands of very low intensity.

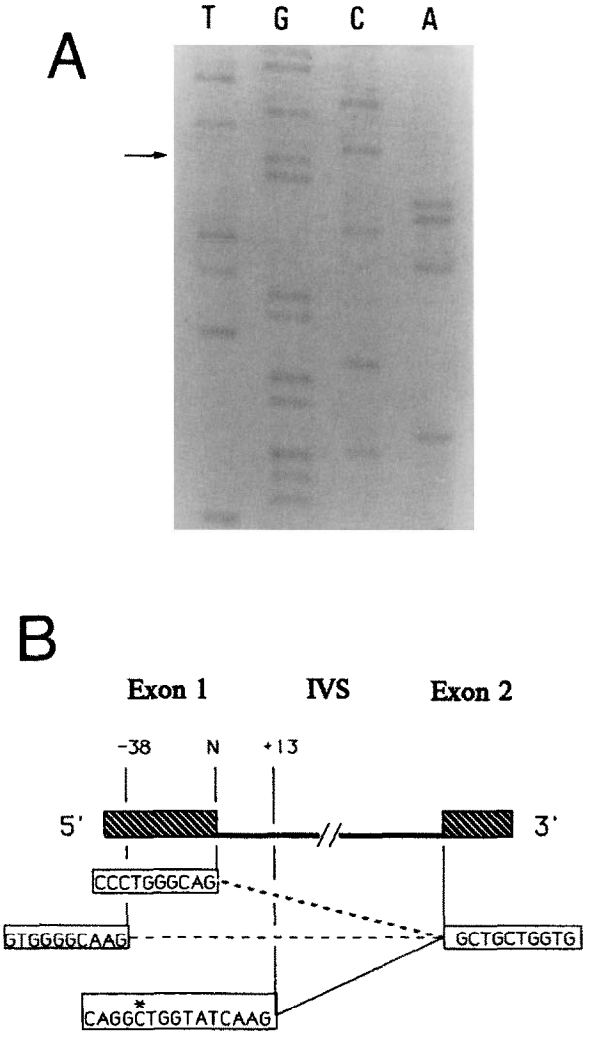

Fig.2. Localization of the splice sites producing β-globin related mRNAs. cDNA was prepared from reticulocyte mRNA of the thalassemic patient. The nucleotide sequence of the β-globin-related mRNA was determined. (A) A sequence flanking the splice junction IVS 1 + 13 (indicated by the arrow) is illustrated. The template was the non-coding strand of the DNA. (B) Diagram showing the position of splicing sites deduced from the determination of the nucleotide sequences of the β-globin-related cDNAs. Three splicing sites were localized: IVS 1-38, the normal splicing site (N) and IVS 1 + 13. Asterisk denotes the T⟶C transition at the 5′ signal of splicing.

polymorphic structure of such an entity could account for its electrophoretic mobility.

The patient studied in the present report was affected by a β-thalassemia that can be related to the almost entire absence of normally spliced β-globin mRNA. The results illustrated in fig.1 indicate that the method used allows the detection of the normal β-globin mRNA while mRNA resulting from splicing at position +16 [12,13] was not detected. Studying the effect of 3 mutations: (IVS 1-1 G⟶A, IVS 1-5 G⟶C and IVS 1-6 T⟶C) in transformed cells, Treisman et al. [12] observed in each case the 3 cryptic splicing sites. The analysis of in vitro transcripts [13] from genes carrying two other mutations (IVS 1-2 T⟶G and IVS 1-1 G⟶C) leads to a similar conclusion. Although slight differences were observed in the degree of activation of cryptic sites, depending on the mutation, the

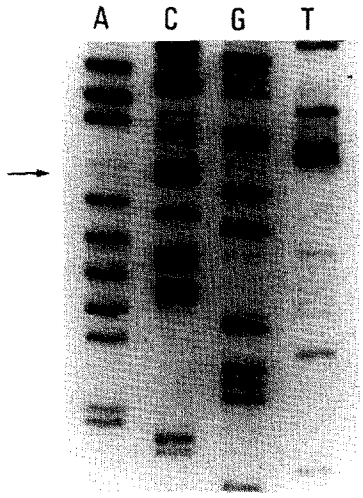

Fig.3. Determination of the composition of the β-globin-related cDNA migrating at an apparent size of 240 base pairs. The sequence obtained from reading bands of greater intensity is indicated and is identical to that of fig.2. The template was the coding strand of the DNA. The simultaneous presence of less intense bands suggests that the analysed DNA is a mosaic of one main product and other less represented products.

results indicated that the same cryptic splicing sites were involved; cryptic site −16 being the most efficient. If we agree with the hypothesis that sequence complementarity with U1 snRNA [14] is responsible for the abnormal splicing, the location of alternative sites should be independent of the mutation at the splice junction. Using the in vivo approach described here on a patient who carried a mutation at the IVS 1 splice junction, we observed that the −38 and +13 cryptic sites were indeed used. We have not observed mature mRNA deriving from effective splicing at position −16. Instability of these other potential transcripts could explain these observations. However, this is not consistent with the sensitivity of the detection method [15]. Better explanations would either be that splicing does not occur at every potential cryptic site or that only the products issued from specific cryptic sites are adequately transferred to the cytoplasm of the cells. Further studies would be needed to decipher the actual mechanism which leads to the selection of this abnormal mRNA.

In any case, our results do not entirely correspond to those obtained in similar situations using transformed cells or in vitro analysis.

The approach developed here is of potential interest in the rapid analysis of the products of many genomic defects. Although homozygosity facilitates accurate analysis of the effect of recessive abnormalities, this approach could be extended to heterozygous or double heterozygous if both genetic defects are sufficiently far from each other to be analyzed separately.

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