Expression of a β thalassemia gene with abnormal splicing

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ABSTRACT:

Expression of a cloned human β thalassemia gene with a single base change at position 5 of IVS 1 has been analyzed 48 hours after transfer of the gene into HeLa cells (transient expression). Little or no normal β globin mRNA accumulates in the presence of the abnormal β gene in contrast to significantly more normal β mRNA produced with other mutations at this same position. By contrast, large amounts of an abnormal β globin mRNA are present; this is due to the use of a cryptic 5' splice site in exon 1 rather than the normal 5' splice site of IVS 1. The results indicate the variability of the effect on RNA splicing of different single base defects within IVS.

INTRODUCTION:

The β thalassemias provide unique models for investigating the relationship between changes in β globin gene structure and function (1, 2). Single nucleotide changes in these disorders have been shown to be associated with defects in promoter function, splicing, codon recognition and translation, and mRNA stability; all result in decreased or absent β globin. We recently described a single nucleotide defect, a G→A change, at position 5 in the small intervening sequence (IVS 1) of the β globin gene associated with β thalassemia in an Algerian patient homozygous for the mutation with a unique haplotype (3). We now report the results of studies of transient expression of this gene in tissue culture cells. Little or no normal β globin mRNA is produced as a result of this mutation, and a single cryptic splice site in exon 1 is largely used in processing the RNA from this gene instead of the normal 5' splice site of IVS 1.

METHODS:

The β thalassemia gene was cloned as originally described (3). The 7.5 kilobase (kb) Hind III fragment containing the entire β gene was subcloned into pBR 322. The 5 kb Bgl II fragment from this subclone was ligated into the plasmid pBSV that had been cleaved with Bam HI and grown in *E. coli* as previously described (4). Purified plasmid containing the mutated gene (β^M) was then used to transfect HeLa cells as previously described (5); 10^7 HeLa cells were mixed with 10 μ g of either β^M or β pBSV containing a normal β globin gene in the same Bgl II fragment as β^M . After 48 hours at 37°C, RNA was isolated by cesium chloride sedimentation (6), and analyzed either by Northern blotting or RNAase protection using one of three SP6 probes. The three probes were: 1) A Bal I to Bam HI fragment extending from the 5' region flanking the β globin gene to the intragenic Bam HI site (Bal/Bam); 2) A Bal I/Hae III probe containing a region of the β globin gene extending from the 5' flanking region to the Hae III site within exon 1; and 3) A composite probe comprised of 5' flanking sequences from the Bal I site to the initiation codon, and then containing cDNA sequences from the initiation codon to the Bam HI site (H β 350) (a kind gift from Dr. Tom Maniatis). SP6 reactions were carried out as described (7). Isoelectric focussing of hemoglobin from the red cells of three untransfused homozygous siblings was performed in polyacrylamide gel using a pH range from 6 to 8 (8).

RESULTS:

The β^+ phenotype is documented by the presence of approximately 10% hemoglobin A seen by isoelectric focussing of blood from three untransfused siblings homozygous for the β thalassemia mutation (data not shown).

When a normal β globin gene is transfected into HeLa cells, the two normal fragments expected with the Bal/Bam probe are seen: A 145 base fragment representing the 5' end and exon 1, and a 209 base fragment containing exon 2 (Figure 1). By contrast, when β^M gene expression is analyzed in HeLa cells, only a small amount of the normal 145 base exon 1-containing

Figure 1:
Analysis of Normal and Thalassemic (β^M) RNA:

RNA was isolated from HeLa cells after transfection as described in the text. A uniformly labelled 553 nt RNA Bal I/Bam HI probe prepared in SP6 was hybridized to 10 μ g total RNA and analyzed after treatment with RNAase as described (7). Lane 1 is RNA from cells with the normal β gene; lane 2, RNA from cells with the β^M gene; lane 3, untransfected HeLa cell RNA; lane 4, 10 ng normal reticulocyte RNA. Lane P is the undigested probe; Lane M, an Hpa II digest of pBR 322 as marker DNA. The top arrow at the left shows the normal exon 2 fragment of 209 nt; the middle arrow is the normal exon 2 fragment of 145 nt; the lower arrow shows the 125 nt abnormal fragment generated as diagrammed.

fragment is seen. A new unique 125 base fragment is the major product along with the expected normal 209 base fragment representing exon 2. The amount of aberrant RNA transcripts

accumulating with the β thalassemia gene is comparable to the amount of normal mRNA present in cells transfected with the normal β gene.

The results indicate that the β^M RNA transcript properly splices at the normal IVS 1-exon 2 junction, but has a different structure between its cap and the 5' end of IVS 1 (the 3' end of exon 1). Two possibilities can explain this result: Either there is downstream initiation 20 nucleotides from the normal cap extending to the normal exon 1/IVS 1 splice junction, or there is normal cap initiation and cryptic splicing using a cryptic splice site in exon 1 at codon 25 as described previously (9). To distinguish between these possibilities, the two other probes were used. Analysis with the Bal I/Hae III probe showed the normal expected 135 base fragment on SP6 analysis with the normal β gene, and only the abnormal 125 base fragment with the mutated β gene (Figure 2). Since the Hae III site is 10 nucleotides 5' to the exon 1/IVS 1 junction, the expected abnormal fragment if downstream initiation occurred with the abnormal gene would have been expected to yield a 115 base fragment instead of a 125 base fragment. The finding of only the 125 base fragment, the same size as that obtained with the Bal/Bam probe, is evidence that the shortened 5' fragment in the abnormal mRNA is due to an abnormal splice in the region of exon 1 as previously described (7) (Figures 1, 2).

When the H β 350 probe was used in SP6 analysis (Figure 3) with the normal gene, the expected 353 base fragment containing cojoined cap to Bam HI sequences in the mRNA with appropriate splicing of exon 1 was obtained. If downstream initiation was the cause of the shortened cap to exon 1/IVS 1 junction in the abnormal gene, then a single shortened fragment protected by this probe would be expected since normal exon 1/exon 2 splicing would be expected. By contrast, the results indicate that the mutated β gene (Figure 3) is abnormally spliced as seen by the presence of the 125 base fragment seen previously representing exon 1, and the 209 fragment representing exon 2. This result further supports an abnormal splice beginning in exon 1 as the cause of the results seen in each of the RNA analyses. This site can be localized to codon 25 as seen in Figure 4.

Figure 2:

Analysis of Normal and Thalassemic (β^M) RNA:

RNA was isolated as described in the text. A uniformly labelled 208 nt Bal I/Hae III RNA probe prepared in SP6 was hybridized to 10 μ g total RNA and analyzed as described (7). Lane 1 is RNA from HeLa cells with the normal β gene; 2, RNA from cells with the β^M gene; lane 3, untransfected HeLa cell RNA; lane 4, 10 ng normal human reticulocyte RNA. Lane P is undigested probe; lane M, Hpa II digest of pBR 322 DNA. The top arrow at the left is the normal 135 nt fragment; the bottom arrow, the 125 nt abnormal fragment as diagrammed.

Similar amounts of plasmid DNA were recovered from HeLa cells transfected with the normal and β^M genes by Southern analysis (data not shown).

Figure 3:

Analysis of Normal and Thalassemic (β^M) RNA:

RNA was isolated as described in the text. A uniformly labelled 527 nt Rsa I/Bam HI RNA probe prepared in SP6 representing 5' β and cDNA sequences was hybridized to 10 μ g total cell RNA and analyzed as described (7). Lane 1, RNA from cells with the normal β gene; lane 2, RNA from cells with the β^M gene; lane 3, RNA from untransfected HeLa cells; lane 4, 10 ng human reticulocyte RNA; Lane P, undigested probe; Lane M, Hpa II digested pBR 322 marker DNA. The upper arrow denotes the 353 nt fragment protected by normal β mRNA; the middle arrow, the normal 209 nt exon 2 fragment; lower arrow, the abnormal 125 nt exon 1 fragment diagrammed from β^M transfected cells.

DISCUSSION:

Studies have been reported of the expression of another β thalassemia gene with a G-C mutation at the same position as the gene described here using a similar system for analysis (9). In

Figure 4:

Diagram of Exon 1-IVS 1 Region of β Gene:

The nucleotide sequence of the region is shown with exon 1 bases in capital letters and IVS in lower case. The two cryptic splice sites described previously (9, 10) are shown. In the β^M gene described here, cryptic splice 2 is predominantly used (Figures 1-3). The G-A mutation in IVS 1 at position 5 is shown.

these experiments, there was a significant amount of normal splicing of IVS 1 and a significant amount of normal β globin mRNA produced, consistent with the β^+ phenotype of this patient. In addition, three cryptic splices were seen: One at codon 18 in exon 1, another at codon 25, and a third at position 13 downstream from the 5' junction in IVS 1 (Figure 4). Similar results for another β thalassemia mutation at this position (G-T) have recently been reported (10).

Our results differ from these in several details. Firstly, while the major abnormal splice is similar to the one shown in these previous reports, the other two splice products are not significantly represented in our studies. Secondly, there is much more abnormally spliced RNA accumulated than in other studies (9, 10). Thirdly, there is much less normally spliced RNA representing exon 1 than in these studies. This latter finding is difficult to explain since the phenotype of this patient and his siblings is β^+ thalassemia. Thus, we must explain the low level of normal splicing of IVS 1 in the presence of in vivo evidence in the patient's cells for the

production of both normal β globin and β globin mRNA. The assay system is capable of detecting normal IVS 1 splicing since the normal gene yields the appropriately spliced products (Figures 1-3). In addition, previous studies from this and several other laboratories have indicated that there is relatively faithful transcription and processing of normal β globin genes and those of patients with β thalassemia. Previous studies of abnormal β thalassemia genes (11, 12) have demonstrated that the abnormal mRNA products are more abundant than these products when seen in erythroid cells. This is presumably due to the fact that HeLa cells allow the accumulation of abnormally spliced mRNAs while these abnormal mRNAs are presumably destroyed by enzymes present in erythroid cells. However, in each of these studies in which there is a β^+ gene, there has been definite evidence for normal β globin mRNA fragments in significant amounts in contrast to the results reported here.

One possible technical problem that might explain our results is that we have not utilized enough probe excess to detect the relatively small amounts of normal β globin mRNA and other cryptic splice products; however, titration with increasing amounts of probe and decreasing amounts of mRNA have eliminated this possibility (data not shown). It is also possible that, in the presence of a large amount of abnormal mRNA, a small amount of normal mRNA processing could not be detected. A mixing experiment in which normal and abnormal mRNA were added to each other eliminated this possibility by demonstrating the expected amount of normal and abnormal fragments with a Bal I/Hae III probe (data not shown). We are, thus, left with the finding that the major product of this aberrant gene is an abnormal mRNA in which splicing is from a cryptic splice site in exon 1, as previously noted. However, in contrast to previous studies with other single nucleotide changes at this position, little evidence for normal splicing is detectable. It is possible that the single base change in this case has led to a difference in either the secondary structure of the RNA precursor, or different splice sequence recognition in the RNA produced from this gene as compared to that of other genes with other mutations at the same position. This could

explain both the lesser amount of normal mRNA and the greater amount of abnormal mRNA produced by this gene.

In summary, the G-A change at position 5 of IVS 1 in a β thalassemia gene leads to the abnormal splicing of the mRNA produced by this gene. The major product is similar to that described with other genes of this type and is an mRNA with a cryptic splice site from codon 25 in exon 1 to the 3' splice junction of IVS 1. By contrast with these previous studies, however, there is little evidence of normal splicing or for the two other cryptic splices previously reported with other single nucleotide changes at this position (9, 10).

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