β-Thalassemia resulting from a single nucleotide substitution in an acceptor splice site

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ABSTRACT

β-globin gene mutations which alter normal globin RNA splicing have confirmed the necessity of invariant nucleotides GT at donor splice sites. Functional consequences of point mutations in the invariant AG acceptor splice site have not been determined. We have isolated and characterized a β-globin gene from a Black patient with β-thalassemia intermedia which has an A→G transition at the usual intervening sequence 2 (IVS2) acceptor splice site. Functional analysis of transcripts produced by this mutant gene in a transient expression vector indicates that the mutation inactivates the normal acceptor splice site and results in some utilization of a cryptic splice site near position 580 of IVS2. This mutation would be expected to produce a β-globin gene which results in no normal β-globin mRNA.

INTRODUCTION

Beta-thalassemia is a common genetic disorder characterized by a marked decrease (β^+) or total absence (β^0) of synthesis of the β-globin chain of hemoglobin (1). Sequence analysis and expression studies of cloned β-globin genes have defined numerous defects associated with this disorder including gene deletion (2,3,4,5), nonsense and frameshift mutations (6,7,8,9), promoter mutations (10,11,12) and single base substitutions resulting in RNA splicing errors (13-21). Functional studies of cloned β-globin genes have confirmed the necessity of the invariant sequences located at the 5' end of introns for normal RNA processing. Similar studies have also demonstrated that alternative patterns of RNA splicing are revealed by mutations that abolish normal splicing signals or create new splice sites in introns or exons (13,19-22). In Greek and Italian populations, mutations which result in altered globin RNA splicing appear to be common causes of β-thalassemia (18). Because little was known about the mutations which cause β-thalassemia in Black populations, we cloned and characterized a β-globin gene from a Black patient with β-thalassemia intermedia. These studies have demonstrated that one of the genes responsible for this condition has an A→G transition at the normal 3' splice acceptor site of intervening sequence 2 (IVS2) and that this mutation

inactivates this acceptor site. Furthermore, the mutation results in
activation of a cryptic acceptor splice site at or near a position 271 base
pairs upstream from the normal acceptor splice site.

MATERIALS AND METHODS

Patient Material: DNA was prepared from peripheral blood nucleated cells of a
Black male patient homozygous for β-thalassemia (β+-thalassemia phenotype)
using a modification of the method of Blin and Stafford (23). Globin chain
synthetic ratios were determined by CMC column chromatography of globin chains
labeled in intact reticulocytes using ^{3}H-leucine.

β-Globin Gene Cloning: High molecular weight DNA was digested to completion
with the restriction enzyme Hind III using conditions recommended by the
supplier (Bethesda Research Laboratory) and directly ligated to Hind III
digested Charon 28 DNA (24). The resulting recombinant DNA was packaged in
vitro into bacteriophage heads (25) and recombinant bacteriophage were
directly screened by the plaque hybridization procedure of Benton and Davis
(26), using as a probe a ^{32}P-labeled Eco R1-Bam H1 fragment of the β-globin
gene encompassing IVS2 (27). Plasmid cloning was performed using standard
methods.

DNA Sequencing: Fragments of the thalassemic β-globin gene were subcloned into
M13-mp8 or mp9 bacteriophage vectors using Bal 1, Hae III, Bam HI, and Eco RI
sites within and around the β-globin gene (28). The recombinants were used to
generate single strand templates for DNA sequence analysis using Sanger's
dideoxy chain termination seqencing method (29). DNA fragments were cloned
into M13 vectors in opposite orientations to allow sequencing of the
complementary strand.

Gene Expression Studies: pBR322-SV40 recombinants containing the thalassemic
β-globin gene (Vβ-thal) and a normal β-globin gene (Vβ-nor) were prepared and
used in these studies. The 5.0 Kb Bgl II fragment containing the thalassemic
or normal β-globin gene were subcloned into an expression plasmid pLTN3B that
was originally constructed in the laboratory of Arthur Nienhuis (30).
Transfection of monkey kidney COS cells or HeLa cells was performed as
described (31) using the technique of calcium phosphate DNA coprecipitation
and glycerol shock. Cells were harvested 48 hours after transfection and total
cellular RNA and DNA were recovered by guanidine-HCl lysis and cesium chloride
density centrifugation as previously described (33,34). S1 nuclease mapping
was performed by a modified Berk and Sharp (35) procedure where total cellular
RNA from COS or HeLa cells was annealed in 50% formamide to either a uniformly

^{32}P-labeled single stranded probe (18) or an end-labeled double stranded fragment of DNA. (See Results for description of specific probes and Fig. 3.) S1 nuclease digestion conditions were identical to those previously described (30) and the protected ^{32}P labeled DNA fragments were analyzed by electrophoresis in 8% or 16% polyacrylamide sequencing gels under denaturing conditions (36).

Primer extension studies were performed using total RNA from HeLa cells transfected with γβ-globin fusion gene recombinants into which a normal (Vγβ-nor) or thalassemic (Vγβ-thal) β-globin gene IVS2 was inserted. 20 μg of RNA was annealed to a 59 bp Eco Rl-Bst Nl fragment derived from exon 3 of the normal β-globin gene and uniquely 5' labeled on the Bst Nl end as described in Favaloro et al (37). Primer extension was performed using reverse transcriptase as described by Triesman et al (17).

All experiments using recombinant molecules were conducted within NIH guidelines.

RESULTS

Cloning of the β-globin Gene: Two bacteriophage recombinants were isolated by screening 4x10^5 plaques by filter hybridization. Both recombinants were shown to contain the 7.5 Kb Hind III fragment which includes the β-globin gene. By restriction endonuclease mapping using the enzymes Bam Hl, Pst 1, Eco Rl and Hind III, we were unable to detect any rearrangements, deletions or inversions in or around these cloned β-globin genes.

Sequence Analysis of the Cloned Gene: One of the two cloned genes was selected for further study by DNA sequencing. The sequence of the gene was determined in its entirety. By comparing the obtained sequence to the published sequence of a normal β-globin gene (38), we found five differences. All of these, except one, have been previously demonstrated to represent polymorphic variation. These include a CAC to CAT change at codon 2, a C to G change at position 16 of IVS2, a G to T change at position 74 of IVS2, and a T to C change at position 666 of IVS2. In addition, a previously unidentified A to G transition at position 849 of IVS2 (the IVS2-exon 3 junction) was found. This base substitution changes the invariant acceptor dinucleotide AG to GG at the 3' splice site of IVS2. (Figure 1)

Gene Expression Studies: Normal and thalassemic globin gene expression were analyzed and compared primarily by examining RNA splicing patterns. The normal and thalassemic β-globin genes were cloned into the Bam HI site in identical orientations in the short term expression vector pLTN3B which has been
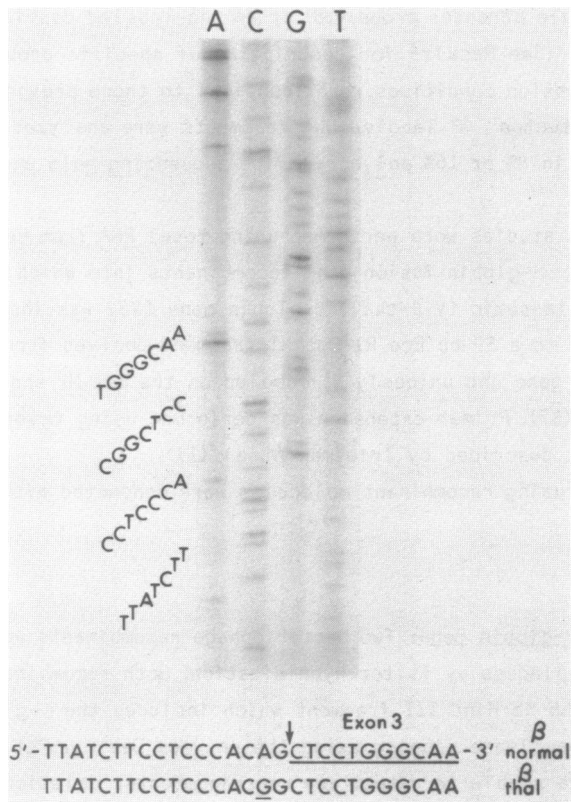
Figure 1. Single base change at IVS2 exon 3 junction. The DNA sequence for the βthal gene is presented below the autoradiograph from which sequence was obtained. The A→G transition is underlined. The normal splice site is identified with a vertical arrow. Exon 3 is underlined in the normal sequence.

previously described by Humphries et al (30) (Fig. 2). In the initial construction of the expression vector, pBR322 sequences which inhibit plasmid replication in eukaryotic cells had been removed and replaced with the SV40 late promoter and origin of replication (40,41). These changes allow this vector to replicate in eukaryotic cells which produce SV40 T antigen (COS cells) (30). The recombinants containing the normal (Vβ-nor) and thalassemic globin genes (Vβ-thal) were transfected into COS and HeLa cells in parallel experiments.

RNA was isolated from cells 48 hours after transfection with these two recombinants. This RNA was analyzed by S1 nuclease analysis using a probe which examines all normal intron-exon splicing of β-globin transcripts. This
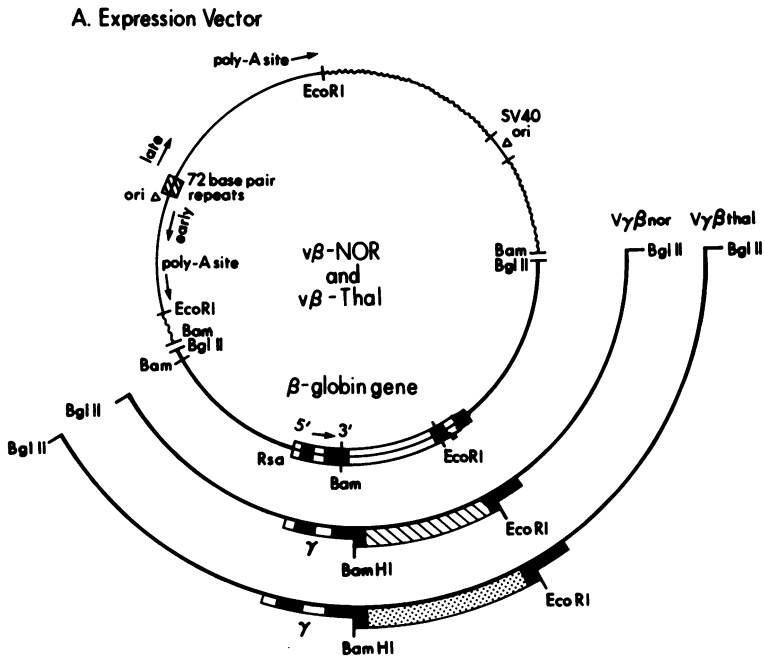
A. Expression Vector

Figure 2. Structure of expression vectors carrying cloned globin genes.
A normal and thalassemic β-globin gene were inserted in the vector pLTN3B. In
this orientation, the β-globin gene utilizes its own promoter under the
effect of the 72 bp SV40 enhancer.
A γβ fusion vector (Vγβ-nor) was prepared by first substituting the Bgl II-Bam HI
fragment which contains 5' flanking, exon I, and exon 2 sequences of the human
γ-globin gene, for the equivalent Bam HI fragment in Vβ-nor. The Bam HI-Eco RI
fragment containing the entirety of IVS2 from this recombinant was substituted
with the equivalent fragment from the β-thalassemia gene to prepare Vγβ-thal.

probe is prepared as a ^{32}P labeled strand synthesized complementary to
β-globin gene sequences extending from a position 63 bp upstream from the
transcription initiation site to the intragenic EcoRl site (See Fig. 3). The
single strand template was isolated as the filamentous form of an M13
recombinant containing the globin gene sequences (18). Fragments of 145, 225
and 49 nucleotides (nt) will be protected from S1 nuclease when hybrids are
made between this probe and normal β-globin mRNA, although only exon 1 and
exon 2 fragments can be visualized in the figure shown here. (Fig. 3, Bone
Marrow). RNA derived from cells transfected with the expression vector
containing the normal β-globin gene produces a pattern of protection of the F
probe similar to that for β-globin mRNA (Fig. 3, Normal). On high percentage
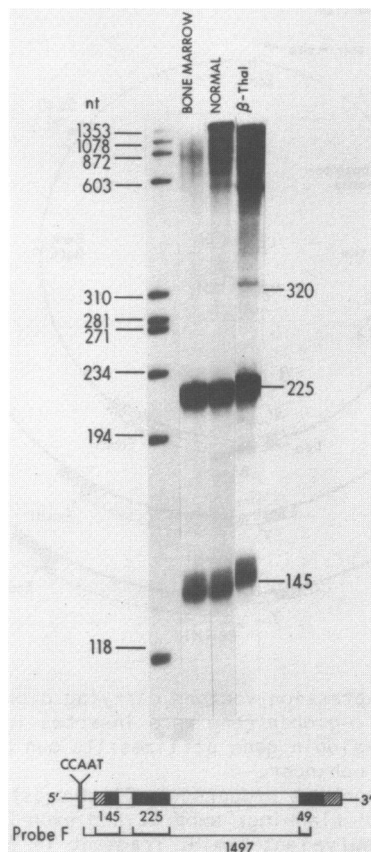acrylamide gels, the 49 nt fragment representing part of exon 3 can be seen.

Figure 3. S1 nuclease study showing use of cryptic splice site.
The F probe is a uniformly ^{32}P labeled fragment of DNA derived from a
region of the normal β-globin gene depicted at the bottom of the figure.
Fragments protected from S1 nuclease by normal β-globin mRNA are accented and
represent exon 1 (145 nt), exon 2 (225 nt), and part of exon 3 (49 nt). The
probe is 1497 nt long and extends 63 nt upstream from the usual transcription
initiation site.
Normal RNA derived from a highly erythoid bone marrow sample demonstrates
protection of exon 1 and exon 2. RNA derived from HeLa cells transfected with
V β-nor (normal) produces a similar pattern. RNA derived from HeLa cells
transfected with Vβthal (β-thal) demonstrates the presence of a 320 nt
fragment not seen in the other lanes, representing utilization of a cryptic
splice site in IVS2.
The 49 nt fragment from exon 3 can not be visualized on this gel, although it
can be seen on other gels in studies using bone marrow RNA and RNA from HeLa
cells transfected with Vβ-nor. Even after long exposures it is not present in
the β-thal lane. (Data not shown.)
Additional bands of approximately 580 and 650 nt are seen in the lanes
containing RNA produced by the expression vectors. The origin of these bands
has not been determined, although they may represent splicing intermediates.

(Data not shown.)

S1 nuclease analysis of RNA derived from COS cells transfected with Vβ-thal (β-thal lane) produced a different pattern. The 225 nt and 145 nt fragments representing protection by exons 1 and 2 are present. Also, a band of nearly 600 nt (42) is present in both Vβ-nor and Vβ-thal although it is more intense in the Vβ-thal study. In the Vβ-thal lane, an additional band at 320 nt is also visible.

The 320 nt fragment is present only in RNA derived from COS or HeLa cells transfected with the β-thal vector. This pattern is similar to that resulting from S1 nuclease experiments by others using β-globin genes which produced transcripts in which IVS2 is improperly spliced (11,19). In these cases, mutations at position 705 or 745 of IVS2 activated a cryptic splice site at position 579 of IVS2. This activation occurs in the 745 position mutant as a result of the generation of a new donor splice site between the cryptic site and the normal 3' acceptor site (11). In the acceptor splice site mutant described here, the 320 nt fragment appears to be due to inactivation of the normal acceptor splice site and some utilization of a cryptic site at or near position 579. No 49 nt fragment could be seen in this experiment using the F probe. (Data not shown.)

In order to further localize the origin of the 320 nt S1 product, we repeated the analysis using a Bam H1-Eco R1 fragment labeled with ^{32}P at the Eco R1 end. This fragment contains the normal IVS2, 9 bases of exon 2, and 49 bases of exon 3 of β-globin gene. This analysis demonstrated that normal β-globin mRNA and RNA from cells transfected with Vβ-nor protected a 49 nt fragment as predicted (Figure 4). However, only a fragment approximately 320 nt was protected by RNA derived from cells transfected with Vβ-thal. This result indicates that the 320 nt fragment is generated by protection of the probe with RNA which includes approximately 270 nt of β-globin IVS2 and 49 nt of exon 3. These data are consistent with utilization of a cryptic splice site at or near position 579 of IVS2.

To determine if the IVS2 splice site is completely inactivated by the mutation in the β-thal gene, we performed additional studies which have a high sensitivity for detecting correctly spliced globin RNA. To limit the study to a comparison of globin RNA splicing in globin genes which differ only in their IVS2, we constructed two recombinant SV40 vectors in which the only differences were the Bam H1-EcoR1 fragments containing the IVS2 sequences from the normal or β-thalassemia genes, respectively (Fig. 2). The vector containing the normal IVS2 has been constructed by substituting the Bam H1
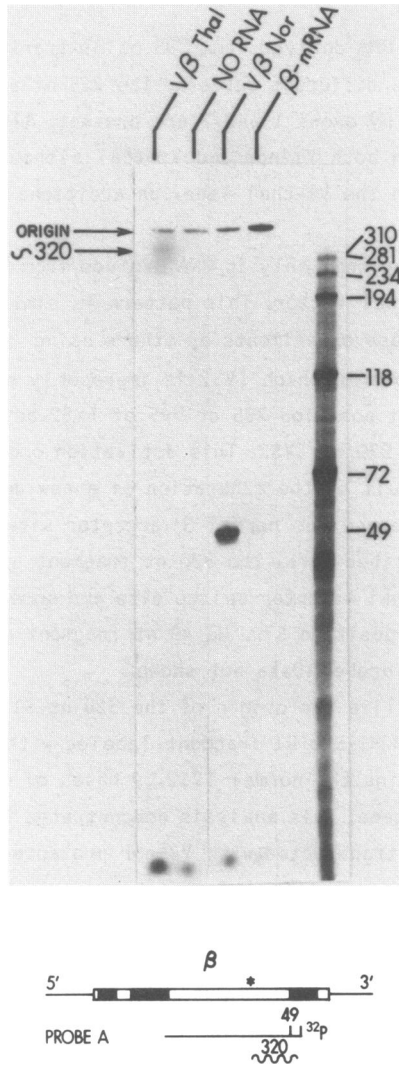
Figure 4. Elimination of functional IVS2 acceptor splice site.
S1 nuclease analysis was performed on RNA derived from transient expression vectors containing the thalassemic β-globin gene, a normal β-globin gene, or β-globin mRNA. A control was performed to which no RNA is added. The probe is a ^{32}P end labeled Bam H1-Eco R1 fragment which includes β-globin gene IVS2 and 49 bp of exon 3.
S1 nuclease analysis of RNA derived from HeLa cells transfected with Vβ-nor or from RNA from marrow cells of a patient with sickle cell disease demonstrate the expected 49 nt band produced by normal utilization of the IVS2 acceptor site. No equivalent band is seen in the analysis of RNA from HeLa cells transfected with Vβ-thal. Instead, a faint band is seen in the region of 320 nt, similar to that seen when using the F probe.
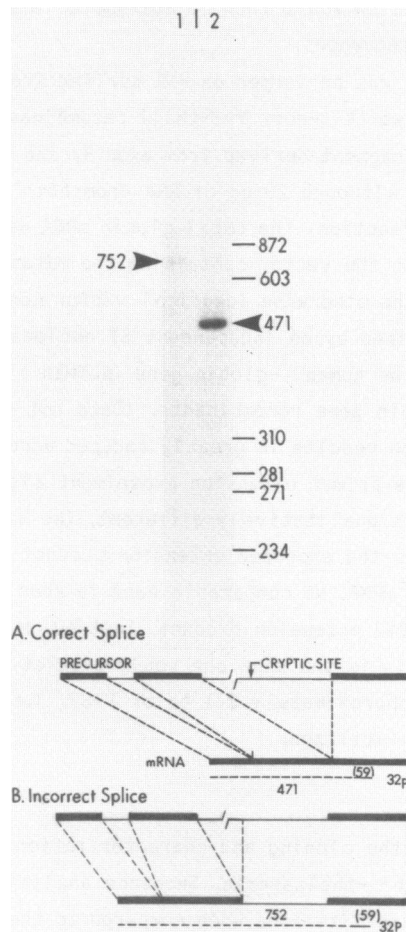
Figure 5. Primer extension analysis of RNA from VγβnorR and Vγβthal.
HeLa cells were transfected with Vγβnor and Vγβthal. 20 g of total RNA
from each transfection was annealed to a ^{32}P labeled 59 nt Eco Rl-Bst Nl
fragment derived from exon 3 (nt 53-112), represented schematically at bottom
of figure. A 471 nt product is expected if the RNA is properly spliced, and is
seen in lane 2 using RNA derived from Vγβnor. No similar sized fragment is
seen in lane 1; instead, a 752 nt fragment is produced representing the
primer extension product of RNA derived from Vγβthal. The difference in size
of the two products is approximately 270 nt, the length of RNA expected to be
included in the processed RNA if the cryptic splice site in IVS2 is utilized.

fragment containing the 5' end of the β-globin gene in Vβ-nor with the Bgl II-
Bam Hl fragment containing the 5' end of the γ -globin gene (Fig. 2). The IVS2
of this γ-β fusion recombinant (Vγβ -nor) is then easily replaced with the
Bam Hl-Eco Rl fragment containing the β-thal IVS2 (Vγβ-thal). Alterations in

splicing or RNA accumulation would then be attributable solely to differences in the two intervening sequences.

A primer extension study was performed on RNA derived from HeLa cells transfected with these two (VγβPβ-nor, VγβPβ-thal) recombinants. The primer was the 59 bp EcoRl-Bst Nl fragment derived from exon 3, and uniquely labeled on the Bst Nl end (Fig. 5). Although 20 ug of RNA from both transfections was used in each annealing reaction, the total globin mRNA was 10-20 times lower in cells transfected with the vector containing the mutant thalassemic intron (Vγβ-thal) compared to the otherwise identical vector containing the normal IVS2 (Vγβ-nor) as determined by an independent S1 nuclease analysis in which a recombinant containing the human α-globin gene (pLTNlh) was co-transfected with each of the γβ-globin gene recombinants. (Data not shown.) Thus the thalassemic IVS2 mutation results in greatly reduced accumulation of globin mRNA. The products of the primer extension experiment also indicate that the mRNA which accumulates is qualitatively different. The single intense band in Lane B, Fig. 5 represents the expected extension product derived from a normally spliced globin mRNA. No comparable band is seen in the lane containing the β-thal IVS2 extension product. Instead, a faint band is seen in the region of 750 nt. This is the size one would predict by improper splicing which failed to remove approximately 271 bp of IVS2, i.e., if the cryptic splice site in IVS2 were utilized.

DISCUSSION

In this paper we report the cloning and characterization of a β-globin gene from a Black patient with β[+]-thalassemia. Sequence analysis of this gene showed five single nucleotide substitutions when compared to the published nucleotide sequence of a normal human β-globin gene (38). One of these changes is a C→ G transversion at position 16 of IVS-2. This is a known polymorphism in Mediterranean and Asian populations (16,39). This transversion abolishes the β-globin intragenic AvaII restriction endonuclease site which is one of the useful polymorphic sites that define different chromosomal haplotypes in the β-globin gene region (16). The other variant bases in codon 2, and IVS2 positions 74 and 666 represent established polymorphic sites in Mediterranean and Asian genes. The base substitution resulting is an A→ G transition at position 849 of IVS-2 changes the AG invariant dinucleotide of the acceptor splice site to a GG. In vitro studies in which this gene was incorporated into a transient expression vector and expressed in COS or HeLa cells demonstrated inactivation of the normal acceptor splice site of IVS-2. As a result of this

inactivation, a cryptic splice site at or near position 579 of IVS2 is
utilized, but at low efficiency.

Mutations which interfere with normal splicing of β-globin mRNA lead to
β⁺-thalassemia when there is partial inactivation of a normal splice site and
to β⁰-thalassemia when the inactivation is complete. The β -thalassemias
that are due to RNA processing errors have been shown to result from mutations
which generate alternative splice sites that are recognized by the splicing
mechanism in favor of or in addition to the normal splice sites
(11,13,14,19,21,22). By comparison, other mutations have been described which
alter the invariant dinucleotide GT of the donor splice site and prevent the
synthesis of any normal β-globin mRNA. For example, G→A transitions in
β-globin genes at position 1 of IVS1 (16) and position 1 of IVS2 (17) have
been associated with β⁰-thalassemia in humans. In vitro gene expression
studies on the first of these genes by Treisman et al demonstrated complete
inactivation of the 5' splice site of IVS1 and utilization of 3 other nearby
cryptic splice sites (11). Similar expression studies using another β-globin
gene with a mutation at IVS2 position 1 (GT→AT) also showed complete
inactivation of this splice site and utilization of a cryptic splice site
within IVS2, in addition to a less abundant RNA species comprised of the
normal first exon spliced directly to the third (17). The results of studies
of naturally occuring thalassemic β-globin genes have been supported by
studies utilizing in vitro mutagenesis directed at the 5' end of the large
intron of rabbit β-globin gene. One of the mutations converted the GT
dinucleotide invariant to an AT and abolished splicing completely at that site
(43). Although it has been postulated that the invariant AG acceptor site
sequences may also play an important role in splicing (30,33,34), there have
been no examples of single base mutations at these sites in naturally occuring
splicing mutants. However, Orkin et al have described a β⁰-thalassemia
mutation resulting from deletion of 25 bp of the β-globin gene including the
IVS1 acceptor splice site (44). In this paper we report an A→G transition at
the acceptor splice site of IVS2 of a β-globin gene from a patient with a
thalassemic phenotype. The expression studies on our cloned β-globin gene
demonstrate that a single base substitution at the first base of the invariant
dinucleotide at the acceptor splice site of IVS2 in this gene completely
abolishes or greatly reduces splicing at that site. Thus the first base of the
AG dinucleotide of the acceptor splice site is necessary for normal RNA
splicing as predicted by sequence comparison studies (45,46,47). Furthermore,
inactivation of the normal IVS2 acceptor splice site results in utilization of

a cryptic splice site at or near position 579 of IVS2. The DNA sequence at the cryptic splice site reveals an AG dinucleotide preceded by a 12-base pyrimidine tract similar to the proposed concensus acceptor splice sites (45). This same cryptic splice site has recently been shown to be activated by mutations at positions 705 (19) and 745 (11) of IVS2. The studies reported here do not establish if the cryptic splice site is utilized during normal β-globin RNA processing. However, we have never been able to identify patterns in S1 nuclease studies of normal β-globin mRNA which suggest utilization of the cryptic splice site.

Expression studies using the cloned β-thalassemia gene reported here fail to demonstrate any normally processed β-globin RNA and, therefore, would be expected to lead to a β^0-thalassemia phenotype. However, globin chain synthesis studies on peripheral reticulocytes from this same patient reveal an α/β synthetic ratio of 9.0 and a β^+-thalassemia phenotype. The most likely explanation for this finding is that this patient is doubly heterozygous for two different mutations which individually would produce either β^+ and β^0-thalassemia. The two β-globin recombinants that we have isolated so far from the patient have been shown, by sequence analysis, to have the same mutations. Blotting studies on genomic DNA from this patient showed heterozygosity at the Ava II and Hgi I restriction sites within the β-globin complex which indicates we have isolated only one of the two β-globin genes from this patient. In view of the mild clinical condition of the patient, the β-globin gene <u>trans</u> to the gene carrying the splice site mutation is expected to carry a mutation which produces a mild reduction in normal β-globin synthesis.

Antonarakis et al have recently reported the characterization of 2 different β-globin genes from Black individuals (48). One had the identical base change at the IVS2 acceptor splice site which we report here. That gene was isolated from a patient with HbS/β^0thalassemia. Their clinical and functional studies of their cloned β-thalassemia gene provide additional evidence that the mutation described here produces a β^0-thalassemia defect.

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