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RNA processing errors in patients with β-thalassemia

(S1 nuclease mapping/M13 phage/splicing)

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ABSTRACT **We have developed a method that permits rapid identification of the consequences of mutations that alter β-globin RNA processing in erythroid cells. S1 nuclease mapping techniques were used to analyze total bone marrow RNA obtained from 15 patients who are clinically homozygous for β-thalassemia and from 5 patients with erythroid hyperplasia from other causes. This analysis was facilitated by the use of single-stranded uniformly labeled DNA probes of high specific activity that were prepared by using recombinant phage M13–β-globin DNA templates. Two abnormalities of RNA processing were found to occur with high frequency in these patients. Nine thalassemic patients were found to have increased levels of an RNA species that retains all sequences transcribed from intervening sequence 1, implying the presence of mutations that decrease the correct splicing of this intron. Seven of 15 thalassemic patients were found to have an abnormally processed RNA species that retains 19 nucleotides transcribed from the 3′ end of intron 1; this abnormality is caused by the G → A substitution in intron 1 that is known to create an alternative splice acceptor site.**

The thalassemias are human diseases characterized by deficient synthesis of either the α- or the β-globin subunit of adult hemoglobin; these diseases are caused by mutations that affect the level of gene expression without altering the structure of the gene product (1–3). Characterization of mutations causing thalassemia would seem likely to identify those portions of globin genes that are critical for a high level of expression.

Functional studies of mRNA metabolism in bone marrow cells of patients with β-thalassemia suggest that mutations that affect processing of the β-globin mRNA precursor are often the cause of deficient β-globin synthesis (4–7). Recently, a thalassemia β-globin gene was shown to contain a single nucleotide (nt) substitution in intron 1, a change that creates an alternative RNA acceptor splice site (8, 9) that functions *in vitro* (10, 11). Another mutation that causes β thalassemia is a single nt substitution (G → A) in the G-T consensus sequence of the 5′ end of the large intervening sequence (IVS) of the β-globin gene (12, 13). Other causes of β-thalassemia include nonsense or frameshift mutations that prevent synthesis of β-globin (14–19) and putative mutations that decrease β-globin mRNA stability (20). Rarely is β-thalassemia due to an actual deletion of all or a portion of the β-globin gene (1–3).

In order to determine the nature and frequency of mutations that alter RNA splicing, we mapped β-globin RNA obtained from bone marrow cells of 15 patients who are homozygous for the β-thalassemia phenotype (such patients may be doubly heterozygous for two distinct mutations, one in each of the two β-globin genes). A family of molecular probes was created by cloning a set of DNA fragments derived from the normal human β-globin gene in the M13 bacteriophage (21). The secreted single-stranded form of each recombinant served as a template for synthesis of a uniformly labeled probe of high specific activity (22). These probes can be used to precisely map the 5′ and 3′ ends of transcripts and the splice sites of β-globin mRNA, using small samples of total bone marrow RNA.

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EXPERIMENTAL PROCEDURES

Preparation of Bone Marrow RNA. After obtaining informed consent, we obtained 1–6 × 10^8 nucleated bone marrow cells from the posterior iliac crest; 2 × 10^7 cells were retained for measurement of globin synthesis (23). DNA and RNA were recovered from the remainder of the cells as described (5).

Construction of M13mp7–β-Globin Templates. Six fragments (Fig. 1) of a normal human β-globin gene (24) were isolated and their ends were converted to *Eco*RI sites (25) for insertion into the *Eco*RI sites of DNA from phage M13mp7. After standard transfections of *Escherichia coli* K-12 (strain JM103), appropriate recombinant clones were selected, each containing the strand of β-globin DNA that would serve as a template for the synthesis of a probe complementary to mRNA. Large amounts of single-stranded template DNA were prepared by inoculating 30 purified plaques derived from the clone of interest into 125 ml of yeast extract/tryptone medium; single-stranded DNA was recovered by using standard techniques after overnight incubation at 37°C.

Preparation of Probes by Using M13–β-Globin Templates. All reagents for probe synthesis were obtained from Bethesda Research Laboratories; all synthetic reactions are based on standard conditions recommended by this supplier. Twenty nanograms of universal 24-bp primer was annealed with 10–20 μg of the single-stranded M13 recombinant; synthesis of the radiolabeled strand was then directed by the large fragment of DNA polymerase I (Bethesda Research Laboratories), using [α-^{32}P]dATP (400 Ci/mmol, Amersham; 1 Ci = 3.7 × 10^{10} becquerels) and unlabeled dTTP, dCTP, and dGTP. Synthesis of the second strand completed a unique *Ava* I site 406 bp downstream from the β-globin insert in each of the six recombinant clones. After synthesis of the second strand, therefore, digestion with *Ava* I released a ^{32}P-labeled single-stranded copy of the globin insert with 406 nt of flanking M13 sequence; this radiolabeled fragment was very different in size from the linearized, unlabeled template strand. The single-stranded radiolabeled probes were easily purified on denaturing gels and recovered by electroelution. Details of these methods are available upon request. All cloning and recombinant DNA propagation were performed under appropriate conditions as suggested by the National Institutes of Health guidelines for recombinant DNA research.

Hybridization Reactions and S1 Nuclease Analysis. Bone marrow RNA (5–10 μg) was annealed with 20,000–50,000 cpm of single-stranded probe for 18 hr at 60°C in a 10-μl reaction

Abbreviations: nt, nucleotide; IVS, intervening sequence; bp, base pair.

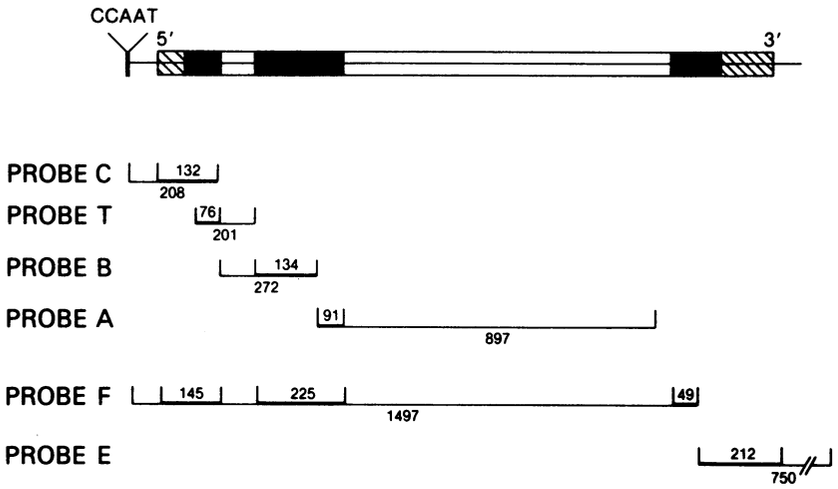

FIG. 1. Derivation of each of the β-globin DNA fragments used in the S1 nuclease mapping experiments. Coding sequences of the human β-globin gene are indicated by black boxes, the hatched areas represent the sequences encoding the untranslated part of the mRNA, and the open areas indicate IVS-*1* and IVS-*2*. CCAAT refers to the conserved sequence located 76 base pairs (bp) upstream from the start of coding sequence. For each of the probes, the number located above the line indicates the number of nt protected from S1 digestion by normally processed mRNA; the number below the line indicates the full length of the human DNA fragment included in the probe.

mixture containing 50% (vol/vol) formamide (4, 5). Each reaction mixture was then digested with 200 units of S1 nuclease (Miles) at 42°C for 30 min (26, 27), and the products were precipitated with ethanol and analyzed by electrophoresis on denaturing polyacrylamide sequencing gels (28).

RESULTS

Patients Studied. Fifteen regularly transfused patients who are clinically homozygous for β-thalassemia were studied. Fourteen are of Italian or Greek ancestry and the other is an American Black (patient 14). Control patients were selected because each had a disease that produced erythroid hyperplasia; four had sickle cell anemia and one had congenital dyserythropoietic anemia, type II.

S1 Nuclease Mapping of β-Globin RNA. Fig. 2 includes results obtained from analysis of control bone marrow RNA (patient 16) and of RNA from a patient with β-thalassemia (patient 4) with each of the six probes. Fig. 1 should be studied in order to clarify the origin of the fragments of each probe protected by mRNA.

Probe C. A 132-nt fragment protected by mRNA initiated at the normal position is the prominent band in both control and

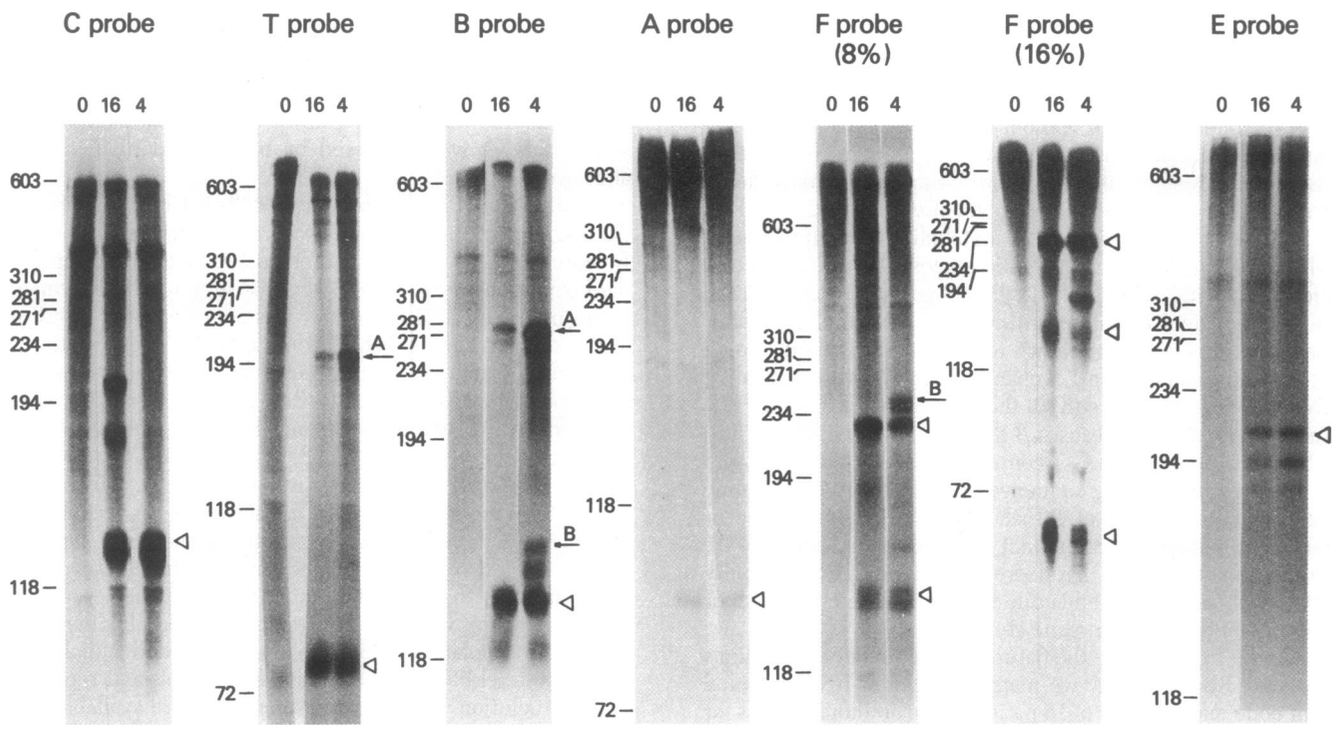

FIG. 2. Results of S1 nuclease mapping analysis from a control patient and a patient with β-thalassemia. Ten micrograms of total bone marrow RNA from a control patient with sickle cell anemia (patient 16) or a patient with β-thalassemia (patient 4) was hybridized with 20,000–50,000 cpm of each single-stranded radiolabeled probe and treated with S1 nuclease. The derivation of the probe used in each panel may be seen in Fig. 1. Lanes marked 0 in each panel indicate that no mRNA was present in the hybridization reaction; lanes 16 and 4 indicate RNA from those patients. The expected lengths of normally processed mRNA species are indicated by ◁ on the right side of each panel. Arrow A, marked for probes T and B, identifies a band derived by protection of the entire human DNA segment of the probe. Arrow B, marked for probes B and F, indicates the presence of a band arising from an abnormally processed RNA species retaining 19 nt from the 3′ end of IVS-*1*. The analysis of probe F fragments on the 16% polyacrylamide gel is designed to reveal the 49-nt fragment protected by correctly processed exon 3; the apparent sizes and relative proportions of the larger bands are distorted on this high-percentage gel. Numbers at the left side of each panel indicate the lengths, in nt, of simultaneously run markers.

thalassemic samples. Additional bands at 208 and 190 nt are also seen in the lane with control RNA. These additionals bands, present upon analysis of all control bone marrow RNA samples and three samples from patients with β-thalassemia, suggest the presence of transcripts initiated upstream from the normal position. This observation will be reported in detail elsewhere.

Probe T. A 76-nt fragment derived from this probe is protected by correctly processed mRNA sequences that are transcribed from the 3′ end of exon 1 in both control and thalassemia RNA samples (Fig. 2). The band of 201 nt arises from protection of the entire globin gene segment of the probe by RNA precursor from which IVS-*1* sequences have not been removed. With RNA from control patient 16 this band is faint, but RNA from patient 4 protects more of this fragment. This band is not caused by hybridization of the probe with contaminating template DNA because no band is seen at this position in the control lane (0) that contained no RNA in the reaction mixture. Furthermore, the full-length probe is 406 nt longer than the β-globin gene segment, because it contains flanking sequences derived from M13.

Probe B. A 134-nt fragment of this probe is protected by normally processed mRNA sequences transcribed from the 5′ end of exon 2. This band is prominent upon analysis of both the control and thalassemic RNA samples (Fig. 2). RNA precursor-containing sequences transcribed from IVS-*1* protects the entire human segment of the probe (272 nt). Note a faint band at this position in the control RNA lane (lane 16) but a very prominent band in this position with analysis of the thalassemic RNA (lane 4). Two additional DNA fragments of approximately 153 and 143 nt are visible in lane 4. The longer one (153 nt) is caused by protection of the probe by an mRNA molecule incorrectly processed to retain 19 nt of the 3′ end of IVS-*1*. The 143-nt fragment presumably arises because of S1 nuclease "nibbling" of the duplex that yields the 153-nt band (10). Fig. 3 presents the results obtained in the analysis of seven thalassemic bone marrow RNA samples with probe B. Note that four patients (nos. 4, 5, 8, and 10) have markedly increased amounts of RNA precursor as indicated by an intense 272-nt band. Two additional patients (nos. 7 and 9) have mildly increased amounts of precursor; one patient (no. 6) has a band of normal intensity. Again, this band does not represent simple hybridization of the probe with contaminating template because there is no corresponding band in the control lane containing no RNA (lane 0). The bone marrow RNA of four patients contains an incorrectly processed RNA species that retains 19 nt transcribed from the 3′ end of IVS-*1*, as indicated by a DNA fragment of 153 nt in lanes 4, 7, 8, and 9. Analysis of reticulocyte RNA obtained from the patient whose gene was sequenced by Spritz *et al.* (8) yields identical bands at 153 and 143 nt, in addition to the band of 134 nt arising from normally processed mRNA (data not shown). RNA from this patient, who is thought to be homozygous for this mutation, generated an intense band at 272 nt.

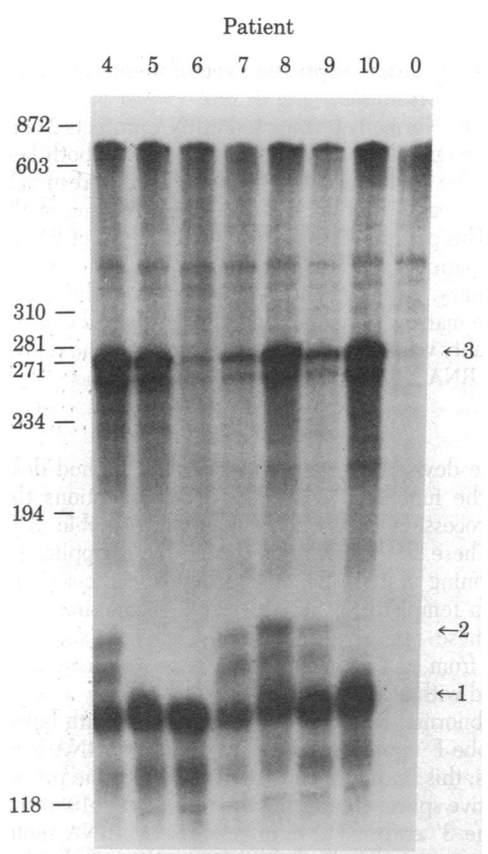

FIG. 3. Analysis of total bone marrow RNA from seven β-thalassemic patients (4–10) with probe B. Ten micrograms of total bone marrow RNA was hybridized with 20,000 cpm of the single-stranded probe and subsequently treated with S1 nuclease. The numbered positions correspond to bands of 134 nt (1), 153 nt (2), or 272 nt (3); these are discussed in detail in *Results*. The positions of phage φX174 *Hae* III marker DNA fragments are indicated on the left (lengths in nt).

Probe A. A 91-nt fragment of this probe is protected by normally processed mRNA transcribed from the 3′ end of exon 2; this band is seen on analysis of both the control and thalassemic bone marrow RNA samples. Prolonged radioautographic exposure of the gel from which the data in Fig. 2 were derived failed to reveal any other DNA fragments; analysis of RNA from 17 additional thalassemic and control patients yielded identical results (Table 1).

A band arising from protection of the entire human DNA segment of the probe by unprocessed precursor would not be seen because of the background radioactivity at the top of this gel. RNA from patients 1–14 and 16–20 was therefore analyzed with a single-stranded probe derived from IVS-*2* sequences only (data not shown). A complex series of bands was seen because of S1 nuclease nicking of the A+T-rich regions in IVS-*2*. This pattern of bands was identical in all patients, however, implying that precursor containing IVS-*2* sequences was indeed identical in all patients studied.

Probe F. Normally processed mRNA should protect three DNA fragments derived from this probe. Sequences transcribed from exon 1 protect a 145-nt fragment, those from exon 2 protect a 225-nt fragment, and sequences transcribed from the 5′ end of exon 3 protect a 49-nt fragment. All of these bands are seen on analysis of RNA from both the control and thalassemic patients (Fig. 2). The 145- and 225-nt fragments were defined by electrophoresis on an 8% polyacrylamide gel; the 49-nt fragment was defined by analysis on a 16% gel. An additional DNA fragment of 244 nt (arrow B), visible with analysis of the thalassemic bone marrow RNA, provides further evidence of the presence of an aberrantly processed mRNA molecule that retains 19 nt transcribed from the 3′ end of IVS-*1*. Fig. 4 shows the data obtained on the analysis of bone marrow RNA from five thalassemic patients and five control patients by using probe F. Note that thalassemic patients 1, 4, and 11 have the aberrantly processed RNA species that retains sequences transcribed from the 3′ end of IVS-*1*. An additional fragment of 165 nt is protected by an RNA species present in a significant concentration in the bone marrow RNA from all of the thalassemic patients tested. This segment of probe F is protected by crossreacting δ-globin mRNA sequences, as verified by study of the RNA transcribed from a cloned human δ-globin gene in

Table 1. Summary of mRNA mapping data

Patient*	Probe C	Probe T		Probe B			Probe A	Probe F			Probe E	Processing errors identified†
				3′ IVS-*1* splice								
	5′ end cap	5′ IVS-*1* splice	Increased precursor	N1	Alt	Increased precursor	5′ IVS-*2*	3′ IVS-*1* Alt	5′ IVS-*2*	3′ IVS-*2*	3′ end	
1 β^+	+	+	−	+	+	−	+	+	+	+	+	B
2 β^0	+	+	−	+	−	−	+	−	+	+	+	0
3 β^0	+	+	+	+	−	+	+	−	+	+	+	A
4	+	+	+	+	+	+	+	+	+	+	+	A + B
5	+	+	+	+	−	+	+	−	+	+	+	A
6	+	+	−	+	−	−	+	−	+	+	+	0
7 β^+	+	+	+	+	+	+	+	+	+	+	+	A + B
8 β^+	+	+	+	+	+	+	+	+	+	+	+	A + B
9 β^+	+	+	+	+	+	+	+	+	+	+	+	A + B
10	ND	ND	ND	+	−	+	ND	−	+	+	ND	A
11 β^+	+	+	−	+	+	−	+	+	+	+	+	B
12 β^+	+	+	−	+	−	−	+	−	+	+	+	0
13 β^0	+	+	+	+	−	+	+	−	+	+	+	A
14 β^+	+	+	−	+	−	−	+	−	+	+	ND	0
15 β^+	+	+	+	+	+	+	+	+	+	+	ND	A + B
16–20 (controls)	+	+	−	+	−	−	+	−	+	+	+	−

The presence (+) or absence (−) of each RNA processing event and the presence or absence of increased precursor-containing sequences transcribed from IVS-*1* are indicated for each patient. ND, not done. N1 indicates a normal 3′ IVS-*1* splice; Alt indicates an alternative splice causing retention of 19 nt from the 3′ end of IVS-*1*.

* β^+ and β^0 were assigned on the basis of measurement of the β-globin synthetic rate or by hemoglobin electrophoresis performed when the patient was not receiving transfusions.

† Processing error A indicates the presence of increased RNA precursor containing sequences transcribed from IVS-*1*. B indicates the presence of an abnormally processed mRNA species retaining 19 nt from the 3′ end of IVS-*1*. 0 indicates no processing errors identified.

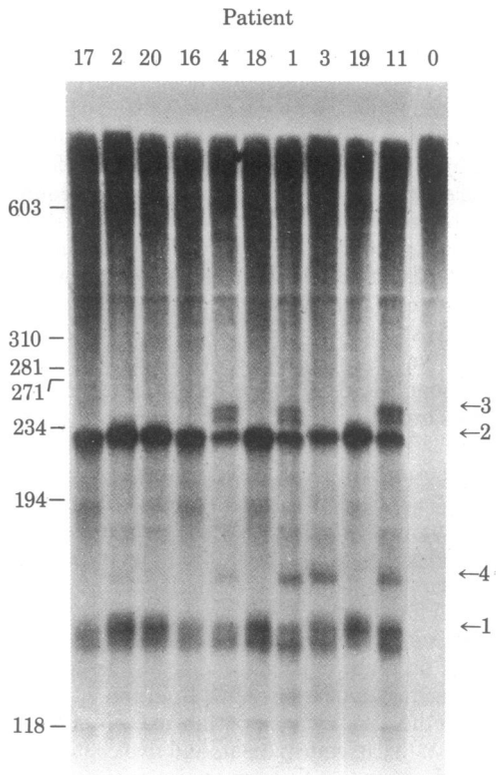

FIG. 4. Analysis of total bone marrow RNA from five thalassemic patients (1, 2, 3, 4, and 11) and five control patients (16–20) with probe F (see Fig. 1). Ten micrograms of total bone marrow RNA was annealed with 50,000 cpm of probe F in each hybridization reaction. The numbered positions correspond to bands of 145 nt (1), 225 nt (2), 244 nt (3), and 165 nt (4); these are discussed in detail in *Results*. The numbers on the left indicate the position of φX174 *Hae*III DNA fragments.

an amplified plasmid expression vector in monkey kidney cells (unpublished results).

Probe E. Normally terminated mRNA protects 212 nt of this probe; a fragment of this length is present in both lane 4 and lane 16. The additional DNA fragments seen presumably arise by S1 nuclease "nibbling" of A+T-rich regions of these duplexes. This pattern was observed with analysis of RNA samples from all patients studied (Table 1).

Summary. Table 1 contains the results of the analysis with the bone marrow samples from all patients and controls. Each abnormality was verified by repeat analysis of the relevant bone marrow RNA.

DISCUSSION

We have devised a generally applicable method designed to detect the functional consequences of mutations that affect RNA processing by precisely mapping β-globin RNA molecules. These studies were facilitated by an application of the M13 cloning system (22), using high-specific-activity probes made on templates of M13–β-globin recombinant DNA. Because these probes are uniformly labeled, sequences transcribed from several portions of a gene can be simultaneously detected with a single probe.

An abnormal RNA species was detected with both probe B and probe F upon analysis of bone marrow RNA from 7 of 15 patients; this finding is consistent only with the presence of an alternative splice site causing the retention of 19 nt transcribed from the 3′ end of IVS-*1* in processed mRNA molecules. A mutation creating such an alternative splice site has been found by DNA sequence analysis of genes cloned from two β^+-thalassemic patients (8, 9). We studied RNA from one of these patients and found the same abnormally processed species. A gene affected by the alternative splice site mutation has been studied in a cellular expression system; although the alternative splice site is used 90% of the time, the presence of normally processed

molecules is consistent with a β^+-thalassemia phenotype (10). Indeed, 6 of the 7 patients in our study with this mutation were documented to have β^+-thalassemia (Table 1).

Analysis of bone marrow RNA with probes B and T reveals that 9 of 15 patients demonstrate a significantly increased concentration of RNA molecules that retain sequences transcribed from IVS-*1*. Several explanations for this observation are possible. In a cellular expression system, study of a β^+-thalassemia gene containing the alternative splice site in IVS-*1* has suggested that this mutation may lead to increased accumulation of RNA precursor that retains all sequences transcribed from IVS-*1* (10). Indeed, analysis of reticulocyte RNA from a patient thought to be homozygous for this mutation reveals a markedly increased concentration of such precursor molecules (unpublished results). Other patients, who may be heterozygous for this mutation, appear to have only a mild increase in unprocessed precursor with IVS-*1* sequences, however (Fig. 3, patients 7 and 9).

We have evidence that at least two of the patients with the alternative splice site mutation have a different mutation in their other β-globin gene that may be expected to affect removal of sequences transcribed from IVS-*1*. These patients (nos. 4 and 8) are both heterozygous for a *Bam*HI polymorphism (unpublished observations) previously shown to be linked to a G → A substitution in the G-T consensus sequence at the 5′ end of IVS-*1* (29). This G-T dinucleotide is thought to be essential for function of the splice site. Each of these patients has markedly increased precursor that contains sequences transcribed from IVS-*1*. The same *Bam*HI polymorphism is also linked to an identical G → A mutation at the 5′ end of IVS-2; we know that patients 4 and 8 did not have this mutation because we were unable to detect any abnormal species in their bone marrow RNA with probe A (Fig. 2 and Table 1).

Previous studies have suggested that bone marrow cells of many patients with β-thalassemia may contain normal or even increased amounts of RNA molecules that contain sequences transcribed from IVS-2 (5, 7); our studies do not allow quantitation of such precursor molecules. We found no evidence in 14 thalassemic patients for one known processing mutation affecting IVS-2, namely the G → A substitution at its 5′ end (12, 13); we were able to detect aberrantly spliced RNA molecules in RNA obtained from an obligate carrier of this gene, however (unpublished results). Nonsense or frameshift mutations that render correctly processed β-globin mRNA nonfunctional are presumably associated with a normal rate of β-globin gene transcription; precursor molecules might therefore be present in normal (or even increased) amounts in patients affected by these mutations. Our studies would not be expected to detect mutations that alter the translatability or stability of normally processed β-globin mRNA molecules.

This method provides direct evidence of the *in vivo* consequences of mutations that affect RNA processing. These studies have also identified two patients (nos. 12 and 14) with β^+-thalassemia and *no* detectable processing errors, who may have as yet uncharacterized mutations that affect β-globin gene expression.

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