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Genetics

Two mutations in the β-globin polyadenylylation signal reveal extended transcripts and new RNA polyadenylylation sites

(β-thalassemia/RNA processing/polymerase chain reaction)

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ABSTRACT **Two mutations in the β-globin poly(A) signal were identified in Israeli patients with β^+-thalassemia by sequence analysis following PCR. One is a point mutation (AATAAA → AATAAG) and the other is a 5-base-pair deletion (AATAAA → A———). The mutant genes were used to investigate the function of the poly(A) signal *in vivo* and to evaluate the mechanism whereby these mutations lead to a thalassemic phenotype. Analysis of RNA derived from peripheral blood demonstrated the presence of elongated RNA species in patients carrying either mutation. Other aspects of RNA processing (initiation, splicing) were unimpaired. RNA obtained from the patients carrying the point mutation contained four discrete, extended RNA species, 1500–2900 nucleotides long, which were found to be polyadenylylated. Some normal cleavage-polyadenylylation was also observed. The 5-base-pair deletion completely abolished cleavage at the normal site. This deletion mutation resulted in a phenotype of β^+-thalassemia, thus providing evidence that the extended mRNAs are translatable *in vivo*. Furthermore, additional transcripts, >5 kilobases, presumably mRNA precursors, were found in all RNA samples, including those of nonthalassemic controls. The extended transcripts of the poly(A) mutants, together with the high molecular weight precursors, suggest that the human β-globin gene transcription unit is significantly longer than previously recognized.**

The mechanism of 3′ end processing of newly formed RNA transcripts in mammals is not well understood. The majority of nonhistone mRNAs in higher eukaryotes are polyadenylylated at their 3′ ends (for reviews, see refs. 1 and 2). The highly conserved sequence AAUAAA is located 10–30 nucleotides (nt) upstream from the poly(A) addition site. It is known that transcription proceeds beyond the poly(A) addition site, but the sites of transcription termination for mammalian genes are mostly unknown. Recent studies have focused on the endonucleolytic cleavage and the ensuing addition of several hundred adenylate residues to the 3′ terminus (3).

β-Thalassemia is an autosomal recessive disease characterized by defective synthesis of the β-chains of the hemoglobin tetramer. The disease can be subdivided into two types: β^+, in which some chains are present, or β^0, in which β-chain synthesis is absent. The disease is caused by any of >90 point mutations, in addition to a smaller number of deletions, which impair β-globin gene function to various degrees (4). Following their discovery, functional studies of many such mutant alleles were undertaken. These studies have led to a wealth of information on critical nucleotide sequences required for proper gene function and clarified the effect of the mutations on the clinical condition. In all, five mutations in the AATAAA sequence have been observed to lead to β^+-thalassemia (4), supporting the crucial nature of this hexanucleotide in 3′ end processing.

Functional analysis of thalassemia alleles can be accomplished by analyzing RNA obtained from patients (5, 6). However, patient RNA is not always available. In addition, these studies are sometimes complicated by the presence of two different thalassemia mutations in the same patient. An alternative approach is to study the expression of the cloned mutant gene in heterologous cells, such as HeLa or COS. However, differences in the utilization of various splice sites and relative β-globin stability in heterologous cells have been reported (7, 8).

In studies on RNA derived from a patient with a point mutation in the human α-1 globin gene, AATAAA → AATAAG (9), decreased levels of mature α-mRNA were seen. Expression studies of the cloned mutant gene in HeLa cells demonstrated extended RNA products, indicating "read-through" past the mutated signal. Another point mutation, in the human β-globin gene, was similarly investigated (10). In this case, extended transcripts were seen in RNA derived from the patient's erythroid cells. The extended transcripts were ≈1500 nt in length, which corresponded to the location of the next AATAAA downstream from the normal signal. The authors suggested that, in the presence of the mutation, this downstream signal became the functional poly(A) signal. Whether the mutant allele contributed to normal-size mRNA production *in vivo* could not be assessed, since the mutation was present in compound heterozygosity with a β^s allele, which produced normal-size mRNA.

More recent investigations focused on the role of auxiliary signals required for proper 3′ end processing. Initially, such signals were suggested by comparison of conserved sequences distal to the poly(A) sites of different genes. Some candidate sequences that were identified were TGTGTTG-GAA (11, 12), YGTGTTYY (13), and the trinucleotide TGT in conjunction with oligo(T) stretches (14). More recently, recombinant DNA techniques were utilized to create various synthetic poly(A) sites and analyze their relative efficiencies (15, 16). It seems that a (G+T)-rich or T-rich element, at a defined distance within 50 nt 3′ to the poly(A) site, is crucial.

We report here the identification and functional analyses of two β-thalassemia mutations in the poly(A) signal. These mutations afforded a unique opportunity to investigate the function of poly(A) signals *in vivo*.

MATERIALS AND METHODS

Sequencing of the Mutant Alleles. Direct genomic sequencing was performed on unknown alleles after purifying the PCR products with a Centricon 30 column, using the dideoxy method, as described (17).

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Abbreviations: nt, nucleotide(s); IVS, intervening sequence.

Isolation of RNA from Peripheral Blood. RNA was isolated from peripheral blood normoblasts or reticulocytes as described (18). All RNA samples were quantitated by absorbance at 260 nm. To ensure that the RNA was not degraded, samples were analyzed by gel electrophoresis and ethidium bromide staining.

Selection of Poly(A)$^+$ RNA. Poly(A) RNA was isolated from total peripheral blood RNA on oligo(dT)-cellulose columns (19). We used a Pharmacia Quickprep mRNA kit.

RNase Protection Studies. RNase mapping (20) was performed using ≈1-μg samples of peripheral blood RNA. These were hybridized with the following probes: 5′ end of α-globin gene (*Hae* II–*Ava* I), 5′ probes of β-globin gene (*Bal* I–*Hae* III and *Bal* I–*Bam*HI), intervening sequence 2 (IVS2) of β-globin gene (*Bam*HI–*Eco*RI), and 3′ end of β-globin (*Eco*RI–*Pst* I) (see Figs. 1 and 2*B*). Probes were labeled with [^{32}P]UTP using the Riboprobe kit (Amersham). The relative amounts of erythroid RNA in the samples were determined based on the intensity of α-globin RNA signal, using densitometry tracings of the autoradiograms, exposed for short periods to ensure linearity of the band intensities. Equal amounts of erythroid RNA were then utilized for mapping with β-globin probes.

Northern Analysis. Northern analyses (21) were performed using ≈1-μg samples of total peripheral blood RNA (except where otherwise indicated), adjusted to equivalent amounts of erythroid RNA as described above. In most cases, the SP6 vector-generated RNA probes described above were utilized (10^6 cpm of probe per ml of hybridization solution). An additional RNA probe corresponded to the *Acc* I–*Hin*cII fragment (coordinates 65,917–66,782) located >2 kilobases (kb) 3′ to the β-globin gene. This probe represents a unique sequence, although it is derived from a region highly rich in repetitive elements (*Alu* and *Kpn*). In addition, a DNA probe was used (*Dra* I fragment, 1087 nt in length, coordinates 63,859–64,946) that was radiolabeled using a random primer labeling kit (Amersham). This also represents a unique sequence from the region 3′ to the normal poly(A) signal of the human β-globin gene (Fig. 3*D*).

For experiments with RNA probes, 1 μg of peripheral blood RNA was used. Hybridization was performed at 55°C and final washes were at 65°C. Standard RNA size markers (purchased from BRL) were run in parallel and visualized by ethidium bromide staining. For hybridization with the DNA probe, 15 μg of peripheral blood RNA per lane was used.

RESULTS

Mutations in the β-Globin Poly(A) Signal. In the course of identification of β-thalassemia alleles in Israeli ethnic groups, two mutations in the poly(A) signal were discovered. One of the mutations is an A → G transition in the last A of the hexanucleotide, AATAAA → AATAAG, discovered in a patient of Kurdish Jewish extraction with transfusion-dependent β-thalassemia major. This allele, which is linked to Mediterranean haplotype VII, was subsequently found to represent about 10% of thalassemia chromosomes in this ethnic group (22, 23). However, homozygotes for the mutation have not yet been identified. In the present study three adult patients (nos. 2, 3, and 4 in Figs. 1 and 3) of two unrelated families were studied. All three patients have been splenectomized and have a considerable number of nucleated red cells (normoblasts) in their peripheral blood. All three are compound heterozygotes for the poly(A) mutation and a frameshift mutation in codon 44 (24), which results in very unstable β-globin mRNA (25). These patients were selected for the RNA studies because expression of the codon 44 allele does not result in a significant mRNA background. The β/α-globin chain synthesis ratios were $\beta/\alpha = 0.3$, 0.25, and 0.14 for patients 2, 3, and 4 (Figs. 1 and 3), respectively.

The second mutation is a 5-base-pair (bp) deletion, AATAAA → A———, found in a young Arab child from Gaza, homozygous for the mutation. She also suffers from transfusion-dependent β-thalassemia major, with chain synthesis ratios $\beta/\alpha = 0.10$ and $\gamma/\alpha = 0.22$. This mutation has not yet been discovered outside the present family. The parents are first cousins. The β-globin haplotype (26) linked to this mutation has not been previously described in thalassemic chromosomes. Using the following polymorphic sites, *Hin*cII ε, *Hin*dIII Gγ and Aγ, *Hin*cII ψβ (two sites), *Ava* II β, and *Bam*HI 3′ to β, the haplotype was −+−−+ ++.

RNase Protection Experiments. Peripheral blood RNA was analyzed using an α-globin probe and four probes spanning the entire β-globin gene. Fig. 1 shows the results of analysis of three patients carrying the point mutation in compound heterozygosity with the frameshift mutation in codon 44 (24). Since the number of cells of the erythroid lineage varies among the various blood samples, hybridization with the α-globin probe was used to standardize the amounts of erythroid RNA in the samples.

The analysis (Fig. 1) demonstrated that the three patients had diminished amounts of β-globin mRNA (lanes 2–4 of each panel) compared to a nonthalassemic control (lanes 1). The levels of β-globin mRNA in the patients were determined, by densitometer tracing, to be 34%, 12%, and 25% of the normal level for patients 2, 3, and 4, respectively. As a control, we analyzed in parallel RNA from a homozygote for the frameshift 44 mutation, which has been previously shown to lead to the formation of unstable transcripts with a very short half-life (25). Only trace amounts of normal-size β-globin RNA are seen (lane 5 of each panel). Therefore the significant amount of normal-size β-globin RNA in the com-

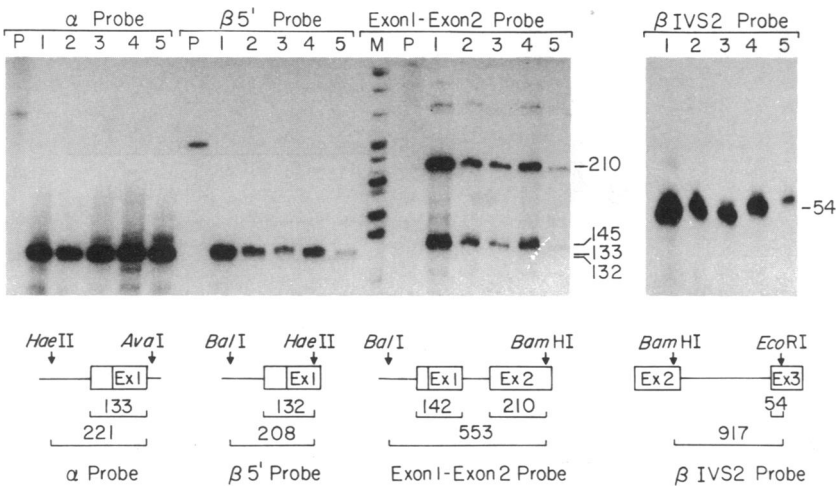

FIG. 1. RNase mapping analysis of the cleavage-polyadenylylation mutations. RNase mapping was performed according to Melton *et al.* (20) with the designated RNA probes. Lanes: 1 (control), a splenectomized nonthalassemic patient with a high reticulocyte count due to immune hemolytic anemia; 2–4, patients 2, 3, and 4, respectively [all three patients are splenectomized, compound heterozygotes for the poly(A) point mutation and the frameshift mutation in codon 44]; 5, splenectomized patient, homozygous for the frameshift mutation in codon 44. M, size marker (in nt) (*Hpa* II digest of pBR322); P, probe control. The probes and expected sizes of normal protected fragments are shown below.

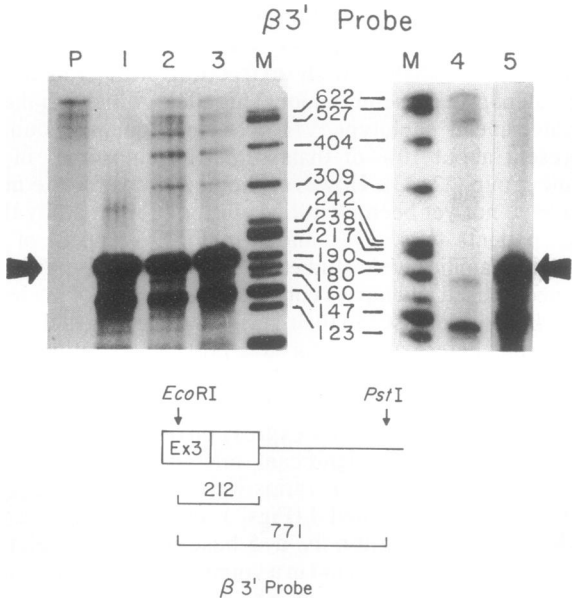

FIG. 2. Analysis of 3′ cleavage. The autoradiogram is overexposed to demonstrate the presence of longer RNA species. Lanes: 1 and 5, control RNA from the nonthalassemic patient shown in Fig. 1; 2 and 3, patients 2 and 3, as in Fig. 1; 4, homozygote for the deletion mutation. The arrows indicate the normal-size protected fragment. M, size marker (in nt). The probe is shown below.

pound heterozygous patients resulted from transcription of the poly(A) mutant allele.

RNase mapping demonstrated that the initiation of transcription (Fig. 1, β5′ probe), splicing out of IVS*1* (exon 1–exon 2 probe), and IVS*2* (β-IVSII probe) were correct. Analysis using the 3′ β-globin probe (Fig. 2, *Eco*RI–*Pst* I probe) revealed the presence of a normal-size protected fragment for the patients with the point mutation (lanes 2 and 3). In addition, bands of higher molecular weight, including full-length protection of the probe, were observed. These longer RNA species were not seen in the nonthalassemic RNA control (lane 1) analyzed in parallel.

In the RNA of the patient homozygous for the 5-bp deletion there was no normal-size protected fragment with the 3′ β-globin probe (Fig. 2, lane 4). Full-length protection of the probe was seen, in addition to the presence of another elongated RNA species, neither of which was present in the controls (lanes 1 and 5).

Northern Analysis of RNA. The RNase protection experiments indicated elongated transcripts that suggested malfunction of the cleavage-polyadenylylation signal. To clarify the nature of these elongated transcripts and to determine their size, analyses by Northern blotting were performed (Figs. 3 and 4). These confirmed the presence of a significant amount of normal-size message, albeit at a decreased level, in the patients with the point mutation (Fig. 3*A*, lanes 2–4). In addition, four longer RNA species, ≈1500, ≈1650, ≈2450, and ≈2900 nt in length, were seen. These longer transcripts were better visualized after a longer exposure of the autoradiogram (Fig. 3*B*, arrowheads).

To confirm that these transcripts were extended beyond the normal cleavage point, we used a probe specific to the region 3′ to the gene (*Dra* I 1087-bp fragment, coordinates 63,859–64,946, Fig. 3*D*). This probe does not include any repetitive sequences (*Alu* or *Kpn*) 3′ to the β-globin gene. As expected, this probe did not hybridize to normal-size mRNA (note absence of signal in Fig. 3*B*, lane 1, *Dra* I panel). However, it did hybridize to the longer RNA species (Fig. 3*B*, *Dra* I panel), proving that these transcripts were extended beyond the normal 3′ end of the β-globin gene.

To determine whether the extended RNA molecules are polyadenylylated, total peripheral blood RNA of one of the patients (patient 2) was separated on an oligo(dT)-cellulose column (19). Analysis of the fractions by gel electrophoresis and ethidium bromide staining revealed that all rRNA was in the high-salt buffer wash. The column fractions were analyzed by Northern blotting (Fig. 3*C*). The results demonstrated that not only the normal-size mRNA but also the four extended transcripts were eluted with the poly(A)$^+$ fraction, indicating that these RNA molecules are poly(A)$^+$. Moreover, the relative intensity of the extended species remained the same after poly(A) selection. No β-globin-specific transcripts were observed in any of the other column fractions.

In addition, all RNA samples, including the nonthalassemic controls, contained various levels of extremely long RNA species, >5 kb (Figs. 3 and 4). These transcripts are seen in large quantities in the RNA of the patient homozygous for the frameshift 44 mutation and in the homozygote for the poly(A) deletion mutation, at lower levels in the sickle homozygote, and at very low levels in the nonthalassemic control and the three patients who were compound heterozy-

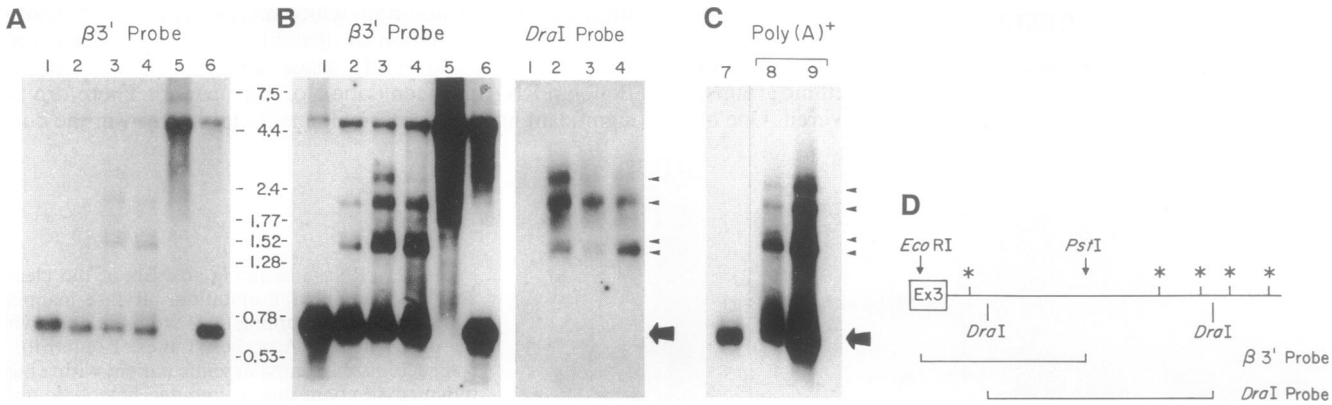

FIG. 3. Northern analysis of the poly(A) point mutation. (*A* and *B*) β3′ Probe. Lanes: 1, nonthalassemic patient control (same as in Fig. 1); 2–4, patients 3, 2, and 4, respectively; 5, homozygote for frameshift 44 mutation (same patient as in Fig. 1); 6, homozygote for hemoglobin S (functionally asplenic). The large arrow points to normal-size mRNA and the small arrowheads point to extended RNA molecules. The probe used in hybridization is designated. The β3′ blot was exposed for 1 day (*A*) and 7 days (*B*). (*B*) *Dra* I blot. The *Dra* I blot was exposed for 4 days. Lanes: 1, nonthalassemic control (same as in Fig. 1); lanes 2–4, patients 2, 3, and 4, respectively. Sizes are in kb. (*C*) RNA selected for poly(A)$^+$. Lanes: 7, nonthalassemic control (as in lanes 1); 8 and 9, RNA of patient 2 (shown in lane 3 of the β3′ panel) after poly(A)$^+$ selection; 9, four times more RNA than lane 8. The probe used was β3′. (*D*) Diagrams of probes. The *Dra* I probe, 1087-nt DNA fragment, was isolated from an agarose gel and labeled using a random primer labeling kit (Amersham). The asterisks indicate AATAAA signals.

gotes for frameshift 44 and the poly(A) point mutations. An attractive explanation is that these represent β-globin mRNA precursors. These transcripts are almost undetectable after poly(A) selection (Fig. 3*C*), suggesting that they are not polyadenylylated.

To test whether these putative precursors were extended in a 3′ direction, the blots were rehybridized to a different probe, an *Acc* I–*Hin*cII fragment (865-nt antisense RNA probe, spanning coordinates 65,917–66,782, not including *Alu* or *Kpn* repeat elements) >2 kb 3′ to the β-globin gene. The probe hybridized to the high molecular weight (≈5 kb) transcripts of all blots (not shown), including the blot previously hybridized with the *Dra* I probe. These findings indicate that those putative mRNA precursors are extended far beyond the normal end of the β-globin gene. We suspect that these bands were not visible on hybridization with the *Dra* I probe because of the considerably decreased sensitivity of the DNA probe. Consistent with this notion, the bands did appear upon rehybridization of the same blot to two additional RNA probes (β-3′ and β-IVS2, results not shown).

In contrast to the findings in the patients with the point mutation, the homozygote for the deletion did not show any normal-size mRNA, even when a 4-fold greater amount of RNA (Fig. 4*A*, lane 2) was used. Instead, the analysis showed a substantial amount of large transcripts, with no apparent discrete bands. We interpret these to be the β-globin mRNA precursor, which is abundant in this patient for unknown reasons. The large amount of precursor present in the RNA of this patient could be masking discrete bands of extended RNA molecules, similar to those of the poly(A) point mutation. Rehybridization of this blot with the distal 3′ probe (*Acc* I–*Hin*cII) demonstrated that the putative precursor is extended in the 3′ direction (Fig. 4*B*). As expected, the normal-size mRNA did not hybridize with this probe (Fig. 4*B*, lane 4).

Computer Analysis. A computer search of the sequence 3′ to β-globin demonstrated the presence of many AATAAA signals in that region (Fig. 5). Those most likely to produce extended species of the sizes estimated from the RNA analyses are indicated in Fig. 5.

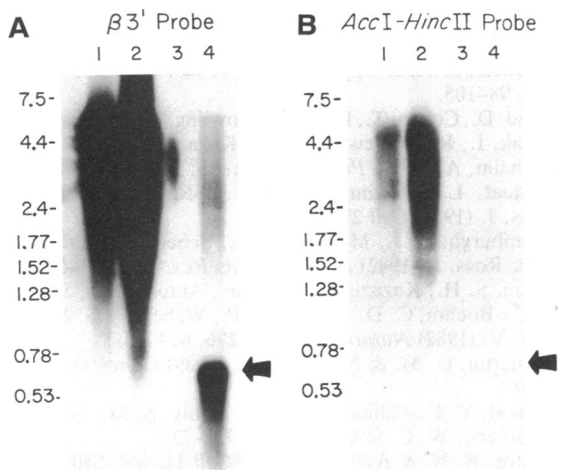

FIG. 4. Northern analysis of the poly(A) deletion mutation. (*A*) Lanes: 1 and 2, peripheral blood RNA from a homozygote for the deletion mutation, 1 μg (lane 1) and 4 μg (lane 2); 3, no RNA; 4, nonthalassemic control with reticulocytosis due to immune hemolytic anemia (this patient has not been splenectomized). The arrow indicates normal-size β-globin mRNA. The probe used was β-3′. (*B*) The same blot as in *A* rehybridized to the *Acc* I–*Hin*cII probe from the region 3′ to the β-globin gene (coordinates 65,917–66,782). The normal β-globin cleavage signal is located at coordinate 63,770. Note the absence of hybridization with normal-size mRNA (arrow). Sizes are in kb.

DISCUSSION

We have analyzed RNA derived from patients' peripheral blood to study the effect of two different mutations in the poly(A) signal on RNA processing and translation. The use of RNA derived directly from peripheral blood allowed analysis of the function of the poly(A) signal *in vivo*, avoiding artifacts that are often encountered in expression studies of exogenous genes in heterologous cells (7, 8). Our analyses showed that the three patients with the point mutation in the poly(A) signal expressed 12–34% of normal β-globin mRNA, consistent with the clinical manifestation of moderate to severe β^+-thalassemia major. The patient with the deletion did not have any normal-size message. Her clinical picture is more severe, with a higher transfusion requirement.

Requirement for AATAAA Signal for mRNA Cleavage. The highly conserved hexanucleotide AATAAA was recognized as essential for proper cleavage, and many subsequent studies have emphasized its importance (2). Our studies demonstrated that an intact AATAAA signal is not an absolute requirement: a significant amount of correct cleavage occurred in spite of the A → G transition. In contrast, the more severe defect, the 5-bp deletion, totally abolished cleavage at the normal site.

Not All AATAAA Signals Are Functional. An intriguing finding is the presence of multiple, discrete, elongated β-globin mRNA species in RNA derived from patients carrying the point mutation (Fig. 3*A*). The shortest of these, which is 1500 nt long, has previously been observed in RNA of an American Black patient with sickle/β-thalassemia, who carries a different mutation in the poly(A) signal, AATAAA → AACAAA (10). However, the three longer RNA species, which are ≈1650, ≈2450, and ≈2900 nt long, have never been reported before. It is possible that the detection of these low-level β-globin transcripts in our studies is due to the increased sensitivity of the RNA probes that were used.

It is of interest that many AATAAA sequences are present distal to the wild-type poly(A) signal, within the range predicted from the sizes of the extended RNA molecules (Fig. 5). However only four extended, poly(A)$^+$ RNA species were observed. This suggests that AATAAA sites are utilized selectively—that is, some signals are functional, whereas adjacent ones are not. In addition, from the intensity of the various bands, it appears that, among the functional poly(A) signals, some are more efficiently utilized than others (or yield more stable RNA). This finding emphasizes, as has been noted by others, that the AATAAA signal is required but not sufficient for cleavage-polyadenylylation.

There have been many reports that attempted to define the accessory signals required for activity of poly(A) signals. In particular, (G+U)-rich regions distal to and within 50 nt downstream to the hexanucleotide were found necessary for

Coordinate		
63,770:	TGCCT AATAAA AAACA	- Normal cleavage signal
64,696:	CTGGA AATAAA ATATG	- +926 nt (~1500 nt)
64,891:	ACAAA AATAAA TTTGA	- +1121 nt (~1650 nt)
65,033:	GGAAA AATAAA GAAGT	
65,220:	AGGAA AATAAA AAAAT	
65,466:	GGATG AATAAA TTCTA	- +1696 nt (~2450 nt)
65,986:	ACCTC AATAAA GTTGA	
66,002:	AGAAT AATAAA AAAGA	
66,043:	TAAAA AATAAA AAAAT	
66,136:	AATAC AATAAA TTTTA	- +2366 nt (~2900 nt)
66,473:	AGTGG AATAAA TAGAA	
66,555:	TTGGA AATAAA AAACA	
66,683:	ACTAG AATAAA AATAT	
66,770:	GGAGA AATAAA TTCAG	

FIG. 5. Computer search for AATAAA signals downstream to the β-globin gene. Functional signals, predicted by RNA analysis, are designated on the right.

proper 3′ end processing to occur (15, 16). However, although these signals are important, they are not as well conserved as the AATAAA signal and have been only vaguely defined as U-rich or (G+U)-rich sequences. It is likely that these downstream sequences participate in forming secondary structures necessary for recognition and/or activity of the cleavage enzymes (3, 27). It is possible that (G+U)-rich elements present further downstream are brought into proximity to the AAUAAA signal by tertiary RNA interactions, as recently shown for the human T-lymphotropic virus type I Rex response element (28). Our computer search for (G+T)-rich regions 3′ to the downstream AATAAA signals did not explain the preferential selection of the particular sites. Further work is required to elucidate the mechanism by which these particular AATAAA sequences are selected.

The Human β-Globin Gene Primary Transcript Is >5 kb Long. All RNA samples, including those of the nonthalassemic controls, contained various levels of extremely long RNA species, >5 kb (Figs. 3 and 4) that are not polyadenylylated. These transcripts are extended in the 3′ direction, as shown by hybridization to the distal 3′ probes. These most likely represent mRNA precursors, usually present at very low levels because they are rapidly processed. The longest human precursors previously reported were ≈2 kb, observed in pulse–chase analyses of RNA derived from peripheral blood and bone marrow of patients homozygous for the frameshift 44 mutation (25). It is of interest that in early experiments in mouse erythroleukemia cells (29) and in avian erythroblasts (30) very long globin precursors (about 5 kb) were demonstrated using total globin cDNA (α and β) as a hybridization probe.

Little is known about transcription termination in mammals. Previous studies have suggested that termination of transcription occurs at multiple sites, extending over hundreds or even thousands of nucleotides (14). Our results suggest that transcription of the human β-globin gene extends for at least 5 kb. These long transcripts are, most likely, very unstable and rapidly processed at the normal cleavage site to give the ≈1600-nt precursor, which is subsequently spliced to yield mature mRNA. In the homozygote for the frameshift 44 mutation, mature mRNA is very unstable (25) and the relative amount of precursor is therefore increased. The patient with the deletion has a substantial amount of unresolved large RNA molecules, which may represent the 5-kb precursor as well as extended transcripts, similar to those seen in the other patients.

mRNA Molecules Extended Beyond the Normal Poly(A) Signal Are Translated *in Vivo*. In spite of the severe defect at the level of RNA processing, the deletion mutation did not totally abolish β-globin chain production. The patient homozygous for this mutation was found to have β^+-thalassemia, with a β/α chain synthesis ratio of 0.10. This implies that she is producing β-globin chains at 10% of the normal level. We therefore conclude that the extended RNA species are translated *in vivo*. The low level of β-chain production may be due to inefficiency of translation of these improperly cleaved transcripts.

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