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Genetics

Base substitution in an intervening sequence of a β^+-thalassemic human globin gene

(RNA splicing/DNA sequence analysis/*in vitro* transcription)

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ABSTRACT **β globin gene fragments from a patient with homozygous β^+-thalassemia have been cloned and subjected to restriction endonuclease, nucleotide sequence, and *in vitro* transcription analyses. Restriction endonuclease mapping of the cloned gene fragments revealed no deletions or other rearrangements, and transcription of the thalassemic gene appeared to be normal *in vitro*. However, nucleotide sequence analysis of the β^+-thalassemic gene fragments permitted identification of a single base change in the body of the small intervening sequence. This nucleotide change creates a sequence much like that of the 3′ splice site of the small intervening sequence. The presence of a potential anomalous splicing site as a result of this base change suggests a mechanism for defective posttranscriptional processing of β globin mRNA precursor molecules in β^+-thalassemia.**

The human β globin gene encodes the β globin chain of normal adult hemoglobin A (subunit structure $\alpha_2\beta_2$). A large number of genetic disorders of the β globin gene are known: hemoglobinopathies, characterized by synthesis of qualitatively abnormal β globin chains, and the β-thalassemias, characterized by quantitatively deficient synthesis of β globin chains.

The β-thalassemias are a clinically and biochemically heterogeneous group of disorders, which probably result from a wide variety of molecular defects. In β^+-thalassemia, the most common type of β-thalassemia, β globin chain synthesis is decreased to approximately 5–30% of normal in the erythroid cells of affected individuals (1, 2). Those β chains that are produced appear normal by both carboxymethylcellulose column chromatography and peptide analysis (reviewed in ref. 3). Moreover, there is a corresponding deficiency of β globin mRNA in β^+-thalassemic erythroid cells (4–6). This mRNA also appears normal by cDNA·RNA hybridization criteria and by its ability to direct translation of normal β globin chains *in vitro* (2). These features suggest that the β^+-thalassemias may result from abnormalities of β globin gene transcription or of processing, transport, or stability of β globin mRNA. Apparent abnormal processing or instability of nuclear β globin mRNA precursor molecules has been observed in some cases (7–10).

Family studies have indicated that most β-thalassemia mutations are allelic with, or tightly linked to, the β globin structural locus (1, 2), suggesting that structural analysis of thalassemic β globin genes might yield insight into the molecular basis of these disorders. Accordingly, we have cloned β globin gene fragments from a patient with β^+-thalassemia, and we have determined the complete nucleotide sequence of this thalassemic β globin gene. Comparison with the nucleotide sequence of a normal human β globin gene (11) reveals only a single divergent nucleotide, which occurs in the internal region of the small intervening sequence. This sequence difference suggests a possible mechanism for abnormal splicing of the thalassemic nuclear β globin mRNA precursor.

MATERIALS AND METHODS

Molecular Cloning. Total DNA was prepared from the spleen of a 12-year-old Greek Cypriot girl with typical transfusion-dependent β^+-thalassemia. The β/α globin chain synthetic ratio obtained after incubation of her erythroid cells with [^{3}H]leucine was 0.14. The erythrocytes of both her parents had elevated Hb A_2 levels of 4.2% and 4.9%, respectively, as well as hypochromia and microcytosis, characteristic of heterozygous thalassemia; the Hb F values in the parents' erythrocytes were 1.3% and 1.7%, respectively. The spleen DNA was digested to completion with *Eco*RI, which cleaves the β globin gene at codon 122, and the fragments were electrophoresed in agarose. A strip of the gel was transferred to nitrocellulose (12), and the 5.2-kilobase (kb) 5′ and 3.6-kb 3′ β globin DNA fragments were identified by hybridization to ^{32}P-labeled nick-translated (13) β globin cDNA from the plasmid clone pJW102 (14). These fragments were eluted from the gel and cloned in the EK-2 λgt*WES* vector system (15). Recombinant β globin clones were identified by the filter hybridization procedure of Benton and Davis (16), using ^{32}P-labeled pJW102 as probe. To facilitate subsequent analyses, the 5′ β globin clone, C6, and the 3′ clone, D47, were subcloned in the plasmid pBR322 (17) and the filamentous bacteriophage M13mp2 (18). All cloning and recombinant DNA propagation procedures were carried out under conditions of the *NIH Guidelines for Recombinant DNA Research* that were applicable at the time.

Restriction Endonuclease Digestion. Restriction enzymes were purchased from New England BioLabs, with the exception of *Eco*RI, *Bam*HI, and *Bgl* II, which were purified as described (19). Digestion conditions were those recommended by the supplier.

***In Vitro* Transcription.** pBR322 plasmids containing the 5.2-kb 5′ *Eco*RI fragment of the thalassemic β globin gene and the 4.4-kb *Pst* I fragment containing a complete normal β globin gene (20) were purified twice by buoyant density centrifugation in CsCl gradients. The sizes of the *Eco*RI fragments containing

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Abbreviations: kb, kilobase(s); snRNA, small nuclear RNA.

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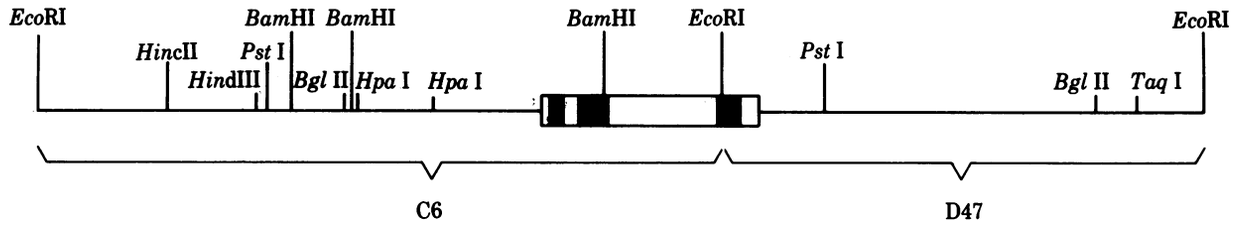

FIG. 1. Partial restriction endonuclease cleavage map of cloned β^+-thalassemic β globin gene fragments C6 and D47. The protein-encoding regions, indicated by solid blocks, are interrupted by two intervening sequences (open regions) located between codons 30 and 31 and between codons 104 and 105. The open regions that flank the gene correspond to the 5′ and 3′ untranslated sequences.

the 5′ portions of the β globin genes were: 5.2 kb for the β^+-thalassemic clone and 6.9 kb (including 3.3 kb of pBR322 DNA) for the normal β gene clone. Equal amounts (2.5 μg) of digested plasmid DNA from the normal and β^+-thalassemic clones were added to separate reaction mixtures and transcribed in a cell-free extract with RNA polymerase II activity, as described by Manley *et al.* (21). The ^{32}P-labeled "run-off" RNA transcripts were denatured with glyoxal, fractionated by electrophoresis in a 1.4% agarose gel in the presence of 0.1% sodium dodecyl sulfate, and subjected to autoradiography (21).

DNA Sequence Analysis. DNA nucleotide sequences were determined by modifications of the methods described by Maxam and Gilbert (22) for 3′- or 5′-^{32}P-labeled DNA fragments; by Maat and Smith (23) for 5′-labeled fragments; or by Sanger *et al.* (24), using pC6-derived primers and single-stranded templates prepared by purification of single-stranded DNA cloned in M13 phage or by degradation of double-stranded plasmid DNA with exonuclease III (New England BioLabs) or with phage T7 exonuclease (25). Sequencing reaction products were fractionated by electrophoresis in 40 cm × 0.5 mm urea-containing 8%, 15%, or 20% polyacrylamide gels or in 80 cm × 0.5 mm gels of 4% or 5% polyacrylamide.

RESULTS

Restriction Endonuclease Mapping of the Cloned β^+-Thalassemic β Globin Gene Fragments. Restriction endonuclease

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gctatggggcctagagatatatcttagagggagggctgagggtttgaagtccaactcctaagccagtgccagaagagccaaggacaggtacggctgtcat

cacttagacctcaccctgtggagccacaccctagggttggccaatctactcccaggagcagggagggcaggagccagggctgggcataaaagtcagggca

gagccatctattgcttACATTTGCTTCTGACACAACTGTGTTCACTAGCAACCTCAAACAGACACCATGGTGCACCTGACTCCTGAGGAGAAGTCTGCCG
                                                                    INIValHisLeuThrProGluGluLysSerAlaV

TTACTGCCCTGTGGGGCAAGGTGAACGTGGATGAAGTTGGTGGTGAGGCCCTGGGCAGGttggtatcaaggttacaagacaggtttaaggagaccaatag
alThrAlaLeuTrpGlyLysValAsnValAspGluValGlyGlyGluAlaLeuGlyArg
                                                  g
aaactgggcatgtggagacagagaagactcttgggtttctgataggcactgactctctctgcctattagtctattttcccaccctaggCTGCTGGTGGT
                                                                                    LeuLeuValVa

CTACCCTTGGACCCAGAGGTTCTTTGAGTCCTTTGGGGATCTGTCCACTCCTGATGCTGTTATGGGCAACCCTAAGGTGAAGGCTCATGGCAAGAAAGTG
lTyrProTrpThrGlnArgPhePheGluSerPheGlyAspLeuSerThrProAspAlaValMetGlyAsnProLysValLysAlaHisGlyLysLysVal

CTCGGTGCCTTTAGTGATGGCCTGGCTCACCTGGACAACCTCAAGGGCACCTTTGCCACACTGAGTGAGCTGCACTGTGACAAGCTGCACGTGGATCCTG
LeuGlyAlaPheSerAspGlyLeuAlaHisLeuAspAsnLeuLysGlyThrPheAlaThrLeuSerGluLeuHisCysAspLysLeuHisValAspProG

AGAACTTCAGGgtgagtctatgggacccttgatgttttctttccccttctttttctatggttaagttcatgtcataggaaggggagaagtaacagggtaca
luAsnPheArg

gtttagaatgggaaacagacgaatgattgcatcagtgtggaagtctcaggatcgttttagtttcttttatttgctgttcataacaattgttttcttttgt

ttaattcttgctttctttttttttcttctccgcaatttttactattatacttaatgccttaacattgtgtataacaaaaggaaatatctctgagatacat

taagtaacttaaaaaaaaactttacacagtctgcctagtacattactatttggaatatatgtgtgcttatttgcatattcataatctccctactttattt

tcttttatttttaattgatacataatcattatacatatttatgggttaaagtgtaatgttttaatatgtgtacacatattgaccaaatcagggtaatttt

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atacaatgtatcatgcctctttgcaccattctaaagaataacagtgataatttctgggttaaggcaatagcaatatttctgcatataaatatttctgcat

ataaattgtaactgatgtaagaggtttcatattgctaatagcagctacaatccagctaccattctgcttttatttttatggttgggataaggctggattat

tctgagtccaagctaggccctttgctaatcatgttcatacctcttatcttcctcccacagCTCCTGGGCAACGTGCTGGTCTGTGTGCTGGCCCATCAC
                                                                    LeuLeuGlyAsnValLeuValCysValLeuAlaHisHis

TTTGGCAAAGAATTCACCCCACCAGTGCAGGCTGCCTATCAGAAAGTGGTGGCTGGTGTGGCTAATGCCCTGGCCCACAAGTATCACTAAGCTCGCTTTC
PheGlyLysGluPheThrProProValGlnAlaAlaTyrGlnLysValValAlaGlyValAlaAsnAlaLeuAlaHisLysTyrHisTER

TTGCTGTCCAATTTCTATTAAAGGTTCCTTTGTTCCCTAAGTCCAACTACTAAACTGGGGGATATTATGAAGGGCCTTGAGCATCTGGATTCTGCCTAAT

AAAAAACATTTATTTTCATTGCaatgatgtatttaaattatttctgaatattttactaaaaagggaatgtgggaggtcagtgcatttaaaacataaagaa

atga
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FIG. 2. Nucleotide sequence of cloned β^+-thalassemic β globin gene. The nucleotide sequence of the "sense" strand of the gene is displayed in the 5′→3′ orientation. Uppercase letters represent the portion of the gene represented in β globin mRNA. Lowercase letters represent the flanking and intervening sequences. The amino acid sequence of β globin is displayed below the protein-encoding regions. The translation initiation (INI) and termination (TER) codons are also indicated. The single divergent nucleotide in the normal β globin gene is displayed above the homologous base in the thalassemic β globin DNA sequence. The 5′ extremity of the known normal β globin DNA sequence (11) is indicated by an asterisk.

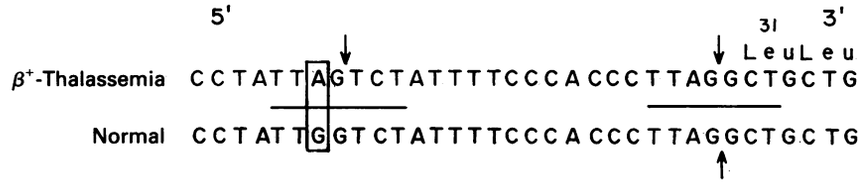

FIG. 3. Nucleotide sequence of the human β globin gene near the 3′ junction between the small intervening sequence and the adjacent protein-encoding sequence. The homologous authentic and putative internal 3′ splice junctions are indicated by the solid lines and the potential sites of intron excision are indicated by the arrows. The single nucleotide change in the β^+-thalassemic DNA sequence is indicated by the box. Normal β globin mRNA codon position 31 is numbered.

mapping of the 5.2-kb 5′ (C6) and 3.6-kb 3′ (D47) β^+-thalassemic gene clones was performed with a number of infrequently cutting enzymes. As shown in Fig. 1, the map thus generated agrees completely with that of the normal human β globin gene locus (20, 26–31).

The thalassemic and normal 5′ β globin *Eco*RI fragments were isolated from the recombinant plasmids pC6 and pHβR5.2 (11), respectively, by digestion with *Eco*RI plus *Bgl* II. The fragments were subsequently purified from their respective plasmid vector DNA, digested with *Hin*fI, *Alu* I, or *Hae* III, and fractionated by electrophoresis in agarose. In each case, the digestion patterns of the normal and thalassemic DNAs were identical (data not shown), indicating that there have been no detectable insertions, deletions, or other rearrangements of the thalassemic β globin locus.

Nucleotide Sequence of the β^+-Thalassemic Globin Gene. The 1904-nucleotide sequence determined (Fig. 2) extends from 216 bases 5′ of the cap site of β globin mRNA to 82 bases 3′ of the poly(A) addition site. The general structure of the human β globin gene is similar to that of other known mammalian genes for β-globin-like proteins (32). The protein-encoding sequence is interrupted by two intervening sequences, confirming previous data obtained by gel blotting of normal DNA (20, 26, 27, 29) and sequence analysis of a cloned normal human β globin gene (11). The small intervening sequence occurs between codons 30 and 31 and is 130 nucleotides long. The large intervening sequence is 850 nucleotides in length, and lies between codons 104 and 105.

Comparison of the nucleotide sequence of the thalassemic β globin gene fragments with that of a normal human β globin gene (11) revealed only a single divergent base. There are no sequence differences in the 5′ or 3′ flanking regions, in the 5′ or 3′ untranslated sequences, in the large intervening sequence, or in the protein-encoding regions of the β globin gene itself. Moreover, all four of the junctions between the coding and intervening sequences are normal in the β^+-thalassemic gene. It should be noted, however, that the coding sequences of both the normal (11) and thalassemic β globin gene fragments differ from the sequence previously reported for normal β globin mRNA (33) at codon Ala-53. The sequence GCA (instead of GCU) previously assigned for this codon (33) was not determined initially by direct sequence analysis of cDNA or mRNA, but was deduced indirectly on the basis of short oligonucleotides present or absent in the cRNA of one individual (33). It is possible that sequence polymorphism exists at this position in different individuals.

The nucleotide sequence of the β^+-thalassemic gene differs from that of the normal human β globin gene at only a single site, in the internal region of the small intervening sequence. Twenty-two nucleotides before the 3′ junction of the intervening sequence with the adjacent protein-encoding sequence, an adenine replaces a guanine. This substitution creates a sequence that is homologous to that at the authentic 3′ splice junction at six of seven bases (Fig. 3), perhaps creating an anomalous splicing signal and thereby resulting in abnormal splicing of some of the β globin mRNA precursor molecules at this site.

Transcription of the Thalassemic β Globin Gene *in Vitro*. The transcriptional capacity of the cloned 5′ *Eco*RI β^+-thalassemic globin gene fragment was assayed *in vitro* by using a cell-free system with RNA polymerase II activity (21). Prior to transcription, the β^+-thalassemic and normal β globin templates were cleaved with *Eco*RI. As shown in Fig. 4, both templates were transcribed faithfully in this system. The β^+-thalassemic transcript is qualitatively and quantitatively identical to that of the normal β globin gene, suggesting that transcription of this thalassemic gene fragment is normal, at least in this *in vitro* system. Similar results have recently been obtained in other laboratories using other cloned β^+-thalassemic genes (35, 36). In addition to the major β globin gene transcription product that runs between the 1.1-kb and 1.8-kb size markers, a number of fainter products of higher molecular weight can be seen be-

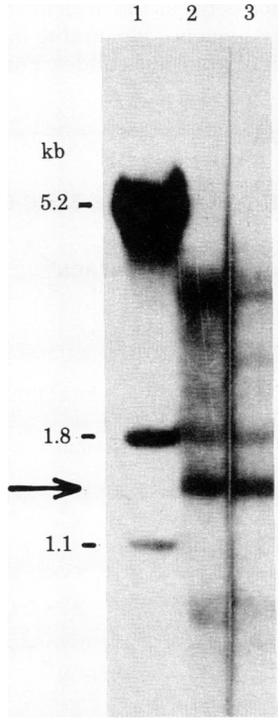

FIG. 4. *In vitro* transcription of normal and β^+-thalassemic 5′ *Eco*RI β globin gene fragments. Lanes: 1, DNA size markers (a mixture of linear DNA molecules from simian virus 40 plus 1.1-kb and 1.8-kb *Hin*dIII subfragments of simian virus 40 DNA); 2, normal; 3, β^+-thalassemic. The RNA transcripts were denatured by glyoxylation and fractionated by agarose gel electrophoresis as described by McMaster and Carmichael (34). Arrow indicates β-globin-specific transcripts. The labeled 1.8-kb fragment in lanes 2 and 3 consists of end-labeled 18S RNA and was also present in control reactions lacking DNA templates.

tween the 1.8-kb and 5.2-kb size markers. These presumably result from end-to-end transcription of the inserted DNA or transcription of pBR322 plasmid DNA sequences (37). Some differences in the size of these larger faint transcripts can be observed between the normal and β^+-thalassemic reactions presumably because of the different sizes of the respective inserted β globin gene fragments.

DISCUSSION

We have cloned β globin gene fragments from a patient with homozygous β^+-thalassemia whose erythroid cells accumulate decreased amounts of cytoplasmic β globin mRNA and, as a result, synthesize reduced amounts of β globin chains. Because the 5′ and 3′ portions of the thalassemic β globin gene were isolated as separate *Eco*RI restriction fragments, we cannot be certain that they derive from the same thalassemic allele. However, β^+-thalassemia in the Greek Cypriot population is extremely homogeneous clinically and biochemically (38), suggesting that the patient is probably homozygous for identical β^+-thalassemic alleles.

Restriction endonuclease mapping of the thalassemic gene fragments revealed no detectable deletions or other rearrangements. Similar results have also been recently obtained by Burns *et al.* (39) in a different case of β^+-thalassemia.

Nucleotide sequence analysis of the thalassemic β globin gene revealed only a single nucleotide difference from a normal β globin gene sequence (11) in the internal portion of the small intervening sequence. This G to A transition 22 bases to the 5′ side of the 3′ junction between the small intervening and coding sequences creates a sequence identical to that at the authentic splice junction at six of seven nucleotides. Furthermore, the one differing nucleotide may not be rigidly specified at the normal 3′ splice junction, because the sequences of the human δ and ε globin genes differ from those of the γ and β genes at this base residue (32). Previous authors have noted striking sequence complementarity between the regions surrounding RNA splice junctions and the 5′ extremity of the small nuclear RNA (snRNA), U-1 (40, 41), and they have suggested that this RNA species may align splice junctions during the RNA splicing process *in vivo*. As shown in Fig. 5, sequence complementarity between U-1 snRNA and the putative abnormal splice site in the thalassemic gene is almost as extensive as complementarity of U-1 snRNA with the authentic 3′ splice junction. Thus, the atypical sequence in the small intervening sequence of the β^+-thalassemic gene may be a functional splice sequence *in vivo*. Splicing at this location might compete or interfere with RNA splicing at the authentic splice site, particularly if recognition of splice sites by the splicing enzyme complex occurs in a processive manner. Any RNAs spliced at the putative abnormal splice site would encode normal β globin amino acid sequences up to codon 29 or 30, depending on the precise site of splicing. These would be followed by a nonsense peptide terminating at an in-phase UAG codon derived from the 3′ border of the small intervening sequence. Such abnormally spliced mRNA precursor molecules containing an in-phase nonsense codon would probably be unstable, or the peptide encoded by this RNA might be unstable, because no abnormal peptide appears on carboxymethylcellulose column chromatography of proteins synthesized by reticulocytes of β^+-thalassemic patients (3). In β^0-thalassemia associated with a nonsense mutation at codon 17 of the β mRNA (43), the predicted abnormal short peptide has not been detected, and the abnormal β mRNA is presumably unstable, because it is present in unexpectedly reduced amounts (43). Atypical or alternative splicing of nuclear mRNA

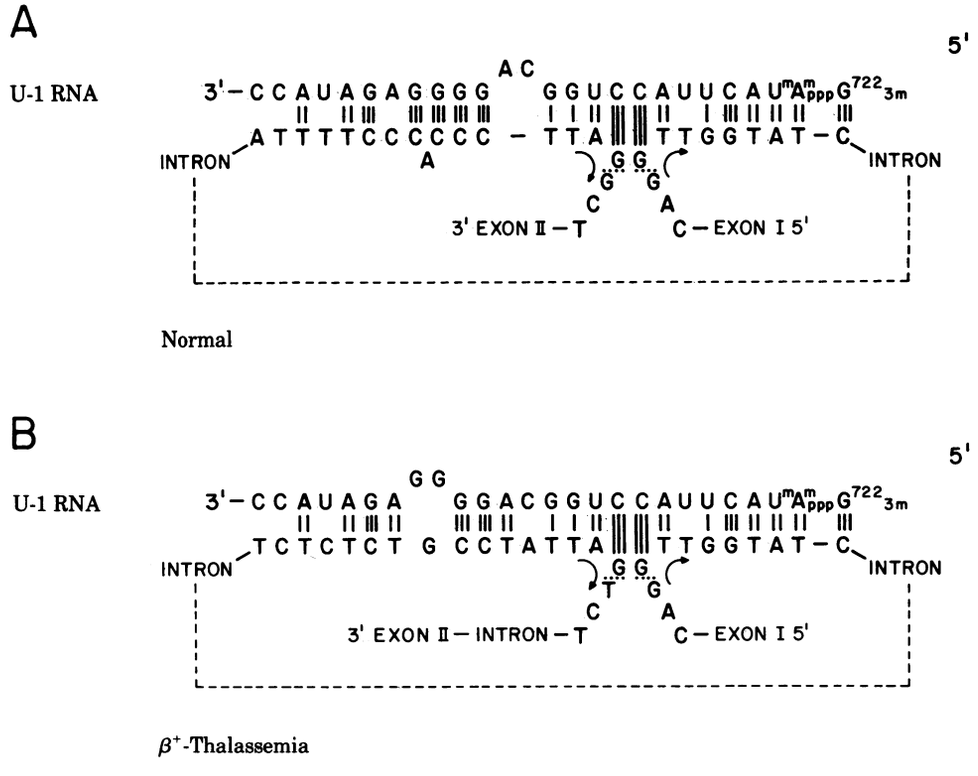

FIG. 5. Possible complementary structures formed between normal and thalassemic β globin nuclear mRNA precursors and U-1 snRNA. The nucleotide sequence of U-1 snRNA is that determined by Branlant *et al.* (42). Base pairing of U-1 snRNA with the authentic 3′ terminus of the normal β globin small intervening sequence or internal region of the β^+-thalassemic small intervening sequence has been maximized by permitting looping out of some unbonded bases. (*A*) Normal β globin mRNA precursor; (*B*) β^+-thalassemic β globin mRNA precursor.

precursor molecules has also been described in the case of human growth hormone (reviewed in ref. 44) and chicken ovomucoid (45).

Transcription of the cloned β^+-thalassemic globin gene by RNA polymerase II indicates that this gene is transcribed normally *in vitro*, suggesting that the single base change in the small intervening sequence does not alter an internal promoter sequence similar to that defined by Brown and colleagues as directing transcription of the *Xenopus* 5S RNA gene *in vitro* by RNA polymerase III (46, 47).

In addition to possibly interfering with splicing of β globin mRNA precursor molecules, the base change that we have identified in the thalassemic gene abolishes an *Eco*PI restriction endonuclease cleavage site [recognition sequence A-G-A-C-C (48)] which is present in the normal β globin gene. However, because cleavage of DNA by *Eco*PI is never complete (48), it is unlikely that restriction endonuclease analysis of amniocyte DNA will be useful for the prenatal diagnosis of this β^+-thalassemic allele.

These data suggest that the basic molecular defect in this patient may be an abnormality of RNA processing, due to the mutation in the small intervening sequence. Alternatively, the molecular defect may be distant from the β globin gene itself. These hypotheses could be tested by analysis of β globin gene expression, using *in vitro* systems that have the ability to process transcribed mRNA precursor molecules into mature mRNA, as well as by sequence analysis of additional β^+-thalassemic globin genes to determine whether the base change that we have identified also occurs in these genes.

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