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Nonsense mutations in the human β-globin gene affect mRNA metabolism

(β-thalassemia/oligonucleotides/site-specific mutagenesis)

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ABSTRACT **A number of premature translation termination mutations (nonsense mutations) have been described in the human α- and β-globin genes. Studies on mRNA isolated from patients with β^0-thalassemia have shown that for both the β-17 and the β-39 mutations less than normal levels of β-globin mRNA accumulate in peripheral blood cells. (The codon at which the mutation occurs designates the name of the mutation; there are 146 codons in human β-globin mRNA.) *In vitro* studies using the cloned β-39 gene have reproduced this effect in a heterologous transfection system and have suggested that the defect resides in intranuclear metabolism. We have asked if this phenomenon of decreased mRNA accumulation is a general property of nonsense mutations and if the effect depends on the location or the type of mutation. Toward this end, we have studied the effect of five nonsense mutations and two missense mutations on the expression of human β-globin mRNA in a heterologous transfection system. In all cases studied, the presence of a translation termination codon correlates with a decrease in the steady-state level of mRNA. The data suggest that the metabolism of a mammalian mRNA is affected by the presence of a mutation that affects translation.**

Many of the steps in β-globin gene expression have been well-studied. Globin mRNA is transcribed from DNA, spliced, polyadenylylated, and then translated into protein in the cytoplasm. Less is known about the determinants of nuclear and cytoplasmic globin mRNA stability and about nuclear–cytoplasmic transport. The β-39 gene causes thalassemia because it encodes a truncated nonfunctional globin protein chain. (The codon at which the mutation occurs designates the name of the mutation.) Although the nonsense mutation at codon 39 affects translation, studies from our laboratory and others suggest that this mutation has an additional unexpected effect on mRNA metabolism (1–8). There is reduced accumulation of the β-39 mRNA when compared to normal human β-globin (β-nl) mRNA in both patients' erythroid cells and in a heterologous transfection system. Since there is also reduced accumulation of β-39 mRNA in the nucleus, it has been hypothesized that the defect in expression of the β-39 gene is intranuclear.

The unusual phenotype of the β-39 mutation suggests that an additional element in the regulation of gene expression may be uncovered by further investigation. To this end, we have constructed a series of nonsense and missense mutations in the human β-globin gene to determine (*i*) if nonsense mutations at other positions in the gene (β-17, β-37, and β-82) would affect mRNA metabolism, (*ii*) if an ochre (UAA) mutation at β-39 would have the same effect as the amber (UAG) mutation at this codon, and (*iii*) if missense mutations at codon 39 would have an effect. In this way we could study the effect of several nonsense and missense mutations in the human β-globin gene on globin mRNA metabolism after expression in a heterologous system.

MATERIALS AND METHODS

Cell Lines. COS-7 (9) cells at low passage (<10) were obtained from the American Type Culture Collection. Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, penicillin (50 units/ml), and streptomycin (50 μg/ml).

Source of Oligonucleotides. All oligonucleotides were synthesized on Applied Biosystems (Foster City, CA) oligonucleotide synthesizers. Sources include the Department of Human Genetics, Yale University (courtesy of Dan DiMaio and Bill Summers); Integrated Genetics (Framingham, MA) (courtesy of Graham Mock); Section of Immunology, Yale University (courtesy of Peter Blier and Al Bothwell).

Selection of Oligonucleotides. Oligonucleotides were designed to create the following nonsense mutations: β-17, β-37, β-82, and β-39 (UAA). The sequences are depicted in Table 1. They were purified according to the directions of the manufacturer (Applied Biosystems). The total yield varied from 300 to 1200 μg per synthesis reaction.

The β-17 and β-82 oligonucleotides contain two mutations. One of these mutations creates a nonsense codon at the designated position; the other creates a silent amino acid substitution. The mutation that creates a silent substitution also creates a new restriction site, which facilitates its detection after mutagenesis. The codon for the silent substitutions was carefully chosen based on a mammalian codon usage to avoid infrequently used codons.

Preparation of M13 Template. Either the 1.8-kilobase (kb) *Bam*HI fragment of the β-nl gene (10) or the β-39 gene (6) was cloned into the *Bam*HI site of M13mp9. Clones that produced single-stranded DNA that was the same sequence as the globin mRNA strand were chosen for mutagenesis. Template was prepared by standard procedures (11).

Site-Specific Mutagenesis. Site-specific mutagenesis was performed according to the method of Zoller and Smith (12) using the strains developed by Kunkel *et al.* (13). M13 host strains JM101 (11) and RZ1032 (13) were used. The mutation frequency was 40–60%.

Analysis of Mutations. The presence of a mutation in the β-globin sequence was confirmed either by the presence of a new restriction site in miniprep M13 replicative form DNA or by direct dideoxy sequencing of the single-stranded DNA template (14). Minipreps of M13 replicative form DNA were prepared by alkaline lysis (15) of JM101 that had been inoculated with one M13 plaque. The presence of the β-17 mutation was confirmed by the presence of a new *Hin*cII site; the β-82 mutation was confirmed by the presence of a new *Kpn* I site. Clones that had the new restriction site were sequenced by the dideoxy method (14). The presence of the

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Abbreviations: SV40, simian virus 40; β-nl, normal human β-globin.
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Table 1. Oligonucleotides used for site-specific mutagenesis and DNA sequencing

β-17 (UAG)	
β-nl sequence	5′-TGGGGCAAGGTGAACGTGG-3′
Oligo	3′-ACCCCGATCCAATTGCACC-5′
β-82 (UAG)	
β-nl sequence	5′-CACCCTCAAGGGCACCTTTG-3′
Oligo	3′-GTTGGAGATCCCATGGAAAC-5′
β-39 (GAG, AAG)	
β-nl sequence	5′-TACCCTTGGACCCAGAGGTTCTT-3′
Oligo	3′-ATGGGAACCTGGTTCTCCAAGAA-5′ C
β-39 (UAA)	
β-nl sequence	5′-CCTTGGACCCAGAGGTTCTT-3′
Oligo	3′-GGAACCTGGATTTCCAAGAA-5′
β-37 (UGA)	
β-nl sequence	5′-GTCTACCCTTGGACCCAGAGGT-3′
Oligo	3′-CAGATGGGAACTTGGGTCTCCA-5′
Sequencing oligo for mutations 5′ to amino acid position 51	3′-CAATACCCGTTGGGATTCCA-5′
Sequencing oligo for intervening sequence 1	3′-CCTCTGTCTCTTCTGAGAAC-5′
Sequencing oligo for 5′ untranslated region	3′-CTCGGTGTGGGATCCCAACC-5′

The β-nl sequences given are those of single-stranded DNA having the same strand sense as the globin mRNA. Oligo, oligonucleotide.

β-37 and β-39 nonsense mutations and the amino acid substitutions at position 39 were confirmed by direct dideoxy sequencing of the single-stranded M13 template.

Subcloning. The vector used in these experiments was prepared by Takeshita *et al.* (6). The 5-kb *Bgl* II fragment of the human β-39 gene was cloned into the *Bam*HI site of PTL9, a simian virus 40 (SV40) vector with an SV40 origin of replication, the SV40 enhancer region, and the ampicillin-resistance gene of pBR322. This vector was cut with *Bam*HI, and the larger vector-containing fragment was gel-purified, religated, and transformed into *Escherichia coli* strain HB101. In this way a PTL9 vector containing β-globin sequences minus the 1.8-kb *Bam*HI fragment was prepared.

The mutated 1.8-kb *Bam*HI fragment was prepared from M13 replicative form DNA by electroelution (15). The 1.8-kb *Bam*HI fragment from each of the mutants was ligated to *Bam*HI-cut vector DNA by standard procedures (15). Colonies were screened by colony hybridization (16) with a nick-translated gel-purified 1.8-kb *Bam*HI fragment. Plasmid minipreps of positive colonies were digested with *Nco* I to check for the correct orientation.

Transfections. Plasmids used for transient expression studies were purified by banding twice in CsCl. Thirty micrograms of each of the β-globin plasmids and 15 μg of an α-globin plasmid (pSV0α1) (17) were cotransfected into a 150-cm^2 tissue culture flask of subconfluent COS-7 cells by the calcium phosphate method (18). After 6 hr, the cells were shocked for 1 min with 20% glycerol and washed, and then fresh medium was added. Transfection of the β-nl and β-39 genes was included for each experiment.

RNA Isolation. Total mRNA was isolated from the transfected cells by the guanidine isothiocyanate procedure (19). The RNA was stored in 70% ethanol at −20°C.

S1 Nuclease Analysis. S1 nuclease analysis on the isolated RNA was performed according to Metherall *et al.* (20). Fifty micrograms of RNA was analyzed with each of the α and β probes per S1 nuclease reaction.

Densitometric Tracing. The autoradiographs of the α- and β-globin-protected fragments were traced vertically with a Beckman DU-8 spectrophotometer.

Statistical Analysis. Using the data from densitometric tracings, the amount of each of the β-globin mRNAs was normalized to the amount of β-nl mRNA; i.e., the amount of β-nl mRNA was set equal to 1.0. Expression of the α-globin genes was treated in a similar manner. For each lane in each experiment, the β-to-α ratio was calculated. The β-to-α ratio for three experiments was averaged, and the SEM was calculated.

RESULTS

The expression of five nonsense and two missense mutant human β-globin genes was analyzed (Fig. 1). We studied amber (UAG) mutations at β-17 and β-82, an opal (UGA) mutation at β-37, and an ochre (UAA) mutation at β-39. We also studied two missense mutations at position 39, GAG and AAG, the codons for glutamic and lysine residues, respectively. These mutations "correct" the nonsense mutation at amino acid position 39 to translatable codons and were constructed from a β-39 DNA template.

Relative expression of the mutant β-globin genes was tested in a transient expression system using COS-7 cells. Expression of each mutant was compared to expression of the β-nl gene. An α-globin gene was cotransfected to control for variability in transfection efficiency. Fig. 2*A* shows that the level of accumulation of the nonsense mutant mRNAs was much lower than the level of accumulation of the translatable mRNAs. This result was confirmed by densitometric tracing of the α- and β-globin autoradiographs. Fig. 2*B* shows that the data fell into two groups. Some mutations gave rise to decreased levels of mRNA, whereas others gave rise to normal levels of mRNA. In the first group, all the nonsense mutations tested gave rise to decreased levels of mRNA. In the second group, the two missense mutations at amino acid position 39 gave rise to levels of mRNA similar to those of the wild-type β-globin gene. This suggests that correcting the amber nonsense mutation at codon 39 to a missense mutation affects the level of mRNA that accumulates. There was some variability in the levels of expression between mRNAs within each group; however, in the case of the nonsense mutations, the variability was not related to the type of codon or to its relative position in the transcript.

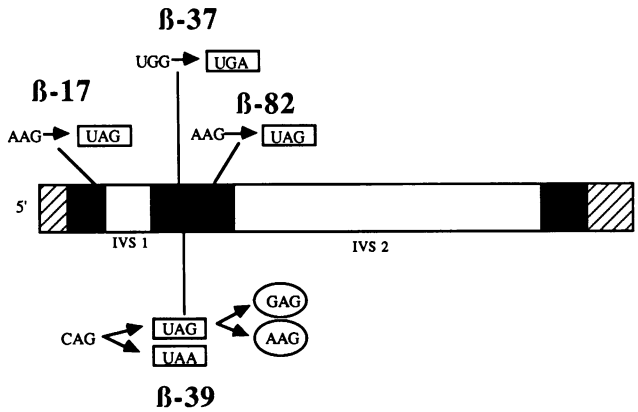

Fig. 1. Origin, location, and type of mutations in the human β-globin gene. A map of the mutations analyzed in the human β-globin gene is shown. Nonsense mutations at β-17, β-37, β-39 (UAA), and β-82 were constructed from the β-nl template (10). The β-nl codon at each position is shown and the nonsense mutations are enclosed in boxes. The missense mutations, which are circled, were constructed from the β-39 amber (UAG) mutant by site-directed mutagenesis. IVS, intervening sequence. The hatched bars indicate 5′ and 3′ untranslated sequences and the closed bars indicate exons.

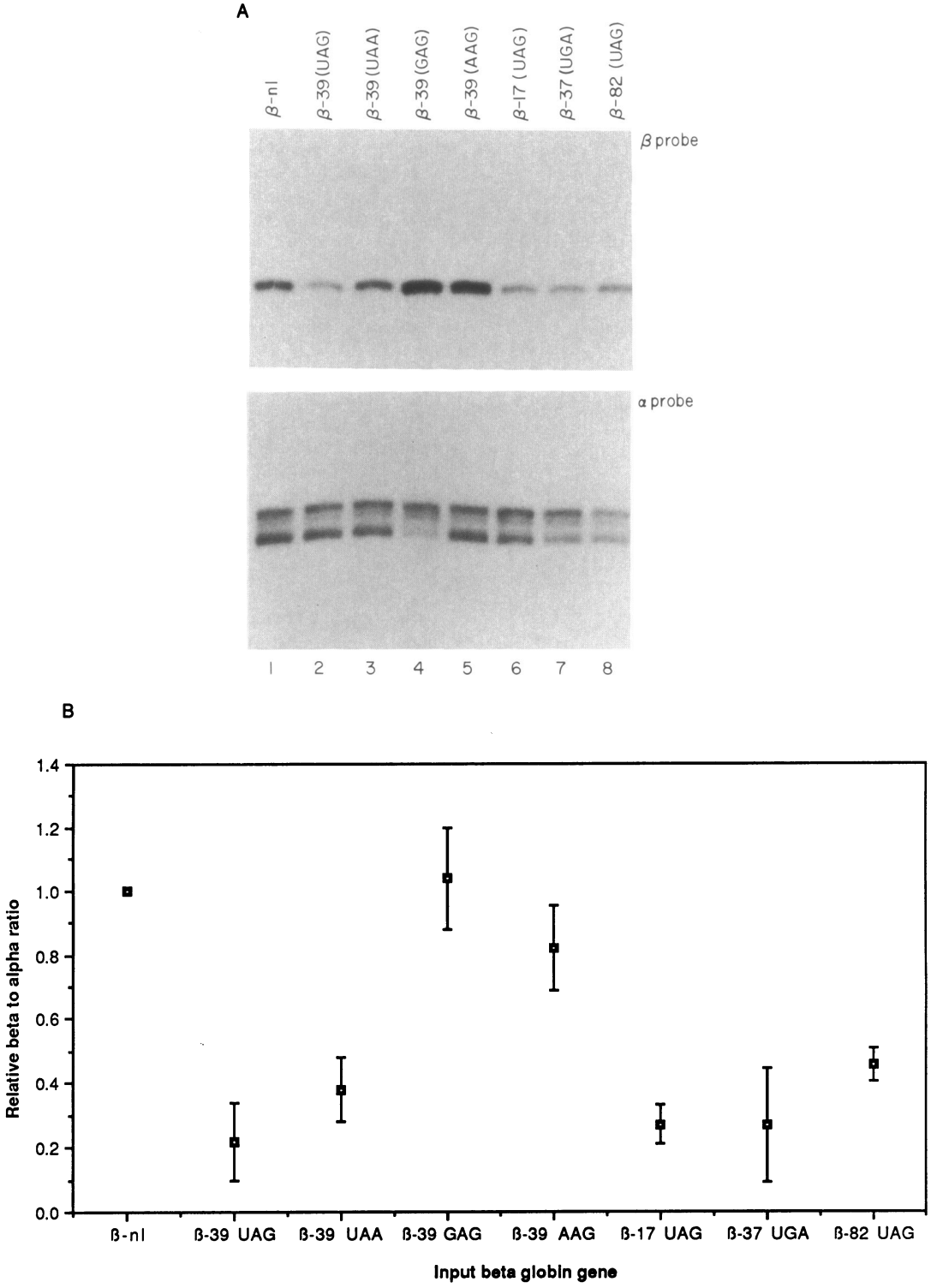

FIG. 2. Relative expression of nonsense and missense mutant β-globin mRNA. (*A*) An analysis of the relative expression of the human β-globin genes was performed (6, 7). The upper gel depicts an autoradiograph of the S1 nuclease analysis of the β-nl, nonsense, and missense mutants, as indicated. In all cases the expected protected β-globin fragment of 209 base pairs was generated. The lower gel depicts an autoradiograph of the S1 nuclease analysis of the α-globin mRNA. The α-globin gene was cotransfected with each of the β-globin constructs to control for transfection efficiency. (*B*) A graph of the relative expression of the nonsense and missense mutant β-globin mRNAs is shown. Autoradiographs of the S1 nuclease analysis of the β-globin and α-globin mRNAs were traced by densitometry. The β-to-α ratio (β/α) was calculated for each construct and then, within each experiment, normalized to the amount of normal β-globin mRNA. The amount of wild-type β-globin mRNA is designated as 1.0. The graph indicates the mean ± SEM for three experiments.

DISCUSSION

Our data suggest strongly that nonsense codon mutations in the human β-globin mRNA give rise to a defect in β-globin mRNA accumulation. Several lines of evidence support this. The reduction in steady-state levels of mRNA is seen with all five of the nonsense mutations but not with either of the missense mutations, even when the missense mutation is

inserted at the same nucleotide. It is interesting that two different nonsense mutations, amber (UAG) and ochre (UAA) at amino acid position 39, give rise to similarly reduced levels of β-globin mRNA. Since the missense mutant genes were constructed using the β-39 (UAG) gene as a template, this experiment confirms that decreased mRNA accumulation is due to the presence of the nonsense mutation and not to any other mutation occurring in that gene or any aberrant features of that construct. Direct DNA sequence analysis demonstrated that the amber (UAG) mutation is the only mutation occurring in the β-39 gene (ref. 6 and S.J.B., unpublished data). In contrast to the missense mutations, two different nonsense mutations, amber (UAG) and ochre (UAA) at amino acid position 39, give rise to similarly reduced levels of mRNA. The defect seems to be independent of type (amber vs. ochre vs. opal) of the nonsense mutation. mRNA accumulation is not affected merely by the occurrence of a mutation at a particular location; rather the occurrence of a translation termination mutation is crucial to the lowered mRNA levels.

In prokaryotic operons, the effect of nonsense mutations on mRNA accumulation is polar; nonsense mutations located toward the 5′ end of an operon reduce accumulation more than those more 3′ (21). From our data we cannot say whether there is a polar effect of nonsense mutations on human β-globin mRNA metabolism in transfected tissue culture cells. The number of samples are too few to include or exclude this hypothesis.

Previous studies have shown that the nontranslatable β-globin mRNA transcribed from this vector initiates, splices, and terminates correctly both *in vivo* and *in vitro* (6, 7). These experiments were performed by S1 nuclease analysis of mRNA isolated from the peripheral blood of patients with β-thalassemia or from stable cell lines expressing the β-39 mRNA.

Low levels of accumulation of nonsense mutant β-globin mRNA have been reported for both the β-17 (22) and β-39 (UAG) mutants (1–5, 8) in mRNA isolated from the peripheral blood of patients with β^0-thalassemia. mRNA accumulation *in vivo* for the β-37 mutant has not been investigated (23). The levels of β-17 and β-39 mRNAs that accumulate are reported to be 15% and 5% of normal, respectively. Our *in vitro* data mimic the *in vivo* patient data to the extent that, for all mutants tested, reduced levels of nonsense mutant mRNA are observed. However, the percent of β-nl mRNA that accumulates is not identical *in vitro*. The β-17 mRNA accumulates to about 30% of normal, and the β-39 (UAG) accumulates to about 20% of normal. The β-82 mutant mRNA accumulates to about 50% of normal.

There are no *in vivo* data on mRNA levels for the missense mutations, although both mutations have been described *in vivo* (24, 25). Neither mutation is associated with thalassemia. The β-39 (AAG) mutation has been described through a voluntary screening program as hemoglobin Alabama and is not associated with any hematologic abnormality. The β-39 (GAG) mutation has been described as hemoglobin Vaasa in a Finnish family. This β-globin chain is mildly unstable and is associated with a very mild hemolytic anemia.

A nonsense mutation has also been described in the third exon of the α-globin gene, α-116 (UAG) (26). The α-116 mRNA accumulates in normal amounts. This is not unexpected since a β-globin mRNA with a nonsense mutation at codon 145 (the normal length of β globin is 146 amino acids) makes normal levels of β-globin protein (27) and, presumably, normal levels of β-globin mRNA.

Moschonas *et al.* (1) were among the first to test the expression of β-39 gene in a transient expression system. They compared the expression in HeLa cells of the β-nl and β-39 genes cloned into a pBR328-SV40 vector. They saw no difference in expression of the two genes, in contrast to the results presented here and to those of Takeshita *et al.* (6) and Humphries *et al.* (7). The reason for this is not known.

We have found, then, that mutations in the human β-globin gene that prematurely terminate translation also affect mRNA metabolism. One possible explanation is that since the mRNA is not completely translated it is not protected by ribosomes and is therefore susceptible to endogenous RNase digestion. Previously published reports from our laboratory (6) and from others (7) have demonstrated that, at least for the β-39 (UAG) mutant, the decrease in mRNA accumulation is not a result of cytoplasmic instability. These results have been confirmed in stable cell lines that contain either the β-39 (UAG) or β-nl genes (28). The defect in metabolism of nonsense mutant mRNAs therefore probably resides in the nucleus. One potential mechanism is that there is feedback from translation on intranuclear events and that the presence of a premature termination codon during translation in the cytoplasm serves as a signal to inhibit transport of the specific mRNA out of the nucleus. It is also possible that the presence of the premature termination codon can be sensed within the nucleus itself.

Nonsense mutations have been found to influence mRNA accumulation in other mammalian systems. For the histone genes, which are synthesized and degraded in a cell cycle-specific manner, the presence of a premature termination codon inhibits cell cycle-specific degradation (29). Nonsense mutations have been described for low density lipoprotein (LDL) receptor genes in patients with hyperlipidemia (30, 31). *In vivo* mRNA studies on one of these genes have not demonstrated reduced levels of LDL receptor mRNA (31). It is possible that the effect that we have observed occurs only with mRNAs that accumulate as a major species in the cell. Further studies will be required to determine if our observations for nonsense mutations in the human β-globin mRNA are a general property of nonsense mutations in eukaryotic genes.

Note Added in Proof. Atweh *et al.* (32) have described a mutation in the β-43 gene that is associated with reduced mRNA levels *in vivo* and *in vitro* when compared to β-nl.

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