Molecular Basis for Nondeletion α-Thalassemia in American Blacks

$\alpha 2^{116GAG \rightarrow UAG}$

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Abstract

An American black woman was found to have the phenotype of moderately severe α-thalassemia normally associated with the loss of two to three α-globin genes despite an α-globin gene map that demonstrated the loss of only a single α-globin gene ($-\alpha/\alpha\alpha$). Several individuals in her kindred with normal α-globin gene mapping studies ($\alpha\alpha/\alpha\alpha$) had mild α-thalassemia hematologic values consistent with the loss of one to two α-globin genes. These data suggested the presence of a nondeletion α-thalassemia defect in this family which segregates with the intact $\alpha\alpha$ gene cluster. An abnormally migrating and highly unstable α-globin gene product was demonstrated by in vitro translation of the reticulocyte mRNA from the proposita and this mutant α-globin protein was mapped to the α2-globin gene by hybrid-selected translation. The abnormal α2-globin gene was cloned and sequenced. A single base mutation that results in a premature termination codon was identified at codon 116 (GAG → UAG). The defined α-globin genotype of the proposita ($-\alpha/\alpha^{116UAG}\alpha$) and the positioning of this nonsense mutation at the α2-globin gene locus are fully consistent with the observed α-thalassemia phenotype.

Introduction

α-Thalassemia is an inherited disorder resulting from diminished synthesis of α-globin (1). This deficit in α-globin results in decreased assembly of the $\alpha 2\beta 2$ hemoglobin tetramers, the accumulation of unstable $\beta 4$ tetramers, and a consequent microcytic, hypochromic, hemolytic anemia. The molecular basis of α-thalassemia is most commonly a deletion of one or both of the two α-globin genes located in adjacent positions on the short arm of chromosome 16 (for review see references 2, 3). These single and double gene deletions result, respectively, in the partial or complete loss of α-globin synthesis from the affected chromosome. The specific size and position of the α-globin gene deletion differs among populations. The predominant deletion in black individuals removes 3.7 kilobases (kb) of DNA from the α-globin gene cluster leaving the affected chromosome with a single active α-globin gene (4–7). ~ 30% of American blacks are heterozygous for this deletion ($-\alpha^{3.7}/\alpha\alpha$), and ~ 2% are homozygotes ($-\alpha^{3.7}/-\alpha^{3.7}$) (4).

Nondeletion defects are less common than deletion defects as a basis of α-thalassemia. To date, a total of five nondeletion

α-thalassemia defects have been defined in Mediterranean (8, 9), Saudi Arabian (10), Algerian (11), and Chinese (12–14) populations. In addition, the α-globin structural variant Hb Constant Spring (15) is a frequent cause of α-thalassemia in Asia, and three related antitermination mutations have been identified in Greek (Hb Icara; 16), East Indian (Koya Dora; 17), and Black (Hb Seal Rock; 18) individuals. Whereas two studies have suggested the presence of additional nondeletion α-thalassemia mutations in blacks (19, 20), they have not been confirmed at the molecular level. The molecular definition of such defects and their correlation with the severity of α-thalassemia should further define the variables affecting the severity of this disease. In the present report we describe the clinical, hematologic, and molecular features of a nondeletion defect in an American black kindred. The molecular defect predicts the total loss of expression of the affected α2-globin gene and suggests a basis for its unusually severe effect upon overall α-globin synthesis.

Methods

Hematologic studies. All peripheral blood counts and red cell indices were determined on a Coulter electronic cell counter. Routine hematologic methods were otherwise employed except as noted.

Hemoglobin analysis. Hemoglobin electrophoresis was carried out by isoelectric focusing in polyacrylamide gels using pH 5–7 ampholines (21), on cellulose acetate membranes at pH 8.6 (22), or on agar gels at pH 6.1. HbA2 and HbF were measured as previously described (23, 24). Globin chain synthesis during in vitro translation was analyzed on Triton acid urea gels (25, 26).

Biosynthetic studies. Freshly drawn blood was adjusted to a packed cell volume of 50% and incubated for 1 h at 37°C in the presence of [³H]1-leucine under conditions previously detailed (27). The globin chains were separated by high performance liquid chromatography (HPLC) on large-pore C4 columns (Zydac Inc.) (28) and the radioactivity incorporated into α- and β-globin chains was determined as previously described (27).

Gene mapping. DNA was prepared from the buffy coat of peripheral blood by the method of Blin and Stafford (29). Restriction enzymes (International Biotechnologies, Inc., New Haven, CT) were used according to the manufacturer's recommendations. The digested DNA was fractionated by electrophoresis in a 0.8% agarose gel, transferred to a nitrocellulose filter, and hybridized to the 1.4-kb Pst I DNA fragment containing the α-globin gene that was ³²P-labeled by nick translation (30, 31).

mRNA analysis. RNA was isolated from reticulocytes by phenol extraction of acid-precipitated polysomes (14). α1- and α2-globin mRNA was quantitated by primer extension analysis (32), and hybrid-selected translation of α1- and α2-globin mRNA was carried out as previously detailed (33). In vitro translations were carried out in nuclease-treated rabbit reticulocyte lysate (Bethesda Research Laboratories, Gaithersburg, MD).

DNA cloning and analysis. Highly polymerized DNA used for cloning was isolated from peripheral leukocytes by phenol extraction of proteinase K-digested buffy coat (34). Bam HI-digested DNA was size fractionated on a 0.8% agarose gel next to molecular weight markers. The gel was stained with ethidium bromide and a gel slice containing DNA in the

13–15-kb range was excised under ultraviolet illumination. DNA in this gel slice was purified by glass bead extraction (35). 0.4 μg of the size-fractionated genomic DNA was ligated to 1 μg of Bam HI digested EMBL3 phage arms (Stratagene Cloning Systems, San Diego, CA) (36). The ligation was carried out in a total volume of 5 μl. Ligation buffer containing additional ligase (200 U) was added and the incubation was then continued for an additional 24 h. 4 μl of the final 10-μl ligation was packaged in bacteriophage particles using a combination of freeze-thaw and sonic phage lysates (Gigapack, Stratagene Cloning Systems). This viral stock was used to infect P2392 bacteria (Stratagene Cloning Systems). Phage plaques were screened by in situ hybridization with a nick-translated α-globin cDNA probe (pMC18; 37) according to standard technique (31). DNA isolated from confluent plate lysates of plaque-purified phage stocks containing the α-globin/vector recombinants was digested with Pst I and ligated into the pSP65 plasmid that had been linearized by Pst I digestion and dephosphorylated by reaction with calf intestinal alkaline phosphatase (New England Biolabs, Beverly, MA). α-Globin gene recombinants were identified by in situ hybridization (38) to nick-translated pMC18 insert probe, and DNA was isolated from 3m1 overnight cultures of these colonies by an alkaline miniprep procedure (31). The α2-globin gene recombinants were differentiated from the α1-globin gene recombinants by restriction analysis with Rsa I which is specific for the 3′ nontranslated region of the α1-globin gene (37). Plasmid DNA used for sequencing was isolated from 1-liter cultures by CsCl isopycnic centrifugation of clarified lysates (31). All sequencing was by the chemical degradation method of Maxam and Gilbert (39).

Results

Proband analysis. The proband, an 81-yr-old nulliparous black woman who had been in good health all her life, was referred for evaluation of a hypochromic, microcytic anemia. The physical examination was normal. The results of hematological studies are shown in Table I (subject I1). Serum, iron-binding capacity, and ferritin were normal. Bone marrow examination showed mild erythroid hyperplasia with normal iron stores. HbA2 and HbF were within normal limits and HbH was not detected by electrophoresis or after brilliant cresyl blue incubation of fresh blood.

Hematologic and globin biosynthetic evaluation of the kindred. Members of three generations of the proband's kindred were studied. The ages and accrued data on these individuals are summarized in Table I and Fig. 1. All four individuals in

Table I. Hematological Studies

Patient	Hb	PCV	MCV	MCH	α/β*
	g/dl		fl	pg	
I-1	10.0	31.4	69	22.0	0.83
2	11.0	33.4	72	23.7	0.75
3	11.3	35.9	68	21.7	0.76
4	13.7	39.4	66	22.9	0.86
II-1	14.8	46.9	84	27.0	0.85
2	11.4	38.5	79	24.0	0.71
3	12.6	40.2	80	25.5	0.84
III-1	11.6	34.3	88	30.0	0.77
Normal	13.5–16	38–55	80–95	27–31	0.85–1.20

* Ratio of radioactivity incorporated into newly synthesized α- and β-globin chains after a 1-h incubation of blood with [³H]l-leucine. Ratios < 0.85 are consistent with α-thalassemia, whereas those > 1.20 indicate β-thalassemia.
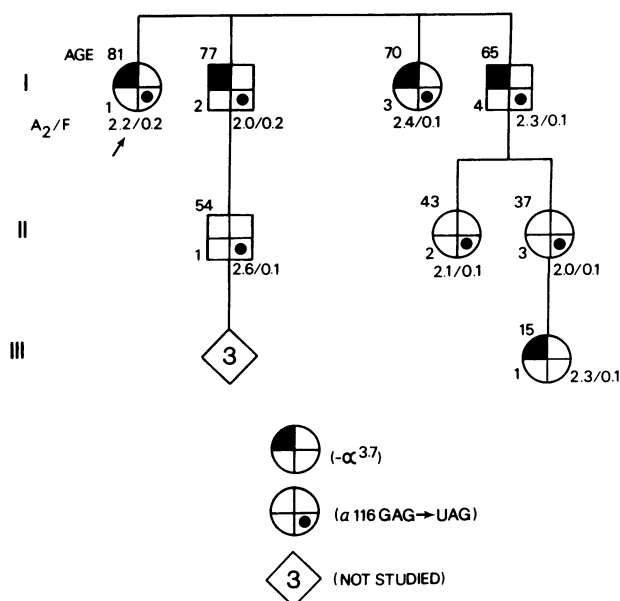

Figure 1. Pedigree of proband's family.

the first generation had marked microcytosis and three of the four had a mild-to-moderate anemia. Two of the three individuals in the second generation had borderline microcytosis and a mild depression of their hemoglobin level while the single individual studied in the third generation had a mild anemia despite a normal MCV. The father of this individual was not available for study. α/β synthetic ratios were measured in the reticulocytes of each of these individuals (Table I, α/β) and a low ratio, consistent with α-thalassemia, was present in six of the eight individuals spanning all three generations.

α-Globin gene mapping. The number and organization of the α-globin genes in each of the members of the kindred was determined by Southern mapping of white blood cell DNA using the restriction enzymes Bam HI and Bgl II. All individuals in the first generation were heterozygous for the 3.7-kb α-thalassemia deletion $(-\alpha^{3.7}/\alpha\alpha)$ as evidenced by α-globin bands of 16.0, 12.5, and 7.0 kb with the Bgl II analysis and bands of 14.0 and 10.0 kb with the Bam HI analysis (6, 40, 41). Each of the individuals in the second generation had a normal α-globin gene map $(\alpha\alpha/\alpha\alpha)$ as evidenced by α-globin bands of 12.5 and 7.0 kb generated by Bgl II digestion and a single band of 14.0 kb generated by Bam HI digestion. Examples of each pattern are shown in Fig. 2.

Relative expression of the α1- and α2-globin mRNA. The relative steady-state levels of reticulocyte α1- and α2-globin mRNA was determined by primer extension analysis (32). The results of a representative study is shown in Fig. 3, which compares the α2:α1 mRNA ratio of a normal individual (lane 2) with that of subject I1 (lane 4). Consistent with previous studies, the ratio in the normal control is 2.6:1 (32, 42). The α2:α1 mRNA in each of the individuals in generation I is 1.0. This value is well within the range measured in a series of black individuals heterozygous for the $-\alpha^{3.7}$ deletion $(-\alpha^{3.7}/\alpha\alpha)$ (42). The α2:α1 ratio in each of the individuals in generation II is ~ 2.6 (data not shown), which is consistent with their normal α-globin genotype.

Identification of the mutant α-globin chain and its encoding gene. Analysis of the hemoglobin from the red cells of the subjects
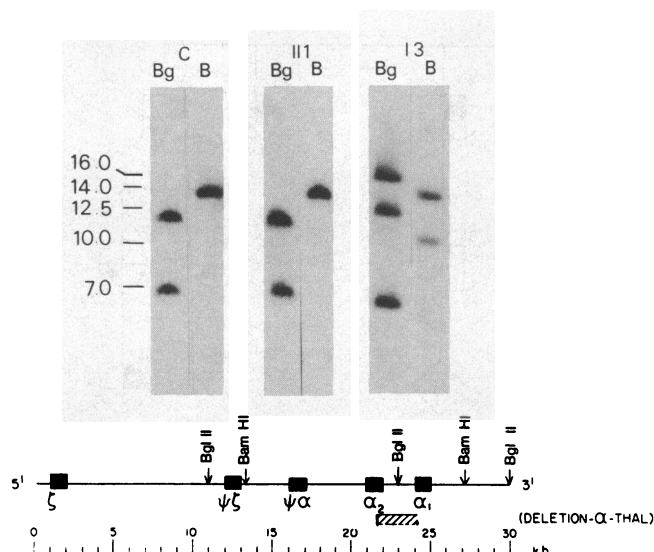
Figure 2. α-Globin gene mapping. Southern blot analysis of the α-globin gene organization in two representative individuals, II1 and I3, and a normal control (*C*) are shown. The map of the involved region of the α-globin gene cluster is at the bottom of the figure and the details of the analysis are given in the text. The size of the hybridizing bands as determined by comparison to standard DNA size markers is shown to the left of the autoradiograph in kb. The approximate positions of five genes in the α-globin cluster and the restriction endonuclease sites (BglII [*Bg*] and Bam HI [*B*]) relevant to the mapping are indicated on the map. The position of the 3.7-kb rightward deletion is shown by the cross-hatched rectangle.

in this family by isoelectric focusing, cellulose acetate, and citrate agar electrophoresis and separation of globin chains by HPLC showed only HbA, HbA2, and HbF (data not shown). Analysis of the globin chains under denaturing conditions on a Triton

Figure 3. Determination of the α2:α1 globin mRNA levels by primer extension mapping. Total reticulocyte RNA isolated from a nonthalassemic control with an αα/αα genotype and from the proband (I1) was reverse-transcribed using a 31-base end-labeled primer complementary to the 3' terminus of α-globin mRNA and analyzed by autoradiography of a 6% acrylamide/8 M urea gel (32). The cDNA product of each of these reactions is shown in lanes *1* and *3*, respectively. The position of the full length 580 nucleotide α-globin cDNA is indicated. An aliquot of the cDNA, shown in lanes *1* and *3*, was digested with Hae III and resolved on the gel in lanes *2* and *4*, respectively. The position of the primer, and the 3' end-labeled α1- and α2-globin mRNA specific bands are indicated to the right of the gel in nucleotides.

acid urea gel similarly showed only the normal α, β, and γ globin chains (data not shown). To determine whether any unstable α-globin structural mutants might be contributing to the α-thalassemia phenotype in this family, we translated reticulocyte RNA in vitro and analyzed the labeled products. The results of this study are shown in lane *1* of Fig. 4. In addition to the bands that migrate with normal α-globin and β-globin, a fast-migrating chain is seen in the in vitro translation. Analysis of an in vitro translation time course demonstrated that this protein was mildly unstable (data not shown). This protein was further characterized by hybrid-selected translation of the reticulocyte mRNA. As seen in lanes *2* and *3* of Fig. 4, the α1-globin mRNA encodes a normal α-globin whereas the α2-globin mRNA encodes the fast-migrating mutant. The α-globin mutant is therefore encoded by the α2-globin gene. Based upon the α-globin gene mapping (Fig. 2) and these translational data, the genotype of the propositus is $-\alpha^{3.7}/\alpha^T\alpha$ (T represents a nondeletion thalassemia mutation). The in vitro translations of the reticulocyte RNA from individuals I2, I3, I4, II1, II2, and II3 also demonstrated the presence of the mutant α-globin protein.

Cloning and sequence analysis of the mutant α2-globin gene. The 14.0-kb Bam HI DNA fragment containing the two adjacent α-globin genes (see Fig. 1) was cloned from the genomic DNA of the proposita in a lambda bacteriophage, and the α2-globin gene was subsequently subcloned on a 1.5-kb Pst I fragment that extends from ~ 400 bp 5' of the gene to 50 bp 3' to the gene (Fig. 5). The entire α2-globin gene was sequenced according to the scheme shown in Fig. 5. The sequence was determined to be identical to that previously reported for the normal α2-globin gene sequence (43, 44) with the exception of a single G → T substitution at the first base position of codon 116. A sequencing gel demonstrating this mutation (C → A in the coding strand) is shown in Fig. 6.

Discussion

The α1- and α2-globin genes are both expressed in normal individuals (32, 45) and encode an identical α-globin protein (46). α-Globin synthesis is therefore the sum of the expression of four α-globin genes. The various α-thalassemia syndromes result from
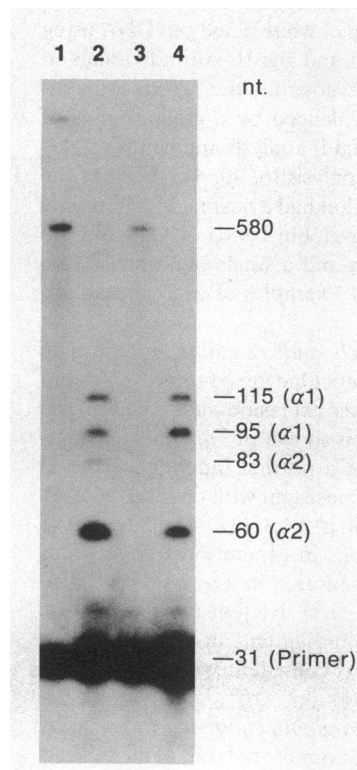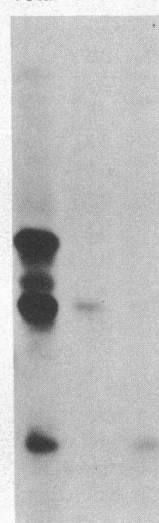

Figure 4. Locus assignment of the mutant globin chain by hybrid-selected translation. Total reticulocyte RNA (*Total*) or hybrid-selected α1- or α2-globin mRNA was translated in vitro in a rabbit reticulocyte lysate, and the [^{35}S]methionine-labeled protein products were analyzed on a Triton acid urea gel. The gel autoradiograph is shown in this figure. The position of the β-, α-, and mutant α-globin bands (α*) are indicated. The additional band just above the normal α-globin band is an α-globin oxidation product.
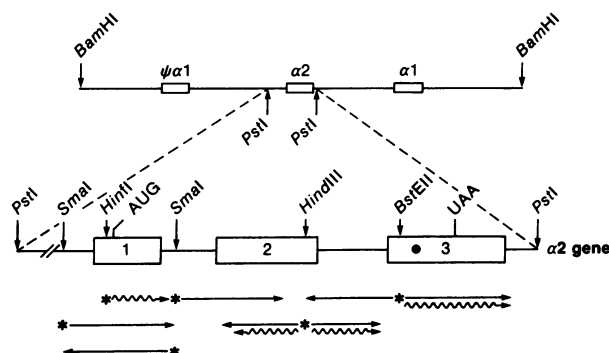
Figure 5. Cloning and sequencing of the α2-globin gene. The 14-kb Bam HI genomic fragment containing the two α-globin genes (*first line*) was cloned in the EMBL3 phage vector. The 1.4-kb Pst I fragment of this insert containing the α2-globin gene (*second line*) was subcloned in the plasmid vector pSP65. The α2-globin gene was then sequenced by the chemical degradation method of Maxam and Gilbert using the sequencing strategy shown at the bottom of the figure. The position of the restriction endonuclease sites relevant to the described steps are shown, as are the positions of the initiation (*AUG*) and termination (*UAA*) codons. The positions of the pseudo α-, α2-, and α1-globin genes are indicated and labeled on the first line, and the positions of the three exonic regions of the α2-globin gene are indicated and labeled on the second line. The labeled end of the fragments used for sequencing is indicated by an asterisk, and the extent and direction of the sequencing is indicated by a horizontal arrow. Fragments sequenced from a labeled 5′ terminus are shown as straight arrows, and those sequenced from a labeled 3′ terminus are shown as wavy arrows. All regions were either sequenced at least twice on a single strand or once on both strands. The position of the mutation at codon 116 is indicated by the dot in exon 3. This mutation was determined on both strands by sequencing from the BstE II site.

the loss of activity of one or more of these genes. The loss of a single α-globin gene is usually undetectable by hematologic studies and erythrocytes maintain a balanced α/β synthetic ratio;
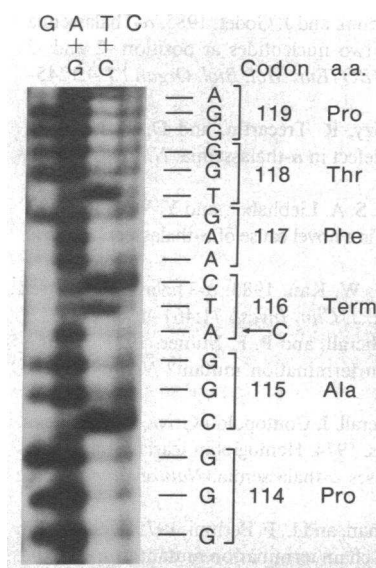

Figure 6. Sequencing gel demonstrating the $\alpha^{116GAG\rightarrow UAG}$ mutation. This sequence of the coding strand of the α2-globin gene demonstrates the base substitution of *C* to *A* at the first position of the codon 116, which results in a *G* to *U* mutation in the mRNA and the resultant termination codon UAG. The fragment was ^{32}P end-labeled at the BstE II site (see Fig. 5) subjected to Maxam and Gilbert chemical degradation reactions and resolved on an 8% acrylamide/8 M urea gel. The base-specific reactions are noted at the top of each lane and the reading of the bases, their codon grouping and amino acid (*a.a.*) translation is noted to the right of the gel. *Pro* is proline; *Thr* is threonine; *Phe* is phenylalanine; *Term* is termination codon; and *Ala* is alanine.

the loss of two genes results in microcytosis usually without significant anemia; the loss of three genes results in a microcytic anemia of variable severity usually associated with the accumulation of β4 tetramers (HbH); the loss of all four genes is not compatible with life (for review see references 1–3). In the subjects of this report, a discordance between the hematological findings and the restriction endonuclease map of the α-globin genes suggested the presence of a nondeletion defect contributing to the α-thalassemia phenotype. Subjects with a normal α-globin gene map (αα/αα) had microcytic red cells and slightly depressed α/β synthetic ratios and subjects with a single α-globin gene deletion ($-\alpha^{3.7}/\alpha\alpha$) had a significantly depressed α/β synthetic ratio and microcytic anemia. These hematologic measurements and reticulocyte protein synthesis studies suggested a loss of α-globin synthesis of the magnitude usually associated with the deletion of two to three α-globin genes.

Correlation of gene mapping data with hematologic studies in this kindred indicated that the loss of α-globin expression cosegregated with the structurally normal α-globin gene cluster (αα chromosome). The genetic studies however could not be used to determine further whether the defect was encoded at the α1- or α2-globin locus. Therefore two additional techniques, determination of the α1- to α2-globin mRNA ratio and in vitro translation of hybrid-selected mRNA, were used to further map and define this mutation. Previous studies of α-thalassemia have demonstrated that, with a single exception (13, 14), α-thalassemic defects are associated with decreased steady-state mRNA levels from the mutant locus (8, 9, 10, 32). The reason for this decrease remains unclear. In an attempt to use this property of α-thalassemia mutations to identify the mutant α-globin gene, we looked for a specific deficiency in α1- or α2-globin mRNA. That the relative levels of α1- and α2-globin mRNA in the individuals in generations I and II were in the range seen for otherwise healthy black individuals with the same αα/αα or $-\alpha^{3.7}/\alpha\alpha$ genotypes (42) indicated that the mutation was not interfering with stable α-globin mRNA synthesis. The only previously described nondeletion thalassemia gene that encodes a similarly normal level of mRNA contains a missense mutation resulting in the synthesis of a highly unstable α-globin (13, 14). To determine whether the thalassemic gene in the present study represented a similar mutation, we translated reticulocyte mRNA in vitro. This study did in fact reveal the presence of an abnormally migrating globin band, which was mapped by hybrid-selected translation to the α2-globin locus (Fig. 3). The absence of this α-globin chain from both the peripheral red cells and radiolabeled reticulocytes (data not shown) was consistent with the synthesis of an unstable α-globin chain. Subsequent sequence analysis of this cloned gene confirmed the presence of a point mutation in the α2-globin gene (Fig. 6) but was somewhat surprising in that this was a termination rather than a missense mutation. Reanalysis of the in vitro translations by sodium dodecyl sulfate–polyacrylamide gel electrophoresis confirmed this result by demonstrating that the aberrantly migrating band is indeed smaller than the normal α-globin (data not shown). The defined mutation, $\alpha2^{116GAG\rightarrow UAG}$, is therefore consistent with the genetic and biochemical analysis and predicts the total loss of expression from the affected α2-globin gene.

The nondeletion α-thalassemia defect in this report has certain unusual and potentially informative characteristics. As the mutation results in the synthesis of an unstable and truncated protein, the entire contribution of the affected α2-globin gene is lost. The individuals in generations I and II should therefore

have lost one of four or one of three α-globin genes, respectively. However, in both cases, the severity of the thalassemia is greater than would be predicted by the number of functioning α-globin genes. This discrepancy may be clarified by recent work in which we have demonstrated that the α2-globin gene encodes two to three times more α-globin than the α1-globin gene (47). The loss of an α2-globin gene therefore results in a major loss of α-globin synthesis. The reason why this has not become evident from previous studies of the α-thalassemias is that the correlation between gene number and disease severity has been based predominantly upon studies in populations in which the most common form of α-globin gene deletion is the $-\alpha^{3.7}$ deletion. The loss of the α2-globin gene in the $-\alpha^{3.7}$ deletion may not be directly comparable to the nondeletion mutation because the net loss of α-globin synthesis in this deletional state appears to be minimized by an associated increase in the expression of the remaining α1-globin gene (42). That the effect of the $\alpha2^{116UAG}$ mutation is more severe than would be predicted is consistent with a nondeletion mutation at the α2-globin locus resulting in a major loss of α-globin synthesis.

The second unexpected result of this study is that a nonsense mutation does not necessarily result in a decreased level in steady-state mRNA from the mutant locus. This is contrary to previous studies in β-thalassemia which indicate that nonsense mutations result in lowered levels of mRNA (48, 49). Our measurements of α2:α1 mRNA ratios in the members of this kindred with normal α-globin gene maps $(\alpha\alpha/\alpha\alpha)$ and single α-globin gene deletions $(-\alpha^{3.7}/\alpha\alpha)$ gave results identical to those for individuals with the same genotype but lacking the nondeletion defect (42). Therefore, no significant loss of α2-globin mRNA occurs within the accuracy of these measurements. If, as suggested by previous studies, the loss of mRNA in termination mutations results from a block in a ribosome-mediated nuclear to cytoplasmic transport (50, 51), it would be expected that this loss should occur in a mutation occurring at codon 116, which is well within the body of the α-globin mRNA. This hypothesis must therefore be somewhat modified. It may be, for example, that the localization of this mutation in the third exon, which distinguishes it from the more 5' nonsense mutations previously defined, allows occurrence of critical nuclear events that do not occur with more proximal mutations. Further studies in this regard will be needed, but it is clear that present concepts regarding the effect of a nonsense mutation upon mRNA levels must be reevaluated.

The prevalence of the $\alpha2^{116UAG}$ nonsense mutation in the black population and/or its presence in other populations is not addressed in the present study. The defined nucleotide substitution at codon 116 neither creates nor destroys a restriction site, which might be useful for diagnosis by conventional Southern gene mapping techniques. At present the best test for this mutation may be detection of the truncated α-globin protein by in vitro translation of reticulocyte mRNA or direct detection of the single nucleotide substitution with a synthetic oligonucleotide probe. While the incidence of this and other nondeletion α-thalassemia mutations is probably far less than the deletional forms, their characterization and identification may be of importance. Nondeletion defects should be considered in instances where a significant loss of α-globin gene expression cannot be reconciled with the results of gene mapping. This may be of particular interest in individuals with unusual clinical courses of β-thalassemia or sickle cell anemia, in which an interaction with an occult form of α-thalassemia may occur. Finally, as in the case of the proband in this report, nondeletion α-thalassemia should be considered in individuals with microcytosis of undefined origin who have a normal α-globin gene complement and no evidence of iron deficiency or β-thalassemia.

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