# α-Thalassemia Caused by an Unstable α-Globin Mutant

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ABSTRACT In a previous study, molecular cloning of the α-globin genes from a patient with nondeletion Hb-H disease (genotype $--/\alpha\alpha$) showed that a single nucleotide mutation (CTG to CCG) in one of the genes resulted in a leucine to proline substitution. This paper describes the approach we used to detect the abnormal α-globin chain. The chain was identified using a cell-free translation system. It turned over rapidly both in vitro and in vivo in the patient's reticulocytes. The unusual feature of this unstable α-globin is that the α-globin deficiency causes α-thalassemia. Simple heterozygotes for this lesion ($\alpha^{Pro}\alpha/\alpha\alpha$) resemble α-thalassemia carriers and do not exhibit the hemolytic anemia usually associated with unstable hemoglobins.

## INTRODUCTION

The human α-globin is encoded by two adjacent genes (α1 and α2) (1–3) on chromosome 16 (4). In the normal diploid state all four α-globin genes are active and produce identical α-globin products (5). Loss of function of one α-globin gene is clinically inapparent; when two genes do not function microcytosis occurs, and the α-thalassemia trait phenotype ensues; three nonfunctional genes cause hemoglobin-H disease (Hb-H), characterized by a moderately severe microcytic hemolytic anemia; functional loss of all four genes produces the lethal hydrops fetalis (6). Loss of α-globin gene function is usually associated with deletion of one or both α-globin loci (2, 3, 6, 7), but can also arise from nondeletion defects in which the α-globin genes are intact, but are expressed at subnormal levels (8–10). Although the reason for the absence of α-globin production in the deletion α-thalassemias is obvious, the molecular mechanisms involved in the nondeletion syndromes are less clear and vary among different populations.

Nondeletion Hb-H disease was initially described in a Chinese family (8). Molecular hybridization analysis and gene mapping studies of DNA from the propositus with Hb-H disease show that both α-globin genes are deleted from one chromosome but intact on the other (2). Since Hb-H disease results from functional loss of three of four α-globin genes, the clinical appearance of Hb-H disease in this patient with two intact α-globin genes implies total dysfunction of one of these genes or partial dysfunction of both. The two α-globin genes from this individual were recently cloned and sequenced (11, 12). The nucleotide sequence of the more rightward gene (α1) is normal, but the more leftward gene (α2) contains a single nucleotide mutation at codon 125 (CTG to CCG) that changes the amino acid from leucine to proline. The α-globin genotype on this α-thalassemia chromosome has been designated or $\alpha^{125Pro}\alpha$ (12).

We previously demonstrated that the abnormal α2-globin gene is transcribed normally (13) and detected trace amounts of a mutant globin chain by isotopic labeling of reticulocyte globin (12). In this study we describe the strategy used to identify the abnormal α-globin chain encoded by the mutant α2-locus. The extremely rapid degradation of this abnormal chain results in a functional loss of the α2-globin gene. The patient is thus left with only one functional α-globin gene (α1) and therefore expresses the Hb-H thalassemia phenotype.

## METHODS

*Patients.* The pedigree of the Chinese family with nondeletion α-thalassemia has previously been described in detail (8). For this study we carried out protein studies on the propositus (II-1) with Hb-H disease (genotype $\alpha^{125Pro}\alpha/--$). To search for evidence of hemolysis in the $\alpha^{125Pro}$ carrier state, we performed additional hematologic studies on one sister (II-5), who has four intact α-globin genes ($\alpha^{125Pro}\alpha/\alpha\alpha$) and a phenotype of α-thalassemia trait.

*Hemoglobin analysis.* Erythrocytes were washed three times in 0.9% NaCl, lysed in 2 vol water, and spun at 12,000 *g* for 5 min. The clarified hemoglobin solution was analyzed by starch gel electrophoresis (pH 7.0 and 8.6), cellulose acetate electrophoresis (pH 8.6), thin-layer isoelectric focusing

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*Received for publication 12 July 1982 and in revised form 4 November 1982.*

(14), and carboxymethyl cellulose chromatography in 8 M urea (15). In addition, 1 μg of clarified hemoglobin was electrophoresed under denaturing conditions (8 M urea) on a 12.5% acrylamide slab gel containing 2% Triton X-100 in 5% acetic acid buffer according to published procedures (16, 17). Gels were run at 9 mA for 14 h, stained for 30 min with Coomassie Blue (0.5% in 2% acetic acid and 30% methanol), and destained overnight in a solution containing 30% methanol and 7% acetic acid. For autoradiography of radiolabeled globin, the gels were treated in "En$^3$Hance" (New England Nuclear, Boston, MA), dried on Whatman 3 MM paper (Whatman Inc., Clifton, NJ) and exposed to Kodak XR5 (Eastman Kodak, Rochester, NY) film at −70°C. Band intensity on autoradiographs of gels containing $^{35}$S- or $^3$H-labeled protein was quantitated by scanning with a soft laser densitometer (LKB Instruments, Rockville, MD).

*mRNA isolation.* RNA was obtained from peripheral reticulocytes by selective lysis of thrice-washed erythrocytes in a buffered isotonic solution containing 0.15 M ammonium chloride and 1 mM ammonium bicarbonate (8). After spinning down unlysed leukocytes and erythrocyte membranes, polysomes were acid-precipitated by titrating to pH 5.1 with 10% acetic acid, and centrifuged at 5,000 rpm for 30 min in a Sorvall HS4 rotor (DuPont de Nemours Co., Sorvall Div., Newtown, CT). The polysome pellet was dissolved in a solution of 0.1 M Tris-HCl (pH 9.0), 0.1 M NaCl, and 1 mM EDTA, and extracted three times with phenol:chloroform:isoamyl alcohol (50:49:1) (18). The RNA was precipitated from 0.2 M sodium acetate (pH 5.1) with 2 vol ethanol. mRNA was purified from the ribosomal RNA by two passages on oligo-dT cellulose (19). The final low-salt peak (polyA$^+$ RNA) was precipitated from 0.2 M sodium acetate with 2 vol ethanol and stored until used.

*Cell-free translation.* Cell-free translation was performed at 30°C in a micrococcal nuclease-treated rabbit reticulocyte lysate (20) (Bethesda Research Laboratories, Inc., Gaithersburg, MD), supplemented with [$^{35}$S]methionine (1,500 Ci/mm, Amersham Corp., Arlington Heights, IL). To test the stability of translation products, unlabeled methionine in 100-fold molar excess of the labeled methionine was added to an aliquot of the reaction after 15 min of translation and incubated for an additional 45 min.

*Identification of the mutant globin chain by hybrid-arrested translation.* 200 ng of the propositus' mRNA was coprecipitated with either 200 ng of a 185-bp fragment of human α-globin cDNA excised from plasmid pRP-9 (21), or 3 μg of human β-globin cDNA pSAR-6 (22) linearized with *Bam* HI. Both pellets were dissolved in a 30-μl hybridization buffer (0.4 M NaCl, 40 mM PIPES [pH 6.4], 1 mM EDTA, 80% deionized formamide), heated to 70°C for 10 min and then incubated at 49°C for 3 h. The hybridization was terminated by precipitating with 3 vol ethanol in the presence of 2 μg yeast tRNA. The pellets were washed with 70% ethanol, dried, dissolved in 20 μl water and divided into two 10-μl aliquots. One was heated to 100°C for 2 min to melt the hybrid, and then both were chilled to 0°C and added to a wheat-germ translation extract (23) (Bethesda Research Laboratories). The reaction was incubated at 25°C for 1 h. 6-μl aliquots of each of the four reactions were applied to the Triton-urea gel.

*Intact reticulocyte incubation.* Reticulocytes were enriched from whole washed blood on a Percoll-Reno M-60 gradient [35% Percoll (Pharmacia Fine Chemicals, Piscataway, NJ); 15% Reno M-60 (E. R. Squibb & Sons, Princeton, NJ); 0.4 M NaCl] (24). 2 ml washed packed cells were applied to the top of a 27-ml gradient and spun at 13,000 rpm for 10 min in a Sorvall SS34 rotor. Erythrocytes were fractionated into eight distinct bands. The reticulocyte-rich top band was removed, washed four times in cold Krebs-Ringer phosphate, pH 7.4, and then resuspended in 0.5 M Krebs-Ringer phosphate, 0.5 ml dialyzed AB, Rh+ serum, 2 mg glucose, 20 nmol each of the 19 nonradioactive amino acids (excluding leucine), and 30 μCi [$^3$H]leucine. This suspension was incubated at 37°C in a gently shaking waterbath. Aliquots were removed at the specified time intervals and quick-frozen in an ethanol/dry-ice bath. When incubation was complete, the cells were lysed in 3 vol sterile, distilled water, the membranes were spun out at 20,000 rpm for 10 min, and clarified aliquots were applied to the gel for analysis.

## RESULTS

*Hematologic data of nondeletion α-thalassemia carriers.* The erythrocyte mean corpuscular volume and α/β-globin synthetic ratios of the propositus and three generations of his family have been reported (8). Hemoglobin levels and MCV of the family members with deletion type α-thalassemia trait (−−/αα) were indistinguishable from those of family members with α-thalassemia trait due to heterozygosity for the nondeletion defect ($\alpha^{125Pro}\alpha/\alpha\alpha$). We studied one of the latter carriers (II-5) in detail for evidence of accelerated hemolysis due to an unstable hemoglobin. Her hematologic values were: erythrocytes (RBC) 5.84 × 10$^6$/μl 14.0 g/dl, Hct 42.5%, mean corpuscular volume 72.8 fl, mean corpuscular hemoglobin 24.1 pg, and mean corpuscular hemoglobin concentration 33.1 g/dl. Her reticulocyte count was 1.3% (normal < 1.5%). Although the erythrocytes were hypochromic, there was no anisocytosis or poikylocytosis. Heinz body preparation was negative and haptoglobin was 96 mg/dl (normal 27–174), ruling out significant hemolysis. These data resembled those associated with α-thalassemia trait (−−/αα) and were inconsistent with the presence of a significant hemolytic process due to an unstable hemoglobin.

*Absence of abnormal α-globin mutant in erythrocyte hemolysate.* Because the DNA sequence of the α2-globin gene predicted a mutant α-globin, we searched for this protein in the propositus' erythrocytes by several procedures. We did not see any abnormal hemoglobin bands after running fresh whole blood lysate on starch gel electrophoresis (pH 8.6 and 7.0), cellulose acetate (pH 8.6), and isoelectric focusing gels. Chromatography on urea-carboxymethyl cellulose columns showed no abnormal elution peaks and electrophoresis on Triton-urea-acrylamide gel also failed to reveal any new bands (Fig. 1a, lanes 3 and 4).

*Detection of an unstable mutant α-globin chain.* The absence of any identifiable abnormal α-globin chain could either be due to its rapid turnover or to our inability to separate it from the normal chain. Because the abnormal α-globin might be less unstable in a cell-free system, we translated the propositus'

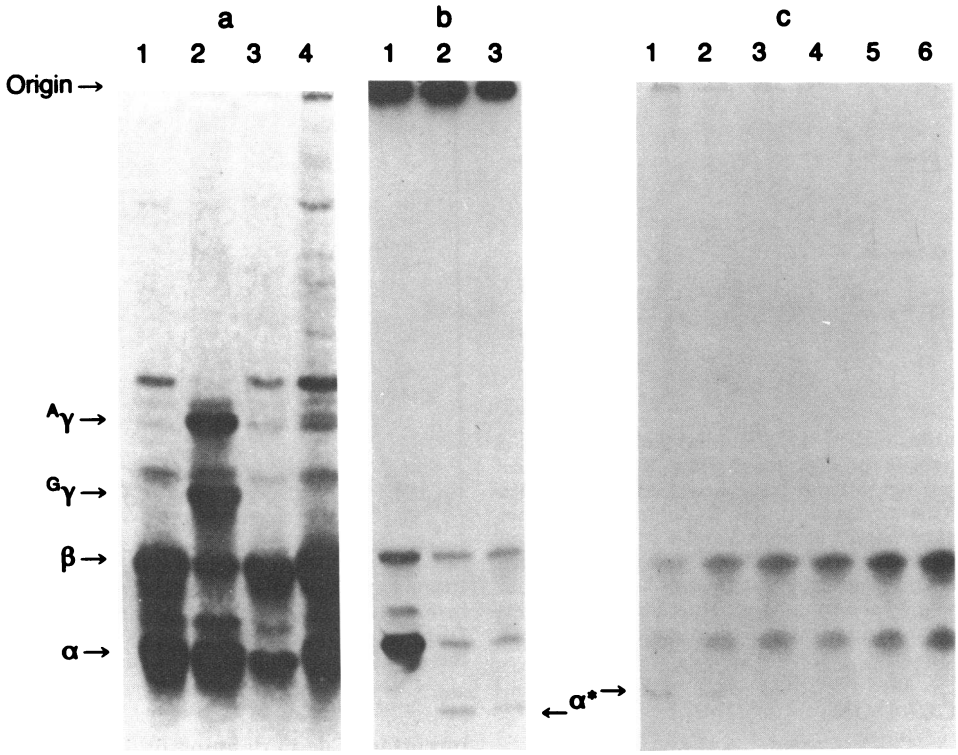


FIGURE 1 Electrophoresis of globin on Triton X100-urea-acrylamide gels. (a) Coomassie Blue staining of lysates from (1) normal adult, (2) normal cord blood, (3) and (4) nondeletion Hb-H patient's blood containing 1 μg and 5 μg hemoglobin. The band just above α-globin seen in all lanes in panel a and in b, lane 1 is an oxidized derivative of α-globin proportional to the duration of storage of the applied sample. (b) Autoradiogram of [$^{35}$S]methionine labeled globin synthesized in a rabbit reticulocyte cell-free system. Synthesis directed by (1) normal globin mRNA, (2) mRNA from the nondeletion Hb-H patient after 15 min incubation, and (3) after adding unlabeled methionine and incubating for an additional 40 min. $\alpha^*$ indicates the $\alpha^{125Pro}$ globin chain. (c) Autoradiogram of [$^3$H]leucine-labeled globin synthesized by the patient's reticulocytes. Lanes 1-6 represent six periods of incubation; 5, 10, 20, 40, 60, and 90 min.

mRNA in a rabbit reticulocyte cell-free system and separated the globin chains on Triton-urea gel. We detected an abnormal fast-moving band in addition to the normal α- and β-globin bands (Fig. 1b, lanes 2 and 3). This abnormal globin was unstable even in the cell-free system. When excess unlabeled methionine was added 15 min after the initial incubation and the mixture was incubated for an additional 40 min, the radioactivity of the fast-moving band diminished faster than that of the normal α- and β-globin chains (Fig. 1b). We identified this new band as an α-globin by hybrid-arrested translation in a wheat germ cell-free synthesis system (24). (Wheat germ was used instead of rabbit reticulocyte lysate because cDNA hybrids arrest translation more effectively in this system, unpublished observation). Synthesis of this band and the normal α-globin was inhibited by hybridizing the mRNA with an α-globin cDNA (Fig. 2). This abnormal α-globin chain was stable in the wheat-germ system, in contrast to its instability in the reticulocyte lysate system.

*Detection of unstable α-mutant in reticulocytes.* After locating the abnormal α-chain on Triton-urea gel using the cell-free translation system, we then searched for newly synthesized chains in vivo. Reticulocytes were incubated in the presence of [$^3$H]leucine, aliquots were removed at timed intervals, quick frozen, lysed, and applied to Triton-urea gels. After prolonged exposure, a rapidly-migrating band was identified at incubation time points up to 20 min at a position identical to the new band seen in the in vitro translations (Fig. 1c). In the incubation system, the rate of incorporation of radioactivity was nonlinear after the initial 10 min (Fig. 3). Although the radioactivity associated with the normal α- and β-globin increased with longer incubation, the rapidly degraded abnormal α-globin was no longer detectable at 40 min (Fig. 4).

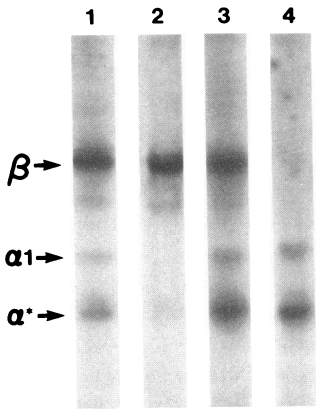


FIGURE 2 Identification of the abnormal globin chain as α in origin by hybrid arrested translation. The propositus' mRNA was translated in a cell-free wheat-germ extract with α-globin cDNA (lanes 1 and 2), and β-globin cDNA (lanes 3 and 4). The experiments in lanes 2 and 4 were performed under hybrid conditions, in lanes 1 and 3 the hybrids were dissociated by heating. Note that synthesis of both the normal and the fast-moving abnormal α-globin chain is inhibited after hybridization with α- but not with β-cDNA.

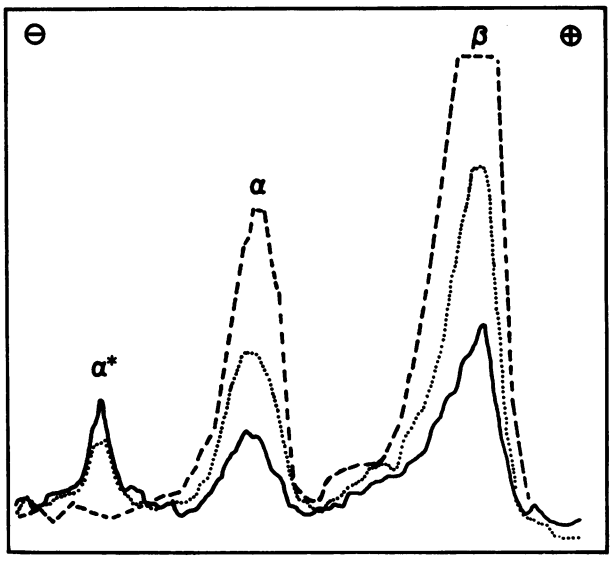


FIGURE 4 Densitometric tracing of autoradiogram shown in Fig. 1c. (——) represent 5 min incubation, (· · ·) show 10 min incubation, and (– – –) is 40 min incubation. Note the rapid turnover of the abnormal α-globin chain (α*).

## DISCUSSION

The delineation of the defect in this patient with nondeletion α-thalassemia illustrates the power of the new DNA technology. By determining the exact genetic mutation one can predict the protein abnormality and subsequently arrive at an understanding of the pathophysiology of the disease. This approach, which proceeds from analysis of DNA at the cellular level to an understanding at the clinical level, has resulted in the delineation of the molecular pathology of many thalassemia lesions (26–32). It will play an increasingly important role in defining other human inherited disorders.

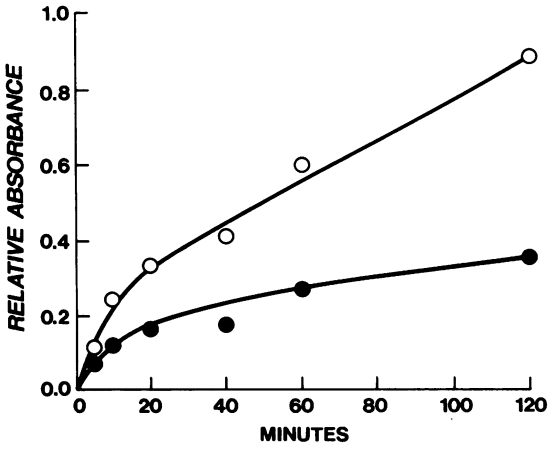


FIGURE 3 Incorporation of [$^3$H]leucine into the α1- (●) and β- (○) globin chains of the propositus' reticulocytes.

The propositus with Hb-H disease has two α-globin genes present, one of which is nonfunctional. His normal α1- and α2-globin reticulocyte mRNA ratio indicates that both genes are transcribed normally relative to each other and that the stability of the mutant α-globin mRNA is also normal (13). The stability of the mutant mRNA distinguishes this patient's lesion from the two previously defined nondeletion α-thalassemias that also result from mutations in the α2-globin gene. In both the α-Constant Spring lesion with a single base change in the termination codon, and the Mediterranean nondeletion α-thalassemia with a small deletion in the first intron, the mRNA from the affected α2-locus turn over rapidly and are not detectable in the reticulocytes (13, 33). In the current study, the propositus' α2-reticulocyte mRNA is probably translated normally, since the quantity of the mutant α-globin and the normal α-globin is approximately equal on short incubation. However, after a longer incubation period, when globin synthesis becomes nonlinear, the mutant α-globin is so unstable that it disappears. The absence of any detectable mutant chain in the mature erythrocytes is the result of its gross instability. These studies also demonstrate that a cell-free translation system can be used to identify proteins too unstable to be detected in vivo.

All the known leucine → proline (Leu → Pro) mutations produce unstable hemoglobin syndromes with

hemolytic anemia (34). For example, Hb Bibba, an $\alpha^{138Leu \rightarrow Pro}$ mutant affecting the same $\alpha$-helix, causes hemolytic anemia from birth (35). Furthermore, a highly unstable $\beta$-globin mutant (Hb Indianapolis) associated with a $\beta$-thalassemia phenotype is accompanied by hemolytic anemia in the heterozygous state (36). In distinction to these cases, the simple heterozygotes for the nondeletion defect ($\alpha^{125Pro}\alpha/\alpha\alpha$) in the family in the present study show no evidence of hemolysis or Heinz bodies, indicating that the mutant chain does not result in an unstable hemoglobin syndrome.

Why does this $\alpha$-globin mutant cause $\alpha$-thalassemia instead of hemolytic anemia? Three $\alpha 1\beta 1$ contact points [122 histidine (His), 123 alanine (Ala), 127 lysine (Lys)] lie close together in the H helix of $\alpha$-globin (37). These contact points are essential for initial $\alpha 1\beta 1$ dimer formation and subsequent hemoglobin tetramer assembly. The substitution of proline for leucine at codon 125 disrupts the H helix, thus altering the spatial arrangements of these contact points and decreasing the affinity of the $\alpha$-globin chain for the $\beta$-globin chain. The free uncombined $\alpha$-globin chains are catabolized rapidly causing $\alpha$-globin chain deficiency. This final outcome resembles heterozygous $\beta$-thalassemia, where the free uncombined $\alpha$-globin chains are rapidly catabolized and are difficult to detect in the reticulocytes.

The identification of the highly unstable $\alpha^{125Pro}$ at the $\alpha 2$-locus explains the pathogenesis of Hb-H disease in the propositus ($\alpha^{125Pro}\alpha/--$), since only one locus ($\alpha 1$) now produces a stable $\alpha$-globin chain. The $\alpha$ thalassemia trait phenotype found in family members with the $\alpha^{125Pro}\alpha/\alpha\alpha$ genotype is unexpected since three out of four genes are still producing stable $\alpha$-globin chains, and these individuals should thus be silent carriers. We do not understand the reason for this discrepancy. Perhaps the abnormal $\alpha$-globin not only causes unstable $\alpha 1\beta 1$ dimer formation, but also affects the stability of transiently assembled tetramers composed of one molecule each of the normal and abnormal $\alpha$ globin. Assuming that the $\alpha$- and $\beta$-globin chains associate randomly, the ratio of $\alpha_2{}^{125Pro}\beta_2$, $\alpha^{125Pro}\alpha\beta_2$, and $\alpha_2\beta_2$ tetramers would be $1^2$, $2 \times 1 \times 3$, and $3^2$, or 1, 6, and 9, respectively. In this event, the amount of loosely associated and readily dissociable tetramer containing $\alpha^{125Pro}$ would approach 50% and the loss of this amount of tetramer would result in microcytosis.

Molecular studies of the thalassemias have disclosed defects at various steps in the process of gene expression. The defect in this patient with Hb-H disease combines two molecular lesions. The first is a double $\alpha$-globin gene deletion commonly associated with $\alpha$-thalassemia and the second is a single base change in the coding region of the $\alpha 2$-gene on the homologous chromosome. The resulting $\alpha$-globin encoded by this gene is unsuitable for hemoglobin tetramer formation and is rapidly catabolized. This posttranslational defect resulting in $\alpha$-thalassemia occurs at the most distal step necessary for the functional expression of the globin genes.

## ACKNOWLEDGMENTS

We thank Marie Doherty for expert technical assistance, Klara Klemen for running the isoelectric focusing gels and Jennifer Gampell for editorial comments.

This work was supported by grants from the National Institutes of Health, the March of Dimes/Birth Defects Foundation, and UNICO National, Inc.

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