Two missense mutations in the beta-globin gene can cause severe beta thalassemia. Hemoglobin Medicine Lake (beta 32[B14]leucine-->glutamine; 98 [FG5] valine-->methionine).

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Research Article

We studied the molecular basis of transfusion-dependent hemolytic anemia in an infant who rapidly developed the phenotype of beta thalassemia major. DNA sequence of one beta-globin gene of the proband revealed two mutations, one for the moderately unstable hemoglobin (Hb) Köln and another for a novel codon 32 cytosine-thymidine-guanine-->cytosine-adenine-guanine transversion encoding a leucine-->glutamine mutation. A hydrophilic glutamine residue at beta 32 has an uncharged polar side chain that could potentially distort the B helix and provoke further molecular instability. This new hemoglobin was called Hb Medicine Lake. Biosynthesis studies showed a deficit of beta-globin synthesis with early loss of beta-globin chains. An abnormal unstable hemoglobin, globin chain, or tryptic globin peptide was not present, demonstrating the extreme lability of this novel globin. Hb Medicine Lake mRNA was present, but an aberrantly spliced message was not. Absence of an abnormal beta-globin gene in the mother makes it likely that a de novo mutation occurred in the proband. The molecular pathogenesis of Hb Medicine Lake illustrates a mechanism whereby the phenotype of a genetic disorder, like the mild hemolytic anemia associated with a hemoglobinopathy, can be modulated by a coincident mutation in the same gene.

Two Missense Mutations in the β-Globin Gene Can Cause Severe β Thalassemia

Hemoglobin Medicine Lake (β32[B14]Leucine→Glutamine; 98 [FG5] Valine→Methionine)

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Abstract

We studied the molecular basis of transfusion-dependent hemolytic anemia in an infant who rapidly developed the phenotype of β thalassemia major. DNA sequence of one β-globin gene of the proband revealed two mutations, one for the moderately unstable hemoglobin (Hb) Köln and another for a novel codon 32 cytosine-thymidine-guanine→cytosine-adenine-guanine transversion encoding a leucine→glutamine mutation. A hydrophilic glutamine residue at β32 has an uncharged polar side chain that could potentially distort the B helix and provoke further molecular instability. This new hemoglobin was called Hb Medicine Lake. Biosynthesis studies showed a deficit of β-globin synthesis with early loss of β-globin chains. An abnormal unstable hemoglobin, globin chain, or tryptic globin peptide was not present, demonstrating the extreme lability of this novel globin. Hb Medicine Lake mRNA was present, but an aberrantly spliced message was not. Absence of an abnormal β-globin gene in the mother makes it likely that a de novo mutation occurred in the proband. The molecular pathogenesis of Hb Medicine Lake illustrates a mechanism whereby the phenotype of a genetic disorder, like the mild hemolytic anemia associated with a hemoglobinopathy, can be modulated by a coincident mutation in the same gene. (*J. Clin. Invest.* 1995. 95:503–509.) Key words: β-thalassemia • hemoglobinopathies • globin • mutation

Introduction

Genetic abnormalities of hemoglobin (Hb)[1] are complex and a paradigm for nearly all inherited diseases. They include hemoglobinopathies, produced by mutations in the coding regions of globin genes and characterized by globins with abnormal primary structure, and thalassemias, typified by reduced globin biosynthesis and generated by many different classes of mutations that impair gene expression (1). Some disorders have features of both hemoglobinopathies and thalassemias (2). Curiously, the phenotype of some hemoglobin disorders caused by a single mutation varies widely, suggesting the presence of additional genetic or acquired factors that modulate the expression of the abnormal gene. For example, sickle cell anemia is clinically heterogeneous (3). Other genetic diseases have similar diversity (4).

Most unstable hemoglobin variants are associated with a mild or moderate hemolytic anemia (1). Rare hyperunstable variants produce a severe disease that is dominantly inherited and resembles the classical thalassemias. These are usually caused by mutations in the third exon of the β-globin gene (2). We studied a child with intense hemolytic anemia who during 1 yr developed the clinical features of severe β thalassemia. While an abnormal hemoglobin protein or a classical thalassemia mutation could not be found, one of her β-globin genes contained two coding region mutations. One coded for the common, moderately unstable hemoglobin variant, Hb Köln, while the other, a novel mutation, was predicted to also result in molecular instability. Kinetic studies of globin synthesis suggested the presence of a labile β-globin chain, but a protein or peptide corresponding to the predicted product could not be detected, implying extreme instability. Together, these mutations produced transfusion-dependent anemia with characteristics of classical β thalassemia, including marked extramedullary hematopoiesis. The molecular pathogenesis of Hb Medicine Lake illustrates a mechanism where the phenotype of a genetic disorder, like the mild hemolytic anemia associated with a hemoglobinopathy, can be modulated by a coincident mutation in the same gene.

Methods

Hematologic tests. Routine hematologic techniques were used. Hemoglobin electrophoresis was done on cellulose acetate membranes, citrate agar gels, and by isoelectric focusing on polyacrylamide gels using both chloroform extracted and unextracted hemolysates (5). Hb F was measured by alkali denaturation and ELISA. Unstable hemoglobins in the hemolysate were evaluated by the isopropanol and heat stability tests (5). Heinz bodies were stained with brilliant cresyl green. Red cell enzyme analysis was done by the Scripps Clinic and Research Foundation (La Jolla, CA) by Dr. E. Beutler. Osmotic fragility and membrane heat stability were evaluated by standard means (1, 5).

Globin gene analysis. DNA was isolated from peripheral blood leukocytes (6). The β-globin gene was amplified by PCR (7). Fig. 1 depicts the location and sequence of the primer pairs used to amplify the regions of interest discussed below. A polymorphism is present at position 74 of IVS II of the β-globin gene, where the wild-type base is guanine (G) and the polymorphic base, thymidine (T). If amplimers specific for either the wild-type sequence or polymorphic sequences were used under stringent conditions, it was possible to specifically amplify β-globin gene fragments with or without this polymorphism.

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1. *Abbreviations used in this paper:* A, adenosine; C, cytosine; G, guanine; gln, glutamine; Hb, hemoglobin; IVS, intervening sequence; T, thymidine.

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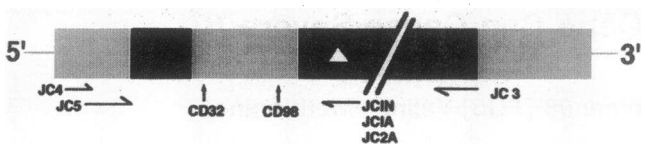

Figure 1. Amplification of the β globin gene fragment containing codons 32 and 98. The PCR primers used to amplify the portion of the β globin gene containing codons 32 and 98 are shown below the diagram that depicts the β globin gene and the relative positions of the primers, mutations, and polymorphisms. Two rounds of amplification were used to obtain a single stranded template for sequencing. For the first round of PCR the 5′ primer was always JC4 and for the second round the 5′ primer was JC5. When the 3′ primer was JC1N, only wild-type sequence was seen at codons 32 and 98. When primers JC1A or JC2A were used only abnormal sequence was present at codons 32 and 98, but when primer JC3 was used, both normal and abnormal sequence was seen at codons 32 and 98. Exons are shown as shaded boxes with codons (*CD*) 32 and 98 designated by arrows and introns shown as open boxes. The IVS II-74 polymorphic site is represented by a triangle. PCR primers: JC1N, 5′ CTG TAC CCT GTT ACT TC 3′ (bp 565–581); JC1A, 5′ CTG TAC CCT GTT ACT TA 3′ (bp 565–581); JC2A, 5′ CCT GTT ACT TAT CCC CTT CC 3′ (bp 556–575); JC3, 5′ GGC ATT AAG TAT AAT AG 3′ (bp 609–625); JC4, 5′ TCC TGA GGA GAA GTC TGC C 3′ (bp 66–84); and JC5, 5′ CTG TGG GGC AAG GTG AAC 3′ (bp 94–112). Annealing temperatures are given in the text.

DNA sequencing was done directly from asymmetrically amplified templates as described previously (8, 9).

α-Globin genotype was determined by Southern blot hybridization after digestion of DNA with EcoRI, BglII, and BamHI, using nonradioactive labeled α-and ζ-globin gene specific probes, as previously described (10, 11).

mRNA studies. Peripheral blood was washed four times with iced normal saline, the buffy coat removed, and the cells lysed with 4 M guanidinium isothyocyanate. Messenger RNA was isolated from reticulocytes of the proband using a rapid total RNA isolation kit (5Prime–3Prime, Inc., Boulder, CO). By means of reverse transcriptase-PCR, two cDNA fragments were isolated. One fragment of 360 bp extended from codon β14 through codon β132 and encompassed the sites of both mutations. A second 96-bp fragment spanned codons β26 to β36. Both cDNA fragments were sequenced directly, as described above, and additionally, the 96-bp fragment was analyzed on Nu-Sieve (FMC Corp., Rockland, ME) agarose gels.

Globin biosynthesis studies. Fresh heparinized blood was washed three times with iced normal saline. After centrifugation at 2,000 rpm for 5 min, portions from the reticulocyte-rich fractions were incubated for 2.5, 5, 10, 20, and 40 min at 37°C with 500 μCi of [^{3}H]L-leucine (sp act 120–190 Ci/mmol) in an incubation mixture containing all essential amino acids except leucine (12). In other studies, 100 μM heme was added to blood and incubated with 500 μCi of [^{3}H]L-leucine at 37°C for 5 and 10 min duration, while another portion of blood was incubated at 22°C for 10 min with [^{3}H]leucine. All incubations were stopped by placing the blood suspension on ice and washing the cells three times with iced saline. The globin chains were then separated by HPLC using a VYDAC C4 column (Hysperia, CA) (13). Radioactivity under the globin protein peaks was counted in a liquid scintillation counter.

Pulse-chase studies. Washed reticulocyte-rich blood was incubated, as detailed above, for 5 min with [^{3}H]L-leucine, placed on ice, and washed four times, with iced saline. The cells were resuspended in an amino acid mixture containing a 100-fold molar excess of cold leucine, and incubated at 37°C. Portions of this suspension were removed at 2.5, 5, 10, 20, and 120 min and the samples placed on ice and washed three times with iced normal saline.

Heat stability of HPLC-separated globin chains. Hemolysate was heated at temperatures from 37°–52°C for 20 min before the separation of labeled globin chains.

Separation of globin tryptic peptides. Normal, unlabeled β^A-globin chains were purified by HPLC, mixed with radioactive protein peaks separated from the hemolysate of the proband, aminoethylated, digested with trypsin, and separated by HPLC as previously described (13–15).

Table I. Hematologic Findings in the Proband and Parents

	Packed cell volume	Hb g/dl	Mean corpuscular volume fl	Reticulocyte percentage	Hb F percentage	Hb A_2 percentage
Proband 9 mo	20.5	6.5	79	16.3	15	—
Proband 19 mo*	26.9	7.8	73	19.5	6.8	3.4
Mother	40.7	13.6	88	1.0	1.0	3.0
Father	48.9	16.3	89	0.8	—	

* These counts were obtained about 3–3.5 mo after blood transfusion.

Results

Case report. A Caucasian female who was anemic from birth, was 19 mo old when last studied. She presented with neonatal jaundice, at least partly attributable to ABO incompatibility. Her first blood transfusion was at 2.5 mo when her hemoglobin was 4.6 g/dl and reticulocytes 12%. By 6 mo, her spleen was 5 cm below the left costal margin. Over the following 12 mo, the spleen enlarged below the umbilicus, the liver extended to five cm beneath the right costal margin, and frontal bossing became apparent. Pigmenturia was not obviously present when the patient first presented, although there were some episodes of dark urine associated with viral infections. These specimens were positive for bilirubin, but negative for heme. The hematologic findings in the proband at ages 9 and 19 mo are shown in Table I. She has received 10 blood transfusions to date. The blood film showed hypochromia, microcytosis, eccentrocytes, elliptocytes, bite cells, target cells, polychromatophilia, basophilic stippling, and nucleated red cells (Fig. 2). Heinz bodies were not observed. At 8 mo, the Hb F measured by ELISA was 20% and isoelectric focusing showed ~ 15% of a hemoglobin band with the migration of Hb F. Hemoglobin electrophoresis and isoelectric focusing of hemoglobin did not show an abnormal hemoglobin. Abnormal hemoglobin bands were not present when heme-specific or protein-sensitive stains were used. A small amount of protein that may represent native or oxidized free α-globin chains was detectable by isoelectric focusing. At 19 mo, isoelectric focusing showed a smaller amount of Hb F, and Hb F measured by alkali denaturation was 6.8%. The Hb A_2 level was 3.4%. Isopropanol and heat stability tests for unstable hemoglobins were negative.

The determination of 21 erythrocyte enzymes showed either normal or elevated levels, consistent with hemolytic anemia. Osmotic fragility tests showed < 5% fragile cells. Heating red cell suspensions at 47°C for 30 min did not cause membrane budding.

Both mother and father were normal hematologically and neither parent had an abnormal hemoglobin detectable (Table I).

β-Globin gene analysis. In the proband, the nucleotide se-

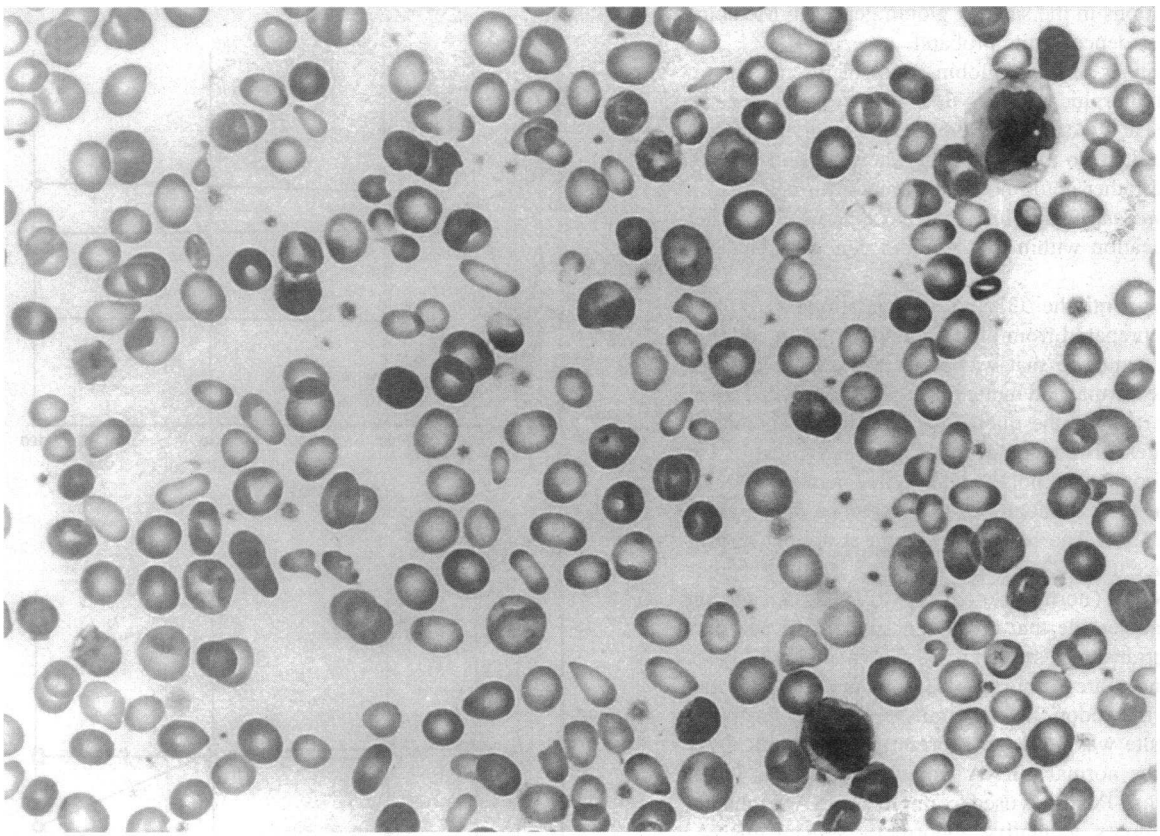

Figure 2. Peripheral blood film of the proband showing hypochromia, microcytosis, and eccentrocytes, bite cells, fragmented cells, tear drop cells, spherocytes, and elliptocytes. Basophilic stippling is prominent.

quence of the β-globin genes and their 5′ and 3′ untranslated regions were normal except at codons 32 and 98. In codon 98 there was a guanine-thymidine-guanine→adenosine-thymidine-guanine (GTG→ATG) transition. This codes for the valine to methionine mutation of Hb Köln, a previously described unstable hemoglobin (16, 17). In codon 32 there was a cytosine-thymidine-guanine→cytosine-adenosine-guanine (CTG→CAG) transversion that would cause a leucine to glutamine substitution. An abnormal hemoglobin with this substitution at codon β32 has not been previously described.

To learn if these mutations were present in *cis* or in *trans* we used allele specific oligonucleotide primers to amplify specifically portions of both β-globin genes. In all PCR reactions, 40 cycles of amplification were done with each cycle consisting of 1 min of denaturation at 94°C, 1 min of annealing at temperatures decided by the oligonucleotide primers used, and 1 min of extension at 72°C. The proband was heterozygous for the G → T polymorphism at intervening sequence (IVS) II, position 74. When an oligonucleotide, JC3, (annealing temperature, 58°C, see Fig. 1) 3′ to the IVS II, position 74 polymorphic site was used as the 3′ primer in PCR, both normal and mutant sequences were present at codons 32 and 98 (Fig. 3, *right panel*). If the oligonucleotide, JC1N (annealing temperature, 45°C), spanning IVS II, position 74 and containing the wild-type nucleotide at this position, was used to prime amplification of a β-globin gene segment that encompassed both codons 32 and 98, both codons contained only normal sequence (Fig. 3, *center panel*). When either oligonucleotides JC1A (annealing temperature, 45°C) or JC2A (annealing temperature, 61°C) containing the polymorphic base at position 74 of IVS II was used to prime amplification, both the codon β32 T→A and codon β98 G→A mutations were present (Fig. 3, *left panel*). These results show that both mutations were present on the same β-globin gene (in *cis*), and were linked to the IVS II-74 polymorphism. We have called the predicted abnormal hemoglobin con-

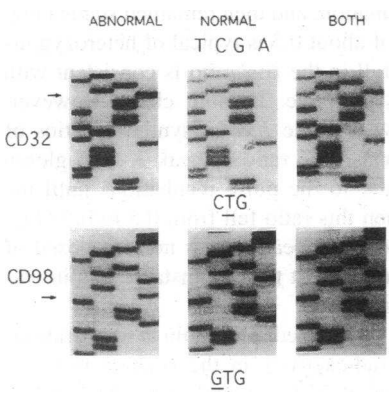

Figure 3. Sequencing gels from the proband showing the Hb Köln and β32 mutations present on the same β-globin gene fragment. When an oligonucleotide, JC3, 3′ to the IVS II, position 74 polymorphic site was employed as the 3′ primer in PCR (see Fig. 1), both normal and mutant sequences were present at codons 32 and 98 (*right panel*). When an oligonucleotide, JC1N, spanning IVS II, position 74 and containing the wild-type nucleotide at this position, was used to prime amplification of a β-globin gene segment that encompassed both codons 32 and 98, both codons contained only normal sequence (*center panel*). When oligonucleotides JC1A or JC2A containing the polymorphic base at position 74 of IVS II were used to prime amplification, both the codon β32 T→A and codon β98 G→A mutations were present (*left panel*).

taining two mutations in the same β-globin gene, Hb Medicine Lake, after the residence of the proband.

Sequence analysis of the β-globin genes of the mother revealed three polymorphic changes in a single β-globin gene, but the sequences at codons 32 and 98 were normal. Therefore, the mother transmitted to the proband the normal wild-type β-globin gene. The father could not be examined further.

α-Globin gene analysis. There was no evidence of either deletion or duplication within the α-globin gene cluster (data not shown).

mRNA Studies. Both the β32 and β98 mutations were present in the cDNA prepared from blood reticulocytes, in addition to the wild type sequence that was expected at these codons. The density of the normal and mutant base bands appeared equal (data not shown). Since the microcytic–hemolytic anemia of the proband suggested reduced globin synthesis typical of the thalassemia syndromes and not the hemolytic anemia that characterizes most unstable hemoglobins, we searched for an aberrantly spliced mRNA. The base substitution at codon 32 produces the sequence ttagGCTGCAGGTG just 3′ to the acceptor splice site of IVS I (normally ttagGCTGCTGGTG) and an additional ag dinucleotide that creates an alternative acceptor splicing site (untranslated sequence is shown in lower case, translated sequences are in upper case, and underlined is the AGG created by the codon 32 T→A transversion). If this putative new splice site was used, an aberrantly spliced mRNA 7 bp shorter than the normal mRNA would be expected. When reverse transcriptase-PCR was used to amplify cDNA fragments that would encompass a putative abnormally spliced mRNA, only normal sized cDNA fragments and sequence was found excluding at the limits of detection of this method, abnormal mRNA splicing.

Globin biosynthesis studies. Globin biosynthesis ratios after incubation times of 2.5 to 120 min and chase periods of 2.5 to 120 min are shown in Fig. 4. Representative elution profiles of HPLC-separated globin chains and their associated radioactivity at 20 and 2.5 min incubation times are shown in Fig. 5. The elution profiles of [^{3}H]leucine labeled globin chains showed a peak of radioactivity, with a consistent small protein peak, labeled X. This pre-β peak was shown to contain only normal β-globin tryptic peptides. The β:α ratio fell from 0.6 at 2.5 min to ~ 0.5 after 20 min incubation, and then remained stable (Fig. 4 *A*). A stable β:α ratio of about 0.5 is typical of heterozygous β thalassemia. An early fall in the β:α ratio is consistent with the presence of a highly unstable β-globin chain. However, pulse–chase studies showed stable β:α biosynthesis ratios of ~ 0.5 for all chase intervals. The ratio of peak X to β-globin chain synthesis was similar to the pulse incubations until the 120 min chase period when this ratio fell from 0.5 to 0.3 (Fig. 4 *B*). These results suggest that peak X was not composed of a highly unstable globin chain, but that an unstable globin was present under the β-chain peak.

When the hemolysate was heated for 20 min at temperatures from 37°C to 52°C, the radioactivity in the β-chain peak remained stable. Incubations at 22°C, or with 100 μM heme at 37°C, did not alter the globin biosynthesis ratios.

Peptide composition of radiolabeled globins. Reversed-phase HPLC of a mixture of aminoethylated trypsin digested β globin chains separated from samples incubated for 2.5 and 120 min showed only normal β globin peptides without an aberrant peak that absorbed at 220 nm (A_{220}) (Fig. 6, *top*). The radioactivity elution profile of tryptic peptides after incubations of 2.5 and 120 min showed only normal β-globin peptides. (Fig. 6, *bottom*) When the radioactive peak X was mixed with normal unlabeled β^A-globin chains only normal β-globin peptides were present indicating that peak X represents the pre-β peak and not an abnormal globin (data not shown).

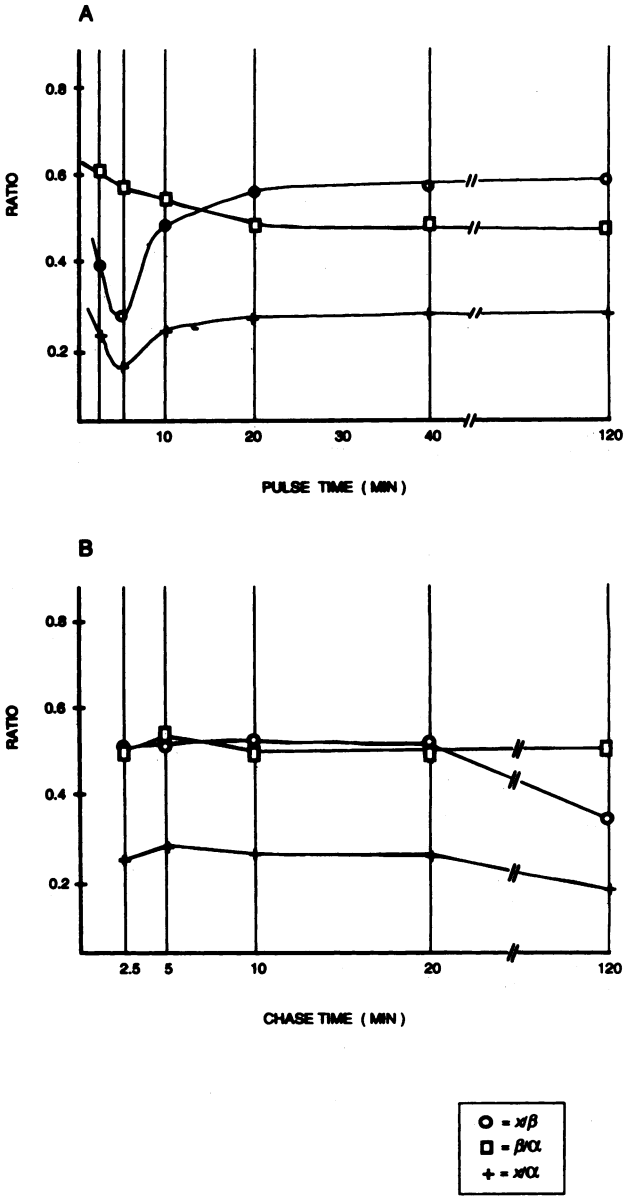

Figure 4. Globin biosynthesis ratios after incubation times of 2.5 to 120 min (*A*). In pulse–chase studies (*B*) incubation for 5 min was followed by chase periods of 2.5 to 120 min.

Discussion

Etiology of the phenotype. Thalassemic hemoglobinopathies are hemolytic anemias with features of classical β thalassemia that are not caused by typical thalassemia mutations, but rather by a select group of hemoglobin variants with different aberrations of their primary structure (2). In the initial example of a hyperunstable hemoglobin variant causing the severe transfusion-dependent β-thalassemia phenotype, the disorder was dominantly

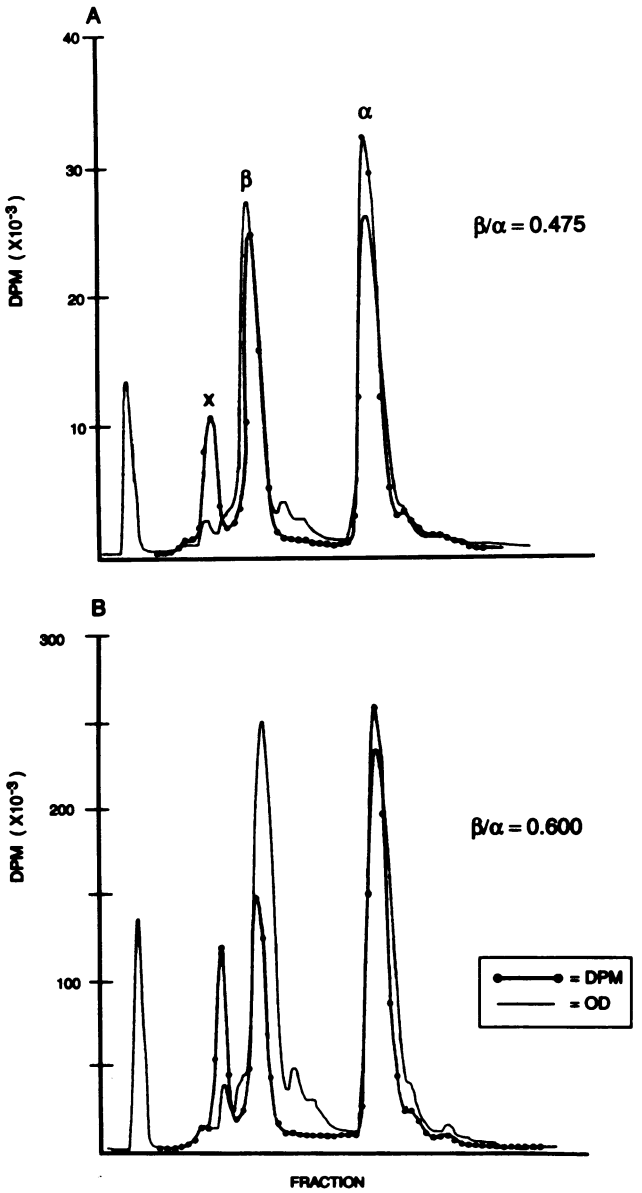

Figure 5. Protein and radioactivity elution profiles, obtained by reverse-phase HPLC after erythrocyte labeling with [^{3}H]leucine for 20 (*top*) and 2.5 (*bottom*) min. The position of β and α chains is shown and the pre-β radioactivity peak is labeled X.

inherited and the affected patients died of their disease (9, 12). While the pathogenesis of this class of disorders is still not totally understood, many, but not all instances have been due to mutations in the third exon of the β globin gene that interfere with the assembly of $\alpha\beta$ dimers. Contact points at the $\alpha_1\beta_1$ interface are largely encoded within the third exon. Proteolysis of redundant normal and unstable globin, or exhaustion of normal proteolytic capacity has been postulated to cause cellular injury (12, 15, 18). Cell damage, induced by the conjoint effects of excessive α-chains and unstable β-chains, may be responsible for the severe phenotype of some thalassemic hemoglobinopathies in contrast to the trivial clinical effects of heterozygous β thalassemia where a single β-globin gene is rendered nonfunctional. As expected in unstable hemoglobinopathies and β thalassemias a small amount of free α-globin chains were detected. We hypothesize that the severe β-thalassemia phenotype of the proband results from the evanescent presence of Hb Medicine Lake and uncombined α-globin. Hb Medicine Lake mRNA is easily detectable in reticulocytes indicating that transcription of this gene is apt to be normal and the resulting message stable. But the β-globin chain, containing both the Hb Köln and the β32 mutation, is likely to be highly unstable and rapidly catabolized within erythroid precursors and in reticulocytes leading to the ineffective erythropoiesis and hemolysis typical of the β thalassemias. Our inability to find an abnormal or unstable protein or peptide in reticulocytes, even after incubations as short as 2.5 min, is consistent with this notion. Heme binding or incorporation into a hemoglobin dimer or tetramer may not occur so that a tetramer never forms and the abnormal globin chain is catabolized nearly instantaneously. Other exceptionally unstable hemoglobins can be undetectable in the hemolysate (2, 12, 15). In distinction to these observations, individuals with unstable hemoglobins have typically mild or moderate hemolytic anemia.

Abnormal mRNA splicing could be an alternative cause of reduced accumulation of a Hb Medicine Lake globin chain. In some thalassemic hemoglobinopathies, the base substitution that changes the primary sequence of globin also activates a new splice site and reduces the accumulation of mRNA (19–22). We were unable to detect an abnormally spliced mRNA at the limits of detection of our reverse transcriptase-PCR method. While the base substitution at codon β32 produces the alternative splice site, ttaggCTGCAGGTG, (AGG created by the codon 32 T→A transversion is underlined; consensus splice sequence is (T/C)^{n}NC/TAGG) the newly created C/TAGG occurs in proximity to another 5′ AG dinucleotide and its utilization is unlikely (23).

Unstable hemoglobins. Hemoglobin Köln was first described in Europeans (16, 17) and has become the prototype and most commonly reported unstable hemoglobin (24–26). Mild anemia, reticulocytosis, splenomegaly, and 10–25% Hb Köln are the major clinical features. Three different mutations have been described at codon β32 (27–29) and all cause molecular instability and hemolysis. The fractional percentage of these variants was between 10 and 20%. Instability was caused by different molecular mechanisms (27–29). Lacking a detectable protein, it is not possible to explore directly the properties of the abnormal hemoglobin containing the β32 CTG→CAG mutation, but some predictions can be made. Since the leucine→glutamine (gln) mutation does not alter the charge of the β subunit, the electrophoretic mobility of Hb Medicine Lake may not differ from Hb Köln, however, the possibility of loss of heme from either Hb Medicine Lake or Hb Köln makes the prediction of electrophoretic mobilities difficult. Retention times of peptides separated by reversed-phase HPLC can be predicted. The abnormal βT-4 of Hb Medicine Lake, containing the β32 gln residue, should elute 2.2 min before β^A T-4 while β T-10, harboring the Hb Köln mutation, should elute 1.2 after β^A T-10. But, an examination of radiolabeled β globin failed to reveal any aberrant peptides. In an intact globin chain, the similar hydrophobicity indices of the two abnormal peptides should cancel each other, causing $\beta^{\text{Medicine Lake}}$ to coelute with β^A on reversed phase HPLC.

Leucine at helical position B14 is an internal residue. Introducing the polar amide of the glutamine residue would allow water molecules into the otherwise nonpolar cavity occupied

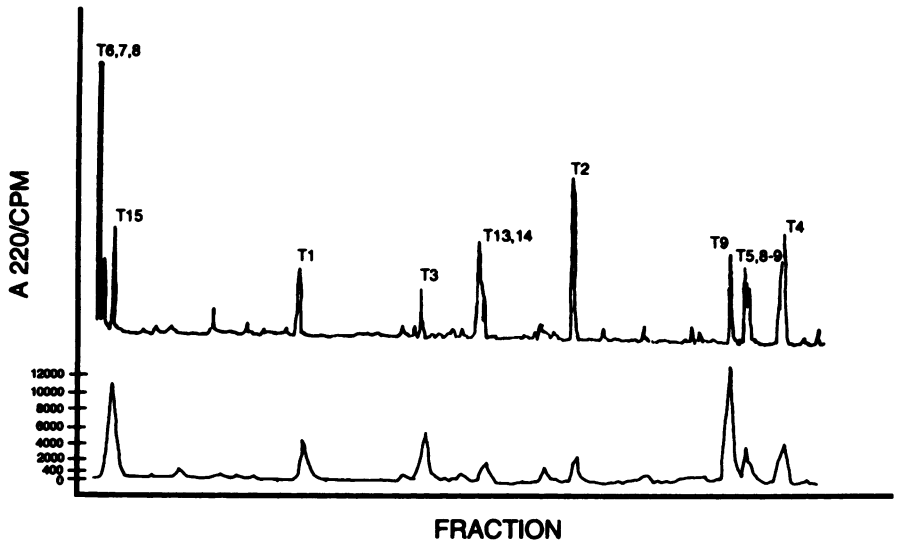

Figure 6. Aminoethylated, tryptic peptides of the β globin chain. (*Top*) A_{220} nm profile of the peptide separation of β globin chains separated from a 2.5 and 120 min incubation sample. Only normal β globin peptides are present. (*Bottom*) Radioactive elution profile of the peptides in this chromatogram. Only peptides that coincide with the A_{220} profile are present indicating the absence of peptides matching the predicted structure of Hb Medicine Lake. The scale indicates dpm.

by the side chain and lead to unfolding of the polypeptide chain. This effect may be more prominent in the presence of the destabilization caused by the Hb Köln mutation (Perutz, M. F., personal communication). Additionally, perturbation of the adjacent β31 leucine that contacts the heme group, could result in instability (27, 28).

Globin chains with two amino acid substitutions. There have been 14 hemoglobin variants described with two mutations in the same polypeptide chain (30). With a single exception, at least one mutation had been previously described. Most of these rare disorders probably arose via homologous crossing-over (30, 31), but this mechanism cannot account for the Hb Medicine Lake gene, as the mother has neither of the β-globin gene mutations present in her daughter. Some doubly substituted hemoglobin variants are unstable.

Genetics of Hb Medicine Lake. Unstable hemoglobins are often the result of new mutations and the many origins of Hb Köln suggest that this codon is a "hot spot" for mutation although the mechanism for this is not known (25). Our inability to relocate and further study the proband's father precludes a definitive assessment of the inheritance of Hb Medicine Lake, but two hypotheses exist. One possibility for the origin of this abnormal hemoglobin is the occurrence of a new mutation in the germ cells of the father or in the proband. Hb Köln is almost always associated with some abnormality of the blood counts and the mother was normal at both β-globin loci. The putative father was hematologically normal while the maternal β-globin allele of the proband had neither the β32 nor Hb Köln mutation. Although the β32 mutation is predicted to cause hemoglobin instability some unstable hemoglobins are clinically silent. Therefore, the father may have transmitted the β32 Gln mutation to the proband and a new mutation may have occurred at codon β98 as often occurs in Hb Köln disease. In rare instances, carriers of Hb Köln are not anemic. If this were the case, Hb Köln may have been inherited from the hematologically normal father with the β32 Gln mutation occurring de novo.

Hb Medicine Lake may be an example of a new mutation in an abnormal hemoglobin gene. Regardless of the origins of the $\beta^{\text{Medicine Lake}}$ gene, the molecular pathogenesis of the disease in the proband illustrates a mechanism by which the phenotype of a genetic disorder, like the hemoglobin instability likely to be present with the β32 mutation or the hemolytic anemia typical of Hb Köln, can be modulated by a coincident mutation. Similar combinations of mutations may explain some heterogeneity of common genetic disorders (32). For example, the presence of two mutations in a single CFTR gene can alter some features of cystic fibrosis (4).

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