Hemoglobin Evanston (α14 Trp → Arg)

An Unstable α-Chain Variant Expressed as α-Thalassemia

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Abstract. A new hematologic syndrome with phenotypic features of mild Hb H disease was identified in three children from two unrelated black American families. Erythrocytes from each of these children contained Hb H (β_4) and Hb Barts (γ_4), as well as a slowly migrating hemoglobin fraction that made up 7–10% of the total hemoglobin. The parents of the affected children all showed mild thalassemia-like changes, with one of the parents in each family also expressing the variant hemoglobin; in the latter individuals the mutant α-chains made up <2% of the total, and were present mainly or exclusively in combination with δ-chains in the form of a slowly migrating Hb A_2. Purified Hb Evanston showed an increased oxygen affinity, but its Bohr effect, cooperativity, and 2,3-diphosphoglycerate effect were normal. The mutant hemoglobin appeared to have normal stability to heat and to isopropanol, and the stability of its α-chain in an extended time course synthesis study also appeared to be similar to that of α^A. However, the results from short-term globin synthesis studies, and from mRNA translation in vitro, suggest that the two types of α-chains were synthesized at relatively equal rates, with a major fraction of the newly synthesized variant α-chains undergoing rapid catabolism. The hematologic data taken in combination with DNA hybridization and globin synthesis findings indicate that the proposita in each of these families has the genotype $-,\alpha^A/-,\alpha^{Ev}$. These observations suggest that two separate mechanisms are contributing to the α-thalassemia-like expression of Hb Evanston: (*a*) the newly synthesized α^{EV}-chains are unstable and are subject to early proteolytic destruction; and (*b*) the mutant α-allele is linked to an α-globin gene deletion.

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Introduction

The α-thalassemia syndromes result from deficient synthesis of the α-globin chains of hemoglobin. The most frequent underlying cause for this group of disorders is the deletion of one or more of the normal complement of four α-globin genes (1, 2). The α-thalassemia phenotype has also been observed in association with certain α-chain structural mutants, and it has further been shown that the thalassemia-like expression of these abnormal hemoglobins may arise by several different mechanisms. In one group of these variants the mRNA products of the mutant

α-globin genes appear to be unstable, causing the mRNA's to be degraded and thereby prematurely terminating their function as templates for globin chain synthesis (3, 4). Other α-globin gene mutations that produce thalassemia-like hematologic changes are expressed by the synthesis of highly unstable α-chains, which undergo proteolytic destruction and are rapidly removed from the erythroid cells (4–6). In a third group of α-chain variants, the mutant α-globin genes exist in linkage to an α-globin gene deletion, so that the linked α-thalassemia determinant is expressed in conjunction with the hemoglobin structural variant (7–12).

In this report we describe a newly identified α-chain mutant that appears to have thalassemia-like expression as a result of two separate mechanisms, i.e., as the result of an instability of the newly synthesized variant α-chain, and because of the linkage of its gene with an α-globin gene deletion.

Methods

Hematologic measurements. Erythrocyte and hemoglobin measurements were made with a model S Coulter counter (Coulter Electronics, Inc., Hialeah, FL), which was calibrated daily using a commercial standard. Other hematologic determinations were by standard laboratory methods (13).

Hemoglobin studies. Stroma-free hemoglobin solutions were prepared from washed erythrocytes as previously described (14). Electrophoresis of hemoglobins and globin proteins, and measurements of electrophoretic mobilities were as described by Schneider and Barwick (15). Hemoglobin (Hb)[1] A_2 measurements were made by DEAE-cellulose microcolumn chromatography (16) and the alkali-resistant hemoglobin fraction was estimated by the method of Singer et al. (17). Isolation and quantification of individual hemoglobins was by the DEAE-cellulose column chromatography procedure of Abraham et al. (18) using glycine-containing buffers. Globin chain separations were by CM-cellulose column chromatography with buffers prepared in 8 M urea (19) or by high performance liquid chromatography (HPLC) according to Shelton et al. (20). For hemoglobin stability testing stroma-free lysates were heated at 50°C in phosphate buffer (21) or at 37°C in buffer containing isopropanol (22); in some of the experiments isopropanol-treated samples and untreated controls were subsequently fractionated by DEAE-cellulose column chromatography, and the percentage of each recovered hemoglobin was determined.

Purified globin chains for structural analysis were digested with trypsin, and the resulting soluble peptides were separated by HPLC as previously described (23). Globin chains and purified peptides for amino acid analysis were hydrolyzed in 6 N HCl at 110°C under reduced pressure for 24 h. The analyses were performed with a Beckman-Spinco amino acid analyzer model 121M (Beckman Instruments, Inc., Spinco Div., Palo Alto, CA).

Hemoglobin functional studies. Oxygen equilibrium curves of whole blood and of purified hemoglobin solutions were obtained using a Hemox-Analyzer (Technical Consulting Services, Southampton, PA). Whole blood determinations were made at 37°C and pH 7.40. Individual hemoglobins for oxygen equilibrium studies were isolated by DEAE-cellulose chromatography in the oxy-form, and were concentrated by sedimentation at 100,000 *g* for 16 h. The hemoglobin solutions were dialyzed against 0.05 M Bis-Tris buffer in 0.10 M NaCl, and adjusted to a final concentration of 1 mg/ml (equivalent to ~60 μM in heme). An enzymatic reducing system (24, 25) was added to each of the samples to minimize methemoglobin formation, and the oxygen equilibrium curves were determined at 30°C. The data were digitized electronically (Tektronix Graphics Tablet; Tektronix, Inc., Beaverton, OR) and were characterized by using the Hill equation, which was fitted by nonlinear regression over the range of 20 to 97% saturation. The smoothness of the sum-of-squared-residuals function allowed the use of a Gauss-method nonlinear regression algorithm (26).

Fluorometric studies of the purified hemoglobins were performed with short path-length cuvettes and by a front surface technique (27). For these determinations the hemoglobins were prepared in Tris-glycine buffer, pH 7.0, at a concentration of 90 μM. The measurements were made with a Perkin-Elmer MPF-44B recording spectrofluorometer (Perkin-Elmer Corp., Instrument Div., Norwalk, CT) with a temperature-controlled cell holder.

Globin synthesis determinations. In studies that used intact reticulocytes, erythroid cell suspensions were prepared from freshly drawn blood samples and were supplemented with glucose, hemin, and a mixture of amino acids excluding leucine, in a Tris-buffered medium at pH 7.4 (28). L-[4,5-^{3}H]leucine with a specific activity of 52 Ci/mmol (New England Nuclear, Boston, MA) was added at a concentration of 10 μCi/ml. The cell suspensions were incubated in a shaking water-bath at 37°C for various times, as indicated for the individual experiments.

For studies of cell-free globin chain synthesis, polysomal RNA was isolated from the reticulocytes that were contained in 5–6 ml of blood, as previously described (6). The cell-free translation experiments using the purified RNA were carried out at 30°C for 60 min, in an incubation mixture containing micrococcal nuclease-treated rabbit reticulocyte lysate (29) (Bethesda Research Laboratories, Gaithersburg, MD), and supplemented with L-[^{35}S]methionine (>600 Ci/mmole; Amersham Corp., Arlington Heights, IL). The newly synthesized globin chains were separated by electrophoresis in 12% polyacrylamide gels containing 2% Triton X-100 and 6 M urea (30). The radioactive protein bands were visualized by fluorography with ENHANCE (New England Nuclear), according to the manufacturer's specifications.

Mononuclear cells for erythroid cell cultures were prepared from peripheral blood samples by the Ficoll-Hypaque density centrifugation technique (31). The cell cultures were grown in methylcellulose according to Iscove and Sieber (32) and Clark et al. (33). The mononuclear cells were plated at a concentration of 10^6/ml in 35-mm Lux standard culture dishes, in a 1-ml mixture containing Iscove's modified Dulbecco's medium, 0.9% methylcellulose, 30% fetal calf serum, 5×10^{-5} M β-mercaptoethanol, 1 μg/ml transferrin, and 2 U/ml of erythropoietin (Connaught Laboratories, Swiftwater, PA). The cultures were incubated for 14 d at high humidity in a 5% CO_2 atmosphere at 37°C. The hemoglobins were labeled by the addition of 100 μCi of L-[^{3}H]leucine to each culture plate 24 h before the completion of the incubations. The erythroid bursts were removed from the methylcellulose by dilution, and were washed with phosphate-buffered saline. The cells were lysed in distilled water, and a mixture of Hb A and Hb Evanston was added as hemoglobin carrier. The labeled globin chains were fractionated by CM-cellulose column chromatography with urea-containing buffers as described above, but two additional purification steps were required before the chromatography to remove a contaminating nonglobin protein that eluted in the position of the mutant α-chain. In the first of these steps the

1. *Abbreviations used in this paper:* 2,3-DPG, 2,3-diphosphoglycerate; Hb, hemoglobin; HPLC, high performance liquid chromatography; kb, kilobase.

hemoglobin preparations in the carboxy form were passed through a 100 × 2.5 cm column of Sephadex G-75 (34). Effluent fractions corresponding to tetrameric hemoglobin were pooled and concentrated, and the globin was isolated by precipitation in acetone-HCl (19). The globin preparations were then subjected to a second gel filtration step, using a 200 × 1.2-cm column of Sephadex G-50 that was equilibrated with 10% acetic acid (wt/vol). Fractions representing the main globin peak were then pooled; and the protein was recovered by lyophilization.

DNA-hybridization studies. DNA was extracted from peripheral blood leukocytes (35, 36). Aliquots of 6–10 μg of the DNA were digested with *Bgl*II (New England BioLabs, Beverly, MA) or with *Bam*H1 (Bethesda Research Laboratories) under conditions recommended by the manufacturers. The DNA fragments were separated electrophoretically in 0.8% agarose gels, and were transferred to nitrocellulose filters (36, 37) using a modification of the method of Southern (38). The filters were hybridized with a ^{32}P nick-translated fragment, either 0.6 or 1.6 kilobases (kb) in length, of the α-globin complementary DNA plasmid JW 101 (39), followed by radioautography. To detect α-globin DNA deletions large enough to involve ζ-globin DNA, blot-hybridization analyses of ζ-specific sequences were also performed with some of the samples. For these determinations the DNA was digested with *Bgl*II, *Bam*H1, and *Eco*R1, followed by hybridization with the ^{32}P nick-translation-labeled 370-base *Hinc*II-*Pvu*II fragment (2, 40) from the plasmid pBRζ1 (2).

Case reports

Family A. The proposita is one of two children in this black American family. A routine examination of the child at 1 yr of age showed no abnormality, but her blood count demonstrated mild microcytic anemia with abnormal erythrocyte morphology (Fig. 1). A trial of oral ferrous sulfate produced no change in her hematologic picture, which prompted a more extensive evaluation of the child and the other members of her family. Representative hematologic values from the family members are shown in Table I.

Family B. The index case is a black infant whose hematologic abnormality was first recognized in the neonatal period. During the pregnancy the mother was noted to have microcytic anemia that was unresponsive to iron. On the first day of life the infant's hemoglobin concentration was 11.2 g/dl, with mean corpuscular volume, 95 fl; erythrocytes, 3.68 × 10^{12}/liter; reticulocyte count, 31.5%; and nucleated erythrocytes, 6.94 × 10^{10}/liter. Her erythrocyte morphology was markedly abnormal, showing anisocytosis, hypochromia, and polychromasia. The direct antiglobulin test was negative, and maternal blood group incompatibility was excluded. The IgM level was also normal, and cultures, as well as viral studies, were all negative.

The neonatal course was marked by increasing anemia and persistent reticulocytosis, but not significant hyperbilirubinemia (the maximum bilirubin level at 1 d of age was 4.6 mg/dl). Hemoglobin electrophoresis (cellulose acetate at alkaline pH) was done at 15 d of age, and was remarkable for the finding of a rapidly migrating hemoglobin (25% of the total) that co-migrated with Hb Barts.

The infant's anemia gradually improved without specific therapy, but the evidence of hemolysis persisted. Her 2-yr-old sister was subsequently also found to have microcytic anemia with reticulocytosis, but without splenomegaly. Both the mother and father also exhibited mild microcytosis and anemia (Table I).

Results

Hemoglobin electrophoresis and chromatography findings. Erythrocyte lysates from the index cases in each of the families contained a slowly migrating hemoglobin fraction that made up between 7 and 10% of the total hemoglobin. The electrophoretic mobility of this variant hemoglobin at alkaline pH was similar to that of Hb S, but sickling tests were negative. The abnormal α-chain exhibited electrophoretic mobility values at acid and alkaline pH similar to those of α-G Philadelphia (Table II). An additional finding in each of these children was the presence of varying amounts of rapidly migrating hemoglobins that had electrophoretic mobilities characteristic for Hb Barts (γ_4) and Hb H (β_4).

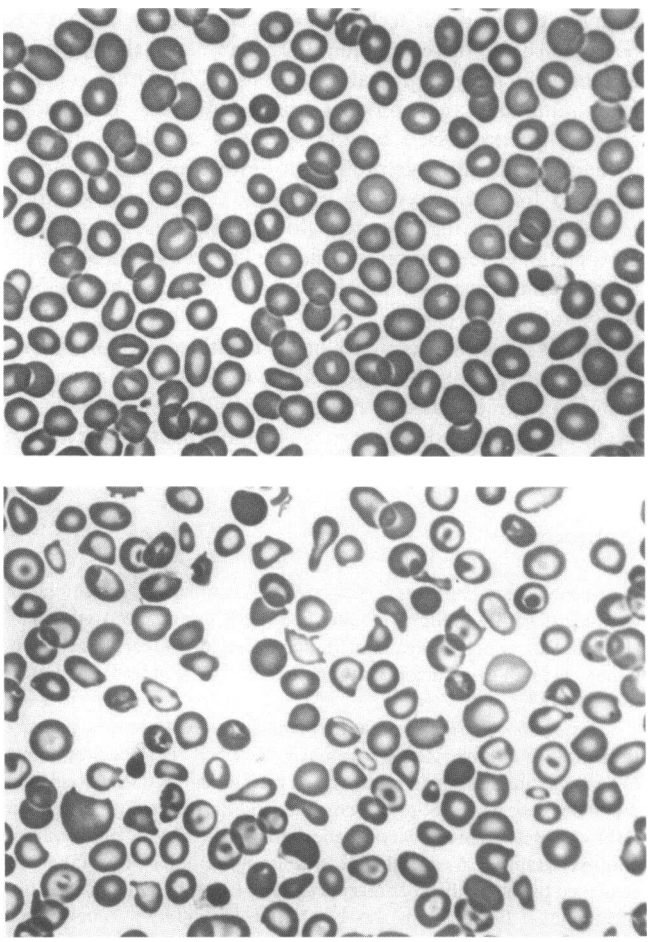

Figure 1. Blood films from the father (top) and proposita (bottom) from family A, demonstrating their abnormal erythrocyte morphology. Erythrocytes from the mother and brother of the proposita were mildly microcytic, but otherwise appeared normal.

The variant hemoglobin eluted immediately before Hb A in DEAE-cellulose chromatography (Fig. 2) and an additional smaller peak emerged ahead of Hb A_2. Determinations of the globin-chain composition of the pre-A_2 peak by electrophoresis and by HPLC indicated that this hemoglobin contained the same abnormal α-chain as was present in the pre-A ($\alpha_2^{var}\beta_2^A$) peak. In hemoglobin samples from the two index cases, the Hb

*Table I. Hematologic and Hemoglobin Values of the Patients and Their Families**

	Age	Hemoglobin concentration	Erythrocyte count	Mean corpuscular volume	Reticulocyte count	Hb A_2	Hb F	Hb Evanston	Hb H (β_4)	Hb Barts (γ_4)	Blood $P^{50}O_2$	2,3-DPG	Serum ferritin
		g/dl	*$\times 10^{12}$/liter*	*fl*	%	%	%	%	%	%	*torr*	*mol/g Hb*	*ng/ml*
Family A													
Proposita	2 yr	9.7	5.59	60	4.4	3.2	2.5						
		(11.0–12.2)	(4.0–5.2)	(70–83)	(0.8–3.0)	(1.3–3.7)	(0.3–2.0)	7.4	tr	tr	29.5	20.0	29
Father		14.1	6.09	71	1.6	3.3	0.3						
		(13.3–17.7)	(4.4–5.9)	(81–100)	(0.8–2.5)	(1.3–3.7)	(0.3–1.0)	0.2	—	—	31.0	19.4	62
Mother		12.0	4.41	85	0.8	2.8	0.6						
		(11.7–15.7)	(3.8–5.2)	(81–100)	(0.8–4.1)	(1.3–3.7)	(0.3–1.0)	—	—	—	28.0	17.8	13
Brother	4 yr	11.7	4.22	86	1.3	3.1	0.8						
		(11.6–13.1)	(4.0–5.3)	(75–89)	(0.8–3.0)	(1.3–3.7)	(0.3–1.8)	—	—	—	29.0	18.4	17
Family B													
Proposita	9 mo	10.1	5.34	61	2.3	2.4	6.1						
		(11.2–12.4)	(4.1–5.2)	(71–85)	(0.8–3.0)	(1.0–3.5)	(0.6–3.0)	9.8	—	<2.0			
Sister	2 yr	9.5	5.01	66	4.9	2.5	6.7						
		(11.0–12.2)	(4.0–5.2)	(70–83)	(0.8–3.0)	(1.3–3.7)	(0.3–2.0)	6.0	tr	3.6			
Mother		11.5	4.97	72	0.9	2.3	1.2						
		(11.7–15.7)	(3.8–5.2)	(81–100)	(0.8–4.1)	(1.3–3.7)	(0.3–1.0)	—	—	—			
Father		13.2	5.35	77	2.2	2.8	1.1						
		(13.3–17.7)	(4.4–5.9)	(81–100)	(0.8–2.5)	(1.3–3.7)	(0.3–1.0)	<2.0	—	—			

tr, trace. * Age-specific normal ranges for the laboratory values are shown in parentheses.

$A_2^{Evanston}$ fraction made up between 13 and 19% of the total of Hb Evanston plus Hb $A_2^{Evanston}$, representing a substantially higher percentage of the Hb A_2 form than would be anticipated from a random association of α^{Ev} chains with β- and δ-chains. Variable quantities of late-eluting hemoglobins were also recovered from the chromatographic separations, and these could be shown by amino acid analyses and globin electrophoresis to be homotetramers of β- and γ-chains.

A representative chromatographic pattern of the hemoglobins from the father from family A is also shown in Fig. 2. In these separations a small hemoglobin peak consistently eluted ahead of Hb A_2, with a yield of ~11% that of the Hb A_2 fraction. Electrophoresis and HPLC analyses of globin from the purified pre-A_2 peak consistently demonstrated the presence of normal δ-chains, together with α-chains that exhibited the characteristic properties of $\alpha^{Evanston}$-chains. These findings therefore suggest that the small quantities of $\alpha^{Evanston}$-chains in the erythrocytes from the father may have been present mainly in combination with δ-chains, in the form of an abnormal Hb A_2.

*Table II. Electrophoretic Mobility of Hb Evanston and Its Mutant α-Chain**

Electrophoresis conditions	Mobility	Reference mobility values
Hemoglobin		
Cellulose acetate, pH 8.6	−4.0	Hb A = 0 Hb A_2 = −10
Citrate agar, pH 6.2	0	Hb A = 0
Globin		
Cellulose acetate, pH 8.6	7.7	α^A = 10 β^A = 20
Citrate agar, pH 6.2	8.0	α^A = 10 β^A = 20

* Electrophoresis methods and calculations of mobility values were according to Schneider and Barwick (15).

Globin structural studies. Globin was isolated from the purified Hb Evanston from an affected child from each of the families, and the α- and β-chains were separated by CM-cellulose column chromatography. The abnormal α-chains in these separations emerged slightly later than α^A. Samples of the purified α-chains were hydrolyzed in 6 N HCl and in 4 N methanesulfonic acid with 0.2% 3-(2-aminoethyl) indole (41). Amino acid analyses demonstrated the presence of an additional residue of arginine, and an absence of the single tryptophan residue that normally occupies the α-14 position. Other samples of the $\alpha^{Evanston}$-chain were digested with trypsin, and their soluble peptides were separated by HPLC. These analyses showed all of the expected α-chain peptides eluting in their normal positions, except for α-T-3, which was replaced by two early-emerging peptide fractions (Fig. 3). The amino acid composition of these peptides (Table III) confirmed the replacement of α-14-tryptophan by arginine, with the arginyl substitution having produced a new trypsin-sensitive site. An additional small peptide was identified

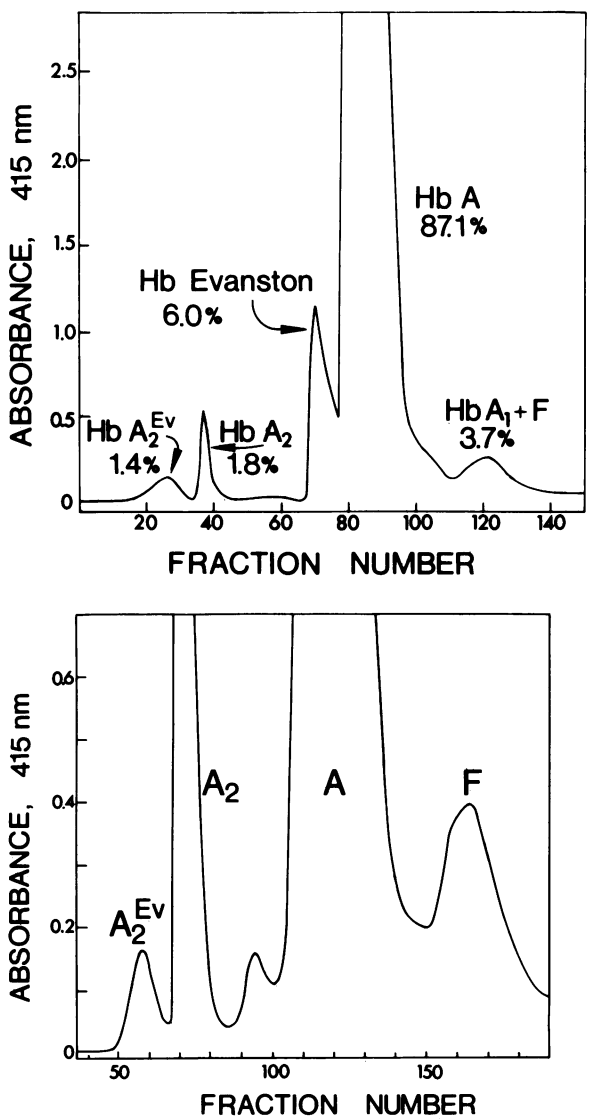

Figure 2. (*Top*) DEAE-cellulose chromatography of hemoglobins from the proposita from family A. Traces of Hb Barts (γ_4) and Hb H (β_4) were also recovered from later fractions, but are not shown in the illustration. (*Bottom*) The hemoglobins from the father from family A.

in the HPLC separation; it emerged immediately ahead of peptide T-4, and was determined by amino acid analysis to represent a T3-b,T-4 dipeptide.

Functional and physical properties of Hb Evanston. The oxygen equilibrium curve of purified Hb Evanston showed a leftward shift in relation to the curve from Hb A (Fig. 4). Its other functional properties, including cooperativity, the Bohr effect, and the change in oxygen affinity in response to 2,3-diphosphoglycerate (2,3-DPG), appeared to be normal or nearly normal.

Absorption spectra of Hb Evanston, both in the oxy- and methemoglobin forms, were determined between 300 and 700 nm. These were indistinguishable from the comparable absorption spectra of Hb A. The fluorescence excitation and emission maxima and intensity of Hb Evanston were also the same as those of Hb A when they were determined by a front surface method (27) or with short path length cuvettes.

Screening tests for an unstable hemoglobin in lysates from the patients' erythrocytes gave negative results by heating (21) or by incubation at 37°C with isopropanol (22). When an isopropanol-treated lysate from one of the patients was fractionated by DEAE-cellulose chromatography, the Hb Evanston fraction was found to make up 6.5% of the recovered hemoglobin; in an untreated sample that was run as a concurrent control, Hb Evanston represented 7.8% of the hemoglobin, which indicated that Hb Evanston is at most only slightly unstable.

Subunit dissociation of the purified Hb Evanston was estimated by Sephadex G-75 gel filtration (34). In this determination the elution pattern of Hb Evanston was indistinguishable from that of a sample of pure Hb A, which suggests that the subunits of Hb Evanston dissociate no more readily than those of normal adult hemoglobin.

Globin synthesis studies. Initial experiments examined the synthesis of hemoglobins and globin chains by reticulocytes. Cells from the proposita of family A were incubated in medium containing radiolabeled L-leucine, and the incorporated radioactivity in each of the hemoglobins was determined after chromatographic fractionation. The Hb Evanston peak contained 12.7% of the recovered radioactivity, with 86.0% present in Hb A. When the labeled Hb Evanston was separated into its α- and β-chains, the incorporated radioactivity in the α^{Ev}-chain was determined to be ~50% greater than that of the β-chain, which is consistent with the hematologic data that suggest that the patient had α-thalassemia (42).

The globin synthesis data from total cell lysates from the members of family A are summarized in Table IV. The α/non-α-globin synthesis ratio from the patient's reticulocytes was 0.72, with the α^{Ev}-synthesis representing ~14% of total α-chains synthesized. In the father's cells, the synthesis of the complementary globin chains was nearly balanced (α/non-α-ratio of 1.09) with α^{Ev}-synthesis making up only ~3% of the total α-chains. The mother and brother of this patient showed α/non-α-globin synthesis ratios within the range characteristic of individuals with mild α-thalassemia.

The patterns of globin synthesis by reticulocytes from the patient and her father were also compared with those from erythroid bursts derived from their circulating erythroid burst-forming units, to allow comparisons to be made with less mature, nucleated erythroid cells. Cell cultures from the patient and from her father synthesized a proportionately greater percentage of γ-chains (data not shown) but the relative synthesis of α^{A}- and α^{Ev}-chains was virtually the same as that of their reticulocytes (Table IV).

Additional experiments with reticulocytes from the patient from family A examined the stability of newly synthesized

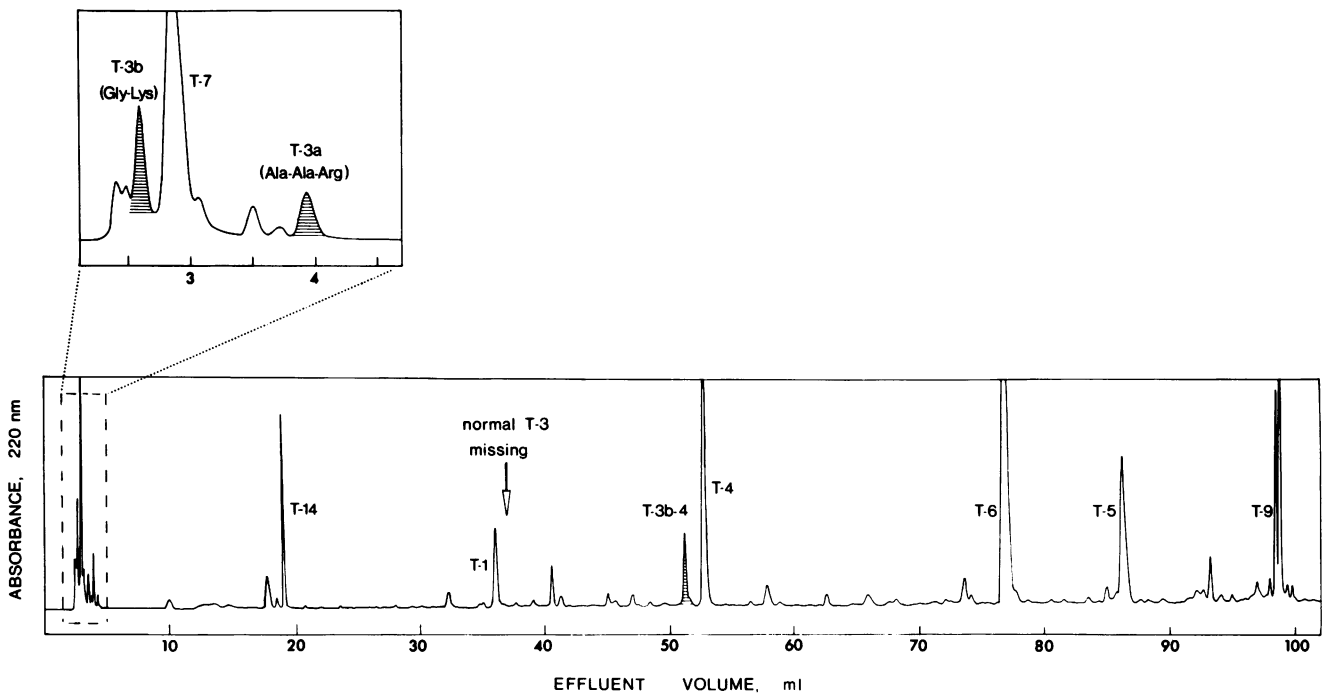

Figure 3. Separation of the soluble tryptic peptides of the Hb Evanston α-chain by HPLC. The normal elution position of α T-3 is shown by the arrow. The abnormal peptides T-3a, T-3b, and T-3b-4 are represented by the cross-hatched peaks.

α^{Ev}-polypeptide chains. Over the course of a 12-h incubation the incorporated radioactivity in the α^{Ev}-chains showed a small decrease in comparison with that of the α^{A}-chains (Fig. 5), which suggests that the α^{Ev}-chains may be slightly less stable than normal. There was a similar change when the cells were incubated for 10 h in nonradioactive medium after an initial 2-h labeling period (data not shown). Fig. 6 illustrates the findings from a very short (2 min) synthesis study. In this experiment the incubation was terminated abruptly by lysis of the cell suspension in several volumes of ice-cold 2.5 mM ε-aminocaproic acid, which was included to retard proteolytic activity (43). Under these conditions, the radioactivity in α^{Ev} made up ~33% of the total recovered α-chain radioactivity, representing a more than twofold increase over that from the preceding experiments.

Translation in vitro of purified reticulocyte mRNA. The results of cell-free translation studies that used reticulocyte mRNA from members of family B are shown in Fig. 7. Compared with a nonthalassemic control (lane N) mRNA from the proposita (lane 3) and her similarly affected sister (lane 2) directed the

*Table III. Amino Acid Composition of Peptides T-3a and T-3b from the $\alpha^{Evanston}$-Globin Chain**

Amino acid	Normal α T-3	α^{Ev} T-3a	α^{Ev} T-3b
Alanine	2	2.11	
Glycine	1		0.91
Lysine	1		1.08
Tryptophan	1	0	
Arginine	0	0.95	

* Indicated values represent the number of amino acid residues per peptide.

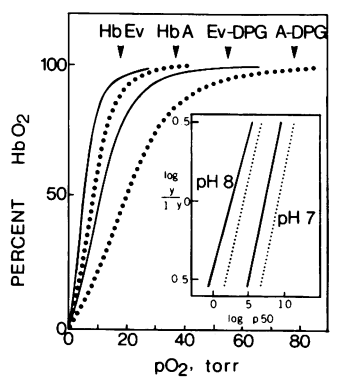

Figure 4. Oxygen equilibrium curves of purified Hb Evanston (——) and Hb A (· · · · ·) from the proposita of family A. The curves were determined at 30°C with the hemoglobins in 0.05 M Bis-Tris and 0.10 M NaCl. Added 2,3-DPG was at a concentration of 1 mM. The $P_{50}O_2$ of Hb Evanston under these conditions was 5.3 mm Hg, and with 2,3-DPG it was 11.2 mmHg. The comparable values for Hb A were 8.6 and 18.9 mm Hg, respectively. The Bohr effect (Δ log P_{50}/Δ pH) shown in insert was −0.47 for Hb Evanston and −0.50 for Hb A. Hill's *n* coefficient for Hb Evanston at pH 7.0 was 2.19, with a comparable value for Hb A of 2.34.

Table IV. Globin Chain Synthesis Data from Family A

	Reticulocytes		Erythroid bursts	
	$\frac{\alpha^{total}}{non\text{-}\alpha}$	$\frac{\alpha^{Ev}}{\alpha^{A}+\alpha^{Ev}}$	$\frac{\alpha^{total}}{non\text{-}\alpha}$	$\frac{\alpha^{Ev}}{\alpha^{A}+\alpha^{Ev}}$
Proposita	0.72	0.14	1.10	0.12
Father	1.09	0.03	1.16	0.05
Mother	0.86	—	—	—
Brother	0.85	—	—	—
Normal control values	1.04±0.05	—	1.16	—

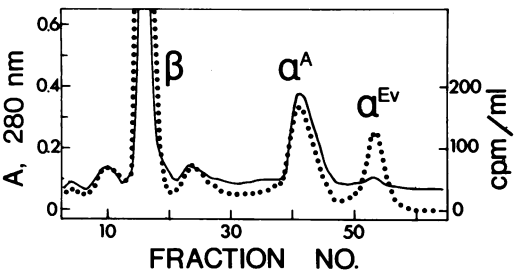

Figure 6. Globin chain synthesis by reticulocytes from the proposita of family A in a 2-min incorporation. (——) globin protein; (· · · · ·) recovered radioactivity.

synthesis of an approximately normal quantity of β-globin, but substantially less of α-globin. The variant α-chain of Hb Evanston, which had a slightly different electrophoretic mobility than that of α^{A}, was also synthesized under these conditions (shown as α^{*} in Fig. 7). Densitometric scanning of the autoradiograms indicated that the ratio of α^{Ev} to total α was approximately 0.33. The α^{*} band was not visualized in studies that used mRNA from the mother (lane 1), who lacked Hb Evanston.

DNA hybridization studies. In determinations to establish the linkage relationship between $\alpha^{Evanston}$ and α-thalassemia, leukocyte DNA was digested with the restriction enzymes *Bgl*II or *Bam*H1, and the fragments were separated by electrophoresis, transferred to nitrocellulose, and hybridized to ^{32}P-labeled α-globin complementary DNA from plasmid JW 101. Normal human α-globin genes are contained in two *Bgl*II fragments of 7.0 and 12.5 kb, or in a single *Bam*H1 fragment of 14.0 kb (44), although the size of the *Bam*H1 fragment may be altered by polymorphic DNA sequences linked to the α-globin genes (45). Single α-globin-gene chromosomes of the "leftward" deletion type yield one *Bgl*II fragment of 7.0 kb; and in the "rightward" deletion, a single *Bgl*II fragment of 15.8 kb (44, 46). With single α-globin-gene chromosomes of either type a 10 kb *Bam*H1 fragment is obtained (44).

DNA from both parents and the brother of the proposita in family A yielded three α-globin *Bgl*II fragments of 7.0, 12.5, and 15.8 kb (Fig. 8), which indicates that each of these individuals is heterozygous for normal and rightward-deletion α-thalassemia-2 chromosomes. However, DNA from the proposita yielded only a single 15.8-kb α-globin fragment, which was reproducibly darker than those from her parents and brother. The proposita is therefore homozygous for rightward deletion α-thalassemia-2 chromosomes, from which it can be concluded that the $\alpha^{Evanston}$ mutation must be contained in the single α-globin gene on a rightward deletion α-thalassemia-2 chromosome. The apparent

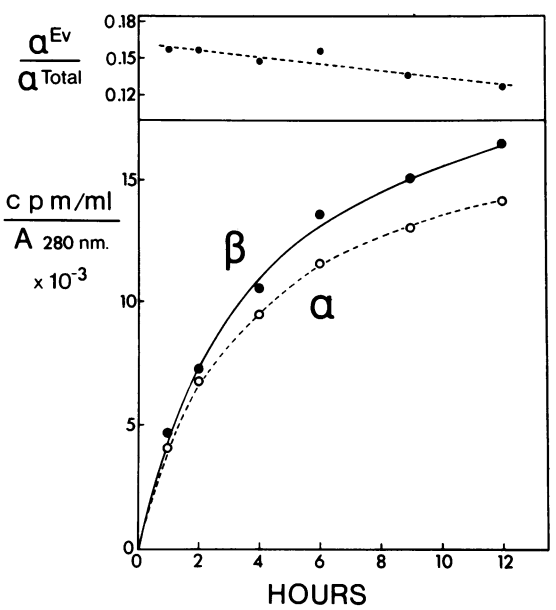

Figure 5. Globin synthesis time course from reticulocytes from the proposita of family A. Specific activity values shown for the α-chains were multiplied by a factor of 0.68 to correct for differences in absorbance at 280 nm. The α^{Ev}/α^{A} ratios were calculated from the total recovered radioactivity from the chromatographic fractions.

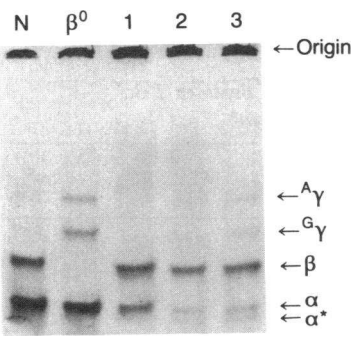

Figure 7. Globin chain synthesis directed by purified reticulocyte mRNA from members of Family B, in a cell-free translation system. The radiolabeled globins (L-[^{35}S]methionine) were separated by electrophoresis in Triton X-100-urea-acrylamide gels, and were visualized by autoradiography. The sources of mRNA were as follows: lane *N*, nonthalassemic control; lane β^{0}, unrelated patient with homozygous thalassemia; lane *1*, mother of the proposita; lane *2*, sister of the proposita; lane *3*, the proposita. α refers to α^{A}, and α^{*} is the α-chain of Hb Evanston.

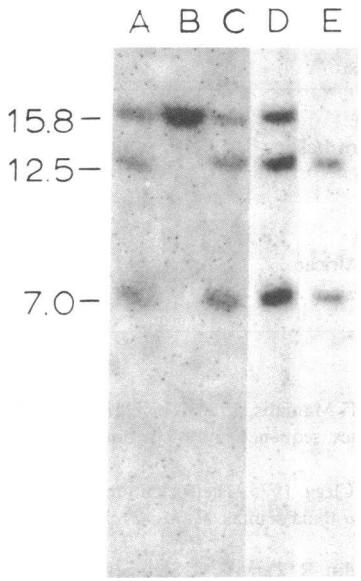

Figure 8. Autoradiogram of α-globin gene-specific fragments obtained by *Bgl*II digestion of leukocyte DNA from the members of Family A. Lane *A:* father; *B:* proposita; *C:* mother; *D:* brother. Lane *E* is from normal human fibroblast DNA. Fragment sizes are indicated in kilobases.

α-globin genotypes of the members of this family are represented in Fig. 9.

Similar studies were performed with DNA from the two affected children and the mother from family B. Each of these individuals had a single α-specific *Bam*H1 fragment of 10 kb and a single *Bgl*II fragment of 15.8 kb (data not shown). Because these findings were compatible with either homozygosity for the α-thalassemia-2 haplotype (−,α/−,α) or heterozygosity for the α-thalassemia-2 and the α-thalassemia-1 haplotypes (−,α/−,−), mapping of the ζ-globin genes was also performed to detect fragments of abnormal size that are generated by many of the α-thalassemia-1 deletions (47). No such abnormal fragments were detected. Although we were unable to perform DNA hybridization studies of the other parent in family B, our mapping data, when interpreted in conjunction with the clinical findings, suggest that the α-globin genotypes of the members of family B are as represented in Fig. 9.

Discussion

The tryptophan residue that normally occupies position 14 of the α-chain fills a large space between the A and E helices (48, 49). The substitution of this position in Hb Evanston by a residue of arginine would leave a gap in this region that would have the effect of loosening the T-quaternary structure of the molecule. This change would be expected to produce a lowering of the oxygen dissociation constant K_T and the allosteric constant L (50) with a resulting increase in the oxygen affinity of the hemoglobin. The functional properties of Hb Evanston appear to be consistent with a mechanism of this kind.

The unusual feature of $\alpha^{Evanston}$-subunits of binding to δ-chains proportionately more than to β-chains cannot, however, be readily explained. The site of the amino acid substitution is not near any subunit contact, and moreover the degree of dissociation of the $\alpha_2^{Ev}\beta_2^A$-tetramer did not appear to be greater than that of normal adult hemoglobin. The finding that the fluorescence properties of Hb Evanston were not different from those of Hb A lends support to the conclusion of Hirsch and Nagel (51) that the predominant intrinsic fluorescence of Hb A is attributable to the tryptophans at position 37 of the β-chains.

The Hb H disease phenotype characteristically results from the deletion of three of the normal complement of four α-globin genes (52). In each of the patients with Hb H in these two families, the DNA hybridization studies demonstrated the presence of two α-globin genes, one of which must carry the Trp → Arg mutation. These considerations therefore suggest that the gene for this structural variant functions as an α-thalassemia determinant. The possibility that this property of $\alpha^{Evanston}$ may be related to an instability of its mRNA, or a premature loss of mRNA during erythroid cell maturation, appears unlikely from the experimental findings. Firstly, the relative rates of synthesis of Hb Evanston and Hb A were virtually the same in erythroid bursts as in more mature reticulocytes, and, in addition, the translation study that used purified mRNA showed generally similar quantities of functional α^A and $\alpha^{Evanston}$ mRNA's.

The $\alpha_2^{Ev}\beta_2^A$-tetramer appeared to be relatively stable, however it can be inferred from the experimental findings that newly synthesized $\alpha^{Evanston}$-subunits are extremely labile and presumably undergo rapid proteolytic breakdown within the erythroid cells. Other unstable variants that produce the α-thalassemia phenotype by an apparently similar mechanism include Hb Petah Tikva (4) Hb Suan-Dok (5) and Hb Quong Sze (6). A related finding is that the individuals who are heterozygous for Hb Evanston (i.e., the father in each family) had virtually undetectable quantities of the mutant hemoglobin in their erythrocytes, whereas in the patients having an additional α-thalassemia deletion gene Hb Evanston made up 6–10% of the total hemoglobin. A possible explanation for this difference is that the excess quantities of uncombined β- and γ-subunits in the erythroid cells of the Hb H patients may promote a more

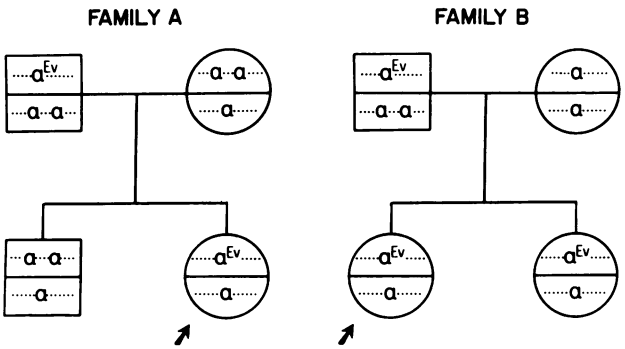

Figure 9. The presumed α-globin genotypes of the members of the two families. The arrows indicate the index cases.

Table V. α-Chain Structural Variants in Linkage with α-Thalassemia Determinants

Hemoglobin variant	Amino acid substitution	α-thalassemia determinant	Population	Reference
Evanston	α14 Trp → Arg	Rightward deletion	African	(This report)
Hasharon	α47 Asp → His	Rightward deletion	Northern Italian	12
G Philadelphia	α68 Asn → Lys	Rightward deletion	African	7
Mahidol (Q)	α74 Asp → His	Leftward deletion	Thai	8
Nigeria	α81 Ser → Cys	Not determined	African	10
J Capetown	α92 Arg → Gln	Not determined	South African	11
J Tongariki	α115 Ala → Asp	Rightward deletion	Melanesian	9

rapid incorporation of newly synthesized α^{Ev}-chains into relatively stable tetramer hemoglobins. A similar situation appears to exist in families with the unstable α-chain variant Hb Petah Tikva (4).

The DNA hybridization determinations from these families indicate that the $\alpha^{Evanston}$-gene also exists on a chromosome *cis* to a gene-deletion α-thalassemia determinant. The mutant allele therefore represents the sole α-globin gene on this chromosome. This variant is one of several known examples of globin structural mutants that exist in linkage with α thalassemia (Table V). Among this group of mutants, however, Hb Evanston appears to be unique in representing two different α-thalassemia-producing abnormalities that exist in coupling on a single chromosome. The α-thalassemia genes associated with the other globin variants shown in Table V are all expressed as the α-thalassemia-2 phenotype (−,α). However, because of the additional rapid posttranslational destruction of newly-synthesized $\alpha^{Evanston}$-subunits, no significant α-globin gene product from this chromosome accumulates in the cells, and the combined abnormalities are expressed as an α-thalassemia-1 determinant (−,−).

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Addendum. A recent report by Moo-Penn et al. (53) has described a third family with Hb Evanston. The affected individuals in this family exhibited hematologic findings very similar to those that we describe. Moo-Penn and his co-workers also demonstrated convincingly that the $\alpha^{Evanston}$-allele was present *cis* to an α-globin gene deletion.

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