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Genetics

Hemoglobin Cranston, an unstable variant having an elongated β chain due to nonhomologous crossover between two normal β chain genes

(frame shift/hemoglobinopathy/mutant hemoglobin/β-globin mRNA/untranslated β mRNA)

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ABSTRACT **An asymptomatic woman was found to have a compensated hemolytic state due to an unstable hemoglobin variant, comprising 35% of the total. Peptide maps of tryptic digests of the abnormal β chain were identical to those of β_A except that tryptic peptide 15 (Tyr-His-COOH) was absent and a new peptide was detected, containing equivalent amounts of Ser, Ile, Thr, and Lys. Its sequence was determined by manual Edman degradation. An additional hydrophobic peptide isolated by counter-current distribution contained: Asx, Ser, Ala, Tyr, 2 Phe, and 3 Leu. Its sequence was determined on an automatic solid phase sequencer. Digestion with carboxypeptidase A confirmed the C-terminal sequence. Hemoglobin Cranston has an elongated β chain with the following structure:**

145 150 155
-Lys-Ser-Ile-Thr-Lys-Leu-Ala-Phe-Leu-Leu-Ser-Asn-Phe-Tyr-COOH

This variant is the first "adult" human hemoglobin known to contain isoleucine. It is likely that hemoglobin Cranston arose because of a nonhomologous crossover of two normal β chain genes, probably during meiosis, with the insertion of two nucleotides (AG) at position 144, resulting in a frame shift.

Hemoglobin Cranston provides new information on the structure of the β chain gene as well as an explanation of both the structure and genetic basis of hemoglobin Tak, the only other elongated β chain variant that has been described.

Studies on mutant hemoglobins have provided a large body of information with important clinical, biochemical, and genetic implications. Over 200 human hemoglobin variants have been described to date. The vast majority are single amino-acid substitutions in a particular globin subunit. In these cases, the structural abnormality can be explained by a single base substitution in the corresponding triplet codon. However, other genetic mechanisms must be invoked to explain the existence of certain mutant hemoglobins. In the past several years, a few variants have been found to contain subunits that are elongated at the C-terminal end. These have attracted considerable attention because they offer new insight into the mechanism of polypeptide chain termination in eukaryotic cells and because they provide specific information about the structure of the 3′ end of the globin messenger RNA (mRNA), and indirectly about the corresponding segment of DNA*. Hemoglobin Constant Spring is an α chain variant containing 31 additional residues beyond arginine 141, the C-terminal residue of the α chain (1, 2). This variant can be explained by a single base substitution (U → C) in the termination codon giving rise to glutamine at position 142 and permitting the translation of a segment of mRNA beyond this point. Recently three other α chain termination mutants have been found: Icaria (3), Koya Dora (4), and Seal Rock (Dr. T. Bradley, personal communication). The genetic basis for these variants has been elegantly confirmed by two additional and independent types of evidence. Hemoglobin Wayne has an elongated α chain containing a unique sequence of eight residues beginning at position 139 (5). From a comparison of the amino-acid sequence of the elongated portion of hemoglobin Constant Spring, Seid-Akhaven *et al.* (5) explained the primary sequence of α_{Wayne} by a frame shift with the deletion of a single nucleotide base at position 139. Thus, a single hypothetical nucleotide sequence of α globulin mRNA can be derived which fits the amino-acid sequences of $\alpha_{Constant\ Spring}$ and α_{Wayne}. Recently, Forget *et al.* (6, 7) have determined base sequences of portions of normal human α and β globin mRNA. They have obtained oligonucleotide sequences for the untranslated region of α chain mRNA which are in perfect agreement with the hypothetical base sequence deduced from the primary structure of these two variants.

In 1970, Flatz *et al.* (8) presented preliminary structural data on hemoglobin Tak, a variant containing 10 additional residues at the C terminal end of the β chain. From the information at hand, no genetic mechanism could be invoked to account for the chain elongation. In this report, we will present structural studies on hemoglobin Cranston, a new unstable variant with an elongated β chain which appears to have arisen because of nonhomologous crossover between two normal β chain genes resulting in a frameshift allowing translation of a normally untranslated portion of β mRNA. The primary sequence of hemoglobin Cranston provides new information about the structure of the β chain gene and offers a plausible explanation for the genetic basis of hemoglobin Tak.

MATERIALS AND METHODS

Preparation of Abnormal Hemoglobins. Blood was collected in heparinized tubes. Hemolysates were prepared by the method of Drabkin (9) and analyzed by isoelectric focusing on polyacrylamide gels (10). Normal and abnormal hemoglobins were purified by chromatography on DEAE-cellulose. Following dialysis of hemolysate against the starting buffer, 600 mg of hemoglobin was applied to a column (2.5 × 20 cm) equilibrated with the starting buffer (0.05 M Tris·HCl, pH 8.1) at 4°. The column was developed over 48 hr with a linear sodium chloride gradient (total volume = 2.0 liters). The adding buffer consisted of 0.05 M Tris·HCl, pH 8.1 and 0.05 M NaCl. Seven milliliter fractions were collected. Hemoglobin peaks were concentrated by pressure dialy-

Abbreviations: A, Cr, and S; normal adult, Cranston, and sickle cell hemoglobins, respectively; Bis-Tris, 2,2-bis(hydroxymethyl)-2,2′,2″-nitrilotriethanol; Tp, tryptic peptide.

* Throughout this report, we will refer to nucleotide bases and base sequences of mRNA only, not DNA.

sis. Globin was prepared by adding hemoglobin dropwise to 2.5% oxalic acid in acetone at room temperature. The precipitate was then washed in cold acetone and dried under N_2. α and β globin subunits were separated by chromatography on CM-cellulose in 8 M urea (11).

Preparation of Peptides. Amino-ethylated subunits were digested in trypsin (TRTPCK, Worthington Biochemical Corp., Freehold, N.J.) (11). Peptide maps were done on cellulose thin layers as described previously (12). Special stains were used for the detection of arginine, tryptophan, histidine, tyrosine, and sulfur-containing amino acids (13). Cellulose powder containing individual peptide spots was scraped off the glass plate and eluted with warm 7% acetic acid. Hand counter-current distribution was employed in order to purify one abnormal tryptic peptide that could not be isolated on thin layer plates (14). Five milliters of 3% acetic acid in water was placed in each of 12 glass-stoppered test tubes. Thirty milligrams of tryptic digest was dissolved in the first tube and 5.0 ml of 1-butanol was added. After thorough extraction (vigorous shaking for 15 min), the organic solvent was passed to the next higher tube and fresh 1-butanol was added to tube no. 1. Twelve such exchanges were carried out. After drying under vacuum, an aliquot of each tube was analyzed by ascending chromatography on cellulose thin-layer plates (see ref. 12 for details). Another aliquot was hydrolyzed in 6 N (constant boiling) HCl and 2% phenol at 110° for 16 hr. Amino acids were quantitated by AAA Laboratories, Seattle, Wash. on a Durrum D-500 amino-acid analyzer. The great bulk of the tryptic peptides remained in the aqueous phase of tube no. 1. βTp 4 was identified in both the aqueous and organic phases of tubes 4–7. This is the most hydrophobic of the normal β chain tryptic peptides. An abnormal hydrophobic peptide was recovered in the butanol phase of tubes 11 and 12 (see below).

Determination of Amino-Acid Sequence. Dansylated derivatives of peptides were prepared in order to identify N-terminal residues (15). Following acid hydrolysis, the mixture of amino acids was analyzed by two-dimensional ascending chromatography on polyamide thin-layer plates (Analtech Inc., Newark, Del.). Dansylated amino acids, identified by their fluorescence, were compared with commercially prepared standards (Sigma Chemical Corp., St. Louis, Mo.).

An abnormal tetrapeptide was sequenced by manual Edman degradation as described by Peterson (16). Trimethylsilyl-phenylthiohydantoin derivatives of amino acids were identified by gas chromatography (17).

A hydrophobic nonapeptide was sequenced by the solid phase method of Laursen (18). This modification of the Edman method involves covalently linking the peptide to an insoluble resin support. Reagents are then pumped over the immobilized peptide. The N-terminal amino group was first blocked using *t*-butyloxycarbonyl azide and then 150 nmol was attached to 50 mg of triethylenetetramine resin support using carbodiimidazole (18). The peptide was automatically sequenced in Dr. Richard Laursen's laboratory by the Sequemat Sequencer (19). The resulting thiazolinones were then converted to phenylthiohydantoins and identified by thin-layer chromatography.

In order to determine the amino-acid sequence at the C-terminal end, intact β-globin subunits were incubated with carboxypeptidase A (Worthington Biochemical Corp., Freehold, N.J.). The reaction was carried out in 0.01 M 2,2-bis(hydroxymethyl)-2,2′,2″-nitrilotriethanol (Bis-Tris) buffer, pH 6.7, at 25° with a substrate:enzyme ratio of 100:1.

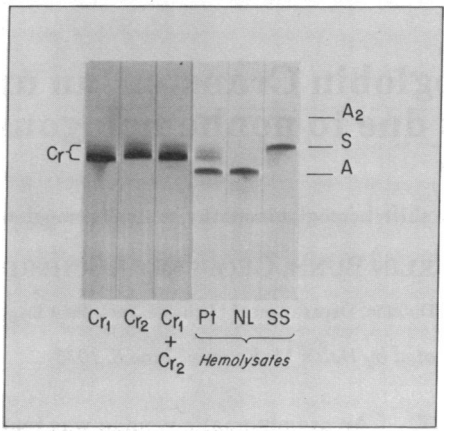


FIG. 1. Gel electrofocusing patterns of hemolysates and purified hemoglobins. From left to right: purified hemoglobin Cr_1; purified hemoglobin Cr_2; mixture of equal amounts of the two purified hemoglobins; proband's hemolysate; normal hemolysate; hemolysate of patient with homozygous sickle cell anemia.

This pH was chosen because the variant β chains were insoluble at pH's between 7 and 10. Norleucine was used as an internal standard. Samples taken at 15, 30, 60, and 120 min were immediately mixed with 1/10 volume of glacial acetic acid and frozen in dry ice. These specimens were applied directly to the Durrum D-500 amino-acid analyzer. The column was developed with the standard sodium citrate buffers used for protein hydrolysates. An aliquot was also run on a Beckman 120 B analyzer, with lithium citrate buffers, permitting the separation and quantitation of serine and asparagine.

Other Methods. Routine hematological methods were employed. Optical spectra of hemoglobin solutions were determined with a Perkin Elmer 350 recording spectrophotometer. Sulfhydryl groups of native hemoglobins were titrated by Dr. Hyman Muss employing the method of Boyer (20). Hemoglobin stability in isopropanol was tested by the method of Carrell and Kay (21).

RESULTS

Clinical Information. The proposita is a 39-year-old woman of Italian–American extraction. During evaluation for a chronic connective tissue disorder, she was found to have a well-compensated hemolytic state. Hemoglobin was 15–17 g/100 ml with normal red cell indices. Reticulocytes were 5–8%. Electrophoresis of her hemoglobin on cellulose acetate, pH 8.6, revealed an abnormal component comprising about 30% of the total and having a mobility close to that of hemoglobin S. No unstable hemoglobin could be demonstrated when hemolysate was incubated at 50° in phosphate or Bis-Tris buffer. However, a precipitate did form when her hemolysate was incubated in isopropanol (21). No Heinz bodies were seen when a fresh specimen of blood was incubated with 1% crystal violet. However, when blood specimens were incubated overnight in the absence of added glucose, the red cells of the proposita developed abundant Heinz bodies, whereas the control preparation did not. The proposita's mother and one of the three children of the proposita had similar hematologic findings and electrophoretically abnormal hemoglobin.

Isolation of Abnormal Hemoglobin. Analysis of the proposita's hemolysate by gel electrofocusing revealed the presence of two closely spaced bands between hemoglobins A and A_2 (Fig. 1). The abnormal hemoglobin was isolated by

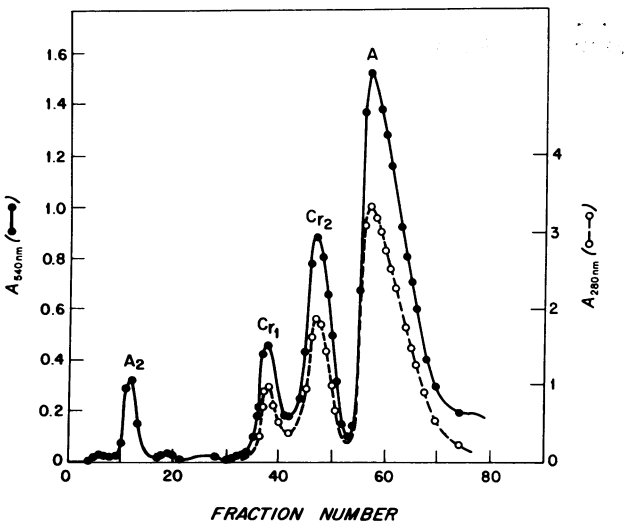


FIG. 2. Elution pattern following chromatography of proband's homolysate on DEAE-cellulose. Heme absorbance: $A_{540\,nm}$ (●—●). Protein absorbance: $A_{280\,nm}$ (O- - -O).

chromatography on DEAE-cellulose. The elution profile shown in Fig. 2 reveals the presence of two abnormal peaks (Cr_1 and Cr_2) that comprise 10% and 30% of the total hemoglobin. Following rechromatography of Hb Cr_2, about 30% of the hemoglobin cochromatographed with Hb Cr_1, the remainder with Hb Cr_2. When concentrated solutions of Hb Cr_1 and Hb Cr_2 were analyzed by gel electrofocusing, they gave nearly identical patterns, shown in Fig. 1. Furthermore, the same banding pattern was obtained when 1:1 (Fig. 1), 1:3, and 3:1 mixtures of Hb Cr_1 and Hb Cr_2 were analyzed. The results obtained by gel electrofocusing and by chromatography on DEAE-cellulose were not affected by storage of blood or hemolysate at 4° for 1–4 weeks.

The optical spectra of Cr_1 and Cr_2 in the visible range were identical to those of hemoglobin A. No methemoglobin or hemichrome (22) could be detected. Furthermore, the fact that hemoglobins Cr_1, Cr_2, and A had identical absorbance ratios at 540 nm and 280 nm ruled out loss of heme groups (23) as an explanation for the chromatographic heterogeneity seen in Fig. 2. The three hemoglobins had nearly identical elution patterns when chromatographed on Sephadex G-100. Titration of free sulfhydryl groups revealed no significant differences between hemoglobins Cr_1, Cr_2, and A.

Table 1. Amino-acid composition of abnormal tryptic peptides

Tryptic peptide 15, eluate from thin-layer plate		Tryptic peptide 16, counter current fractions 11 + 12	
Residue	Residues/mole	Residue	Residues/mole
Ile	1.0	Ala	1.0
Lys	1.0	Asx	0.9
Ser	1.0	Glx	0.1
Thr	1.0	Gly	0.2
		Leu	3.1
		Phe	1.9
		Pro	0.2
		Ser	0.9
		Thr	0.1
		Tyr	0.9
		Val	0.3

Structure of Hemoglobin Cranston. Purified hemoglobins Cr_1, Cr_2, and A were each mixed with an equal amount of canine hemoglobin and incubated overnight at pH 4.5, 4°. Gel electrofocusing patterns indicated that Hb Cr_1 and Hb Cr_2 contained abnormal β chains. Peptide maps of tryptic digests of aminoethylated β_{Cr} showed the absence of normal β tryptic peptide (βTp) 15 (tyrosylhistidine) and the presence of a new peptide with a lower R_f value that was negative for all the specific amino-acid stains, including tyrosine and histidine. Otherwise, the fingerprints of β_{Cr1} and β_{Cr2} were indistinguishable from that of β_A. The amino-acid composition of this abnormal peptide is shown in Table 1. These results indicated that hemoglobin Cranston cannot be explained by a single amino-acid substitution. The amino-acid composition of β_{Cr} Tp 14 was identical to that of β_A Tp 14. Thus, it can be assumed that β_{Cr} has the same structure as β_A through Tp 14 (position 144). The total amino-acid compositions of β_{Cr1}, β_{Cr2}, and β_A are shown in Table 2. There were no significant differences in the amino-acid content of β_{Cr1} and β_{Cr2}. These abnormal subunits lacked one His residue. They contained one additional Ala, Asx, Ile, and Thr residues and two additional Leu, Phe, and Ser residues. These results indicated that hemoglobin Cranston contains β chains that are elongated at the C-terminal end. Six carboxypeptidase A analyses were performed. They revealed the C-terminal sequence to be -Phe-Tyr-COO^-. In addition, the following residues were also consistently released during incubation with the enzyme: Leu, Phe, Ala, Asn, Ser, Lys, Thr, and Ile. However, the pattern of release of these amino acids was not sufficiently reproducible to permit the establishment of any further sequence. Both the total amino-acid composition and the carboxypeptidase experiments indicated that β_{Cr} contained at least one additional tryptic peptide consisting of predominantly hydrophobic residues. We were not able to isolate this peptide on fingerprints. At the suggestion of Dr. William Konigsberg, tryptic digests of β_{Cr1} and β_{Cr2} were analyzed by hand counter-current distribution as described in *Materials and Methods*. In the organic phase of

Table 2. Amino-acid composition (residues/chain) of normal and variant β chains*

	β_A		β_{Cr1}	β_{Cr2}	Difference:
	Expected	Found	Found	Found	β_{Cr} vs β_A
Ala	15	15.0	16.0	15.7	+1
Arg	3	3.0	3.0	3.1	
Asx	13	13.5	14.4	14.0	+1
Glx	11	11.8	12.0	11.8	
Gly	13	13.2	13.3	13.3	
His	9	8.7	8.2	8.0	−1
Ile	0	0	1.0	1.0	+1
Leu	18	18.6	21.6	21.0	+2–3
Lys	11	12.0	11.6	12.0	
Met	1	1.0	1.0	1.0	
Phe	8	8.1	10.0	9.7	+2
Pro	7	7.2	7.3	7.2	
Ser	5	5.3	7.2	6.7	+2
Thr	7	7.0	8.2	8.0	+1
Tyr	3	2.9	2.5	2.5	
Val	18	17.9	18.5	18.1	

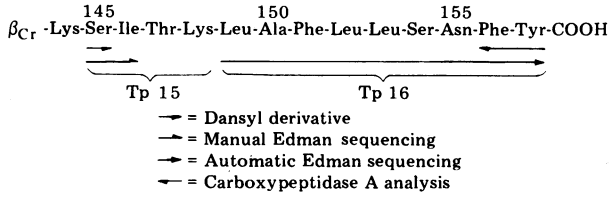
* β subunits were hydrolyzed in 6 N HCl at 110°. Aliquots were taken at 24, 48, and 96 hr for amino-acid analysis. Serine and threonine values are extrapolations to zero time.

the last two tubes, a hydrophobic peptide was isolated with about 85% purity and a yield of 25%. Attempts to purify it further by thin-layer chromatography were unsuccessful. Its amino-acid composition is shown in Table 1.

The tetrapeptide (β_{Cr} Tp 15) was sequenced by manual Edman degradation. The N-terminal sequence was shown to be Ser-Ile. Since it is a tryptic peptide, its C-terminal residue must be lysine. The hydrophobic C-terminal peptide (β_{Cr} Tp 16) was sequenced by the solid phase sequencer (see below). The recovery of the serine and asparagine derivatives in the 6th and 7th cycles was very low. However, good yields of phenylalanine and tyrosine were obtained in the 8th and 9th cycles.

It is unlikely that β_{Cr} contains any other abnormal tryptic peptides. The total amino-acid analysis does not permit additional residues which are not accounted for in β_{Cr} Tp 15 and β_{Cr} Tp 16. Furthermore, the carboxypeptidase A experiments show continuity between the two abnormal peptides. Finally, no other unidentified tryptic peptides were seen on the fingerprints.

From the results on the amino-acid sequences of the two abnormal tryptic peptides, β_{Cr} appears to have the following structure:

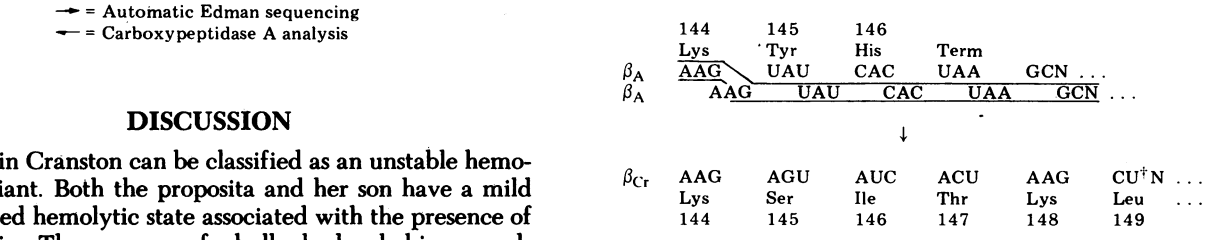


DISCUSSION

Hemoglobin Cranston can be classified as an unstable hemoglobin variant. Both the proposita and her son have a mild compensated hemolytic state associated with the presence of Heinz bodies. The presence of a bulky hydrophobic appendage at the C-terminal end of the abnormal β chains probably accounts for decreased solubility of hemoglobin Cranston and its precipitation within red cells. The elongation of the β chain also results in a marked increase in oxygen affinity. Functional and biosynthetic studies of hemoglobin Cranston will be presented in a subsequent report.

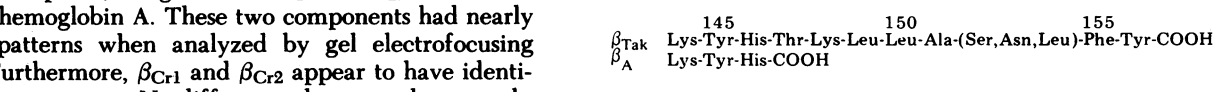
Heterogeneity of Hemoglobin Cranston. Hybridization experiments readily established that hemoglobin Cranston contained normal α chains and abnormal β chains. Since there are two β chain genes, individuals who are heterozygous for a β chain hemoglobin variant should have two main components: hemoglobin A and the variant hemoglobin. Chromatography on DEAE-cellulose consistently revealed two abnormal peaks, designated as Cr_1 and Cr_2, which eluted before hemoglobin A. These two components had nearly identical patterns when analyzed by gel electrofocusing (Fig. 1). Furthermore, β_{Cr1} and β_{Cr2} appear to have identical primary structures. No differences between the two subunits could be detected in: (*a*) the total amino-acid composition of the whole subunits; (*b*) fingerprints of tryptic digests; (*c*) the amino-acid composition of the two abnormal tryptic peptides shown in Table 1; (*d*) the pattern of amino acids released by carboxypeptidase A. Spectroscopic analysis of the peaks eluted from the DEAE-cellulose column failed to reveal any oxidation of heme groups, hemichrome formation, or loss of heme groups. Furthermore, neither hemoglobin Cr_1 nor hemoglobin Cr_2 had blocked sulfhydryl groups or any gross abnormality in molecular weight. Such changes have been reported in some of the other unstable hemoglobin variants (22, 23). We do not have a conclusive explanation for the chromatographic and electrophoretic heterogeneity of hemoglobin Cranston. The two chromatographic species may be conformers which are in slow equilibrium with each other. The elution patterns on rechromatography and the gel focusing experiment shown in Fig. 1 are consistent with this interpretation.

Genetic Basis of Hemoglobin Cranston. β_A and β_{Cr} can be assumed to have identical primary structures from residues 1 through 144. Beginning at residue 145, β_{Cr} has a unique sequence not previously encountered in any hemoglobin, resulting in a subunit that is 157 residues in length, 11 more than β_A. Normal human globin mRNAs are about 30% larger than that required for translation of their gene products (24). By analogy with hemoglobin Constant Spring and the other two variants having elongated α chains, it is likely that the β chain also has a segment of translatable genetic material beyond its termination codon. The chain elongation of β_{Cr} can be explained by the insertion of the repeated nucleotide pair AG into β mRNA between the triplet codon for 144 lysine (AAG) and 145 tyrosine (UAU). This would result in a frameshift and change the reading frame of the mRNA to give the precise amino acid sequence we have obtained for hemoglobin Cranston. This mutation may have resulted from a nonhomologous crossover between two normal β-chain genes, perhaps during meiosis. However, other genetic mechanisms are possible.



The base sequence shown in this diagram is taken from the data of Forget *et al.* (6, 7). The amino-acid sequence at positions 147–148 supports their finding that UAA is the termination codon for β mRNA. Furthermore, the presence of Leu at position 149 confirms their assignment of the C in the triplet codon following the UAA termination codon. The odds that this match of 13 bases in sequence could be due to chance are very small indeed. Thus, some other genetic mechanism for the formation of β_{Cr} is most unlikely.

The only other variant known to contain an elongated β chain is hemoglobin Tak (8). β_{Tak} appears to have the same primary sequence as β_A but has additional residues beyond β146 His. A preliminary report (8) indicates that its structure is:



Hemoglobin Tak cannot be the β chain analog of hemoglobin Constant Spring. As Flatz *et al.* (8) have pointed out, no single base substitution of a termination codon (UAA, UAG, UGA) could give a triplet which codes for threonine (ACN). It appears as if hemoglobin Tak, like hemoglobin Cranston, contains a hydrophobic C-terminal tryptic peptide which is difficult to recover from peptide maps. The amino-acid

† Deduced from the genetic code.

composition of this peptide in β_{Tak} is very similar to what we have obtained for the C-terminal tryptic peptide of hemoglobin Cranston. Furthermore, the C-terminal sequences of the two chains are identical: -Phe-Tyr-COOH. It is very likely that these two peptides are identical. Accordingly, the structure of hemoglobin Tak would be:

	145				150					155				
β_{Tak}	Lys-	Tyr-	His-	Thr-	Lys-	Leu-	Ala-	Phe-	Leu-	Leu-	Ser-	Asn-	Phe-	Tyr-COOH

It is likely that hemoglobin Tak arose because of a nonhomologous crossover at position 146 (two residues beyond the postulated crossover of $\beta_{Cranston}$) with the insertion of a repeated nucleotide base pair AC between the 146 His codon (CAC) and the UAA termination codon:

	144	145	146			
	Lys	Tyr	His	Term		
β_A	AAG	UAU	CAC	UAA	GCN	...
β_A	AAG	UAU	CAC	UAA	GCN	...
			↓			
β_{Tak}	AAG	UAU	CAC	ACU	AAG	...
	Lys	Tyr	His	Thr	Lys	...
	144	145	146	147	148	

Recently, Fitch (25) suggested that hemoglobin Tak could be due to the insertion of AC at position 146. This postulated mechanism involves a match of only five bases in sequence. However, the similarity in structure between β_{Tak} and β_{Cr} strongly supports the contention that there is a crossover at position 146. If this explanation is correct, β_{Cr} and β_{Tak} would differ by only two residues (positions 145 and 146). It is unlikely that in hemoglobin Tak the oxygen-linked salt bonds at β146 His would be preserved, because of the adjacent large hydrophobic group. Accordingly, hemoglobin Cranston and hemoglobin Tak should have very similar functional properties. It is interesting, however, that individuals having hemoglobin Tak are hematologically normal, while those with hemoglobin Cranston have a compensated hemolytic state.

The structure of hemoglobin Cranston provides new information on the base sequence at the 3′ end of human β mRNA. In the following paper, Forget *et al.* (26) present nucleotide sequences from normal human β chain mRNA which fit the C-terminal amino-acid sequence of hemoglobin Cranston. It is interesting to note that the UAA termination codon of both human α and β globin mRNA is followed by the same sequence of three bases: GCU. However, beyond this point, there is no obvious homology between the apparent base sequence of the α and β mRNAs deduced from the structures of variants with extended chains (ref. 25, this report) and from sequence studies on human globin mRNA (6, 7, 26).

The untranslated portions of globin mRNA may be important in stabilizing the mRNA or in the control of transcription or translation. It is of interest that individuals who are heterozygous for hemoglobins Cranston and Tak produce a relatively large amount of the abnormal β chain. It appears as if reading through the β chain termination codon has no drastic effect on synthetic rate. In contrast, variants with elongated α chains (hemoglobins Constant Spring, Icaria, Koya Dora, Seal Rock, and Wayne) appear as minor components in the hemolysates of affected individuals.

Note Added in Proof: Following submission of this paper, we learned that the structural analysis of Hb Tak has been completed (27). The sequence of its C-terminal tryptic peptide is identical to that of Hb Cranston. This finding strongly supports the proposal that both variants are frameshift mutants.

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