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Hemoglobin Grady: The First Example of a Variant with Elongated Chains Due to an Insertion of Residues

(alpha chain/gene duplication/amino-acid sequence)

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ABSTRACT **A black 25-year-old woman and her father have a fast-moving α chain variant in an amount of 8% (the father) and 18% (the daughter). Structural data indicate that this chain has been elongated by the addition of three amino-acid residues to give the sequence: -Pro-(114)-Ala(115)-Glu(116)-Phe(117)-Thr(118)-*Glu-Phe-Thr*-Pro(119)-Ala(120)-. The underlying genetic alteration responsible for hemoglobin Grady appears, therefore, to be a tandem duplication of nine base pairs which may have arisen by a process of mismatched intragenic crossing over. Functional and physicochemical properties of the variant are not greatly altered, and hematological data are normal.**

Comparative phylogenetic studies of sequences of many proteins suggest that intragenic duplications have played an important role in the evolution of structural genes and proteins. The evidence for such duplications has been reviewed elsewhere (1). We report here the first case of tandem repetition of a short sequence of amino-acid residues occurring in the α chain of a human hemoglobin (Hb). The variant, termed Hb-Grady, was observed in a 25-year-old black woman and her father. It has an increased electrophoretic mobility at alkaline pH, and subsequent structural analyses showed that its α chains were elongated with three amino-acid residues because the sequence Glu-Phe-Thr in positions 116–118 was repeated. Since this variant has been detected thus far in only two members of one family, it may be assumed that the tandem intragenic duplication is of recent origin. The nature of the duplication is such as to suggest that it may have arisen by unequal crossing-over.

EXPERIMENTAL

Source of Blood. Samples from the propositus and from five relatives were collected in EDTA and analyzed in Atlanta or shipped by air to Augusta, Ga.

Identification Procedures. Solutions of Hb were prepared by mixing one volume of washed erythrocytes with one volume of distilled water and 0.5 volume of carbontetrachloride. Debris was removed by centrifugation. Initial identification of the Hb types was made by cellulose acetate electrophoresis in Tris–EDTA–borate buffer (pH 8.4) (2), and by starch gel electrophoresis with the Tris–EDTA–borate discontinuous buffer system, pH 9.0 (3). Alkali-resistant Hb was measured by the method of Betke *et al.* (4). Chromatographic analyses made use of columns of DEAE–Sephadex (5, 6) or DEAE–cellulose (7).

Isolation of Hb Variant and Abnormal Chain. The variant was isolated by chromatography on DEAE–Sephadex (5, 6). Hb-Grady was contaminated with the normally occurring minor Hb-A_1, but was used without further purification because the abnormal α chain of Hb-Grady was readily separated from the the normal α chain of Hb-A_1 on 1.7 × 15-cm columns of carboxymethylcellulose (8).

Digestion of the α Chain. The chain was digested with trypsin for 4 hr at room temperature in a pH stat at pH 9.0 with addition of enzyme at zero and at 1 hr in an enzyme:protein ratio of 1:100. The pH was lowered to 6.5, and the insoluble core was isolated by centrifugation. The pH of the solution of soluble peptides was lowered to 2.5, and the solution was dried by lyophilization.

Digestion of the Core. The core was oxidized with performic acid (9), and digested with chymotrypsin at room temperature in a pH stat at pH 9.0. Three digests were prepared; the times of digestion were 8, 16, and 40 hr. Enzyme was added at zero, at 2 hr, and at 4 hr (first digest), at 8 hr (second digest), and at 24 hr (third digest) in an enzyme:protein ratio of 1:50. After the pH was adjusted to 2.5, the digests were lyophilized.

Isolation of Peptides. Tryptic as well as chymotryptic peptides were separated on 0.9 × 60-cm columns of chromobead resin type P (Technicon Instruments) at 37° (10). Volatile developers were used, and the gradients between these pyridine–acetic acid buffers of different molarities and pH were: 666 ml of 0.1 M buffer, pH 3.1, and 333 ml of 1.0 M buffer, pH 5.0 (first gradient), and 166 ml of 1.0 M buffer, pH 5.0, and 332 ml of 2.0 M buffer, pH 5.0 (second gradient). Several peptides were purified by chromatography on a 0.6 × 60-cm column of Dowex-1 (11).

Amino-acid Analyses. Peptides were hydrolyzed for 24 hr, and occasionally for 72 hr, at 110° under reduced pressure with 6 M HCl. The analyses were made with a Spinco model 121 automated amino-acid analyzer (Beckman Instruments) equipped with high-sensitivity cuvettes and an Infotronic model CRS-110A integrator. Sequential degradation of selected peptides by the Edman phenylthiohydantoin procedure followed the method of Schroeder (12).

Other Procedures. Hematological evaluation was made with standard laboratory methods (13).

RESULTS

The Propositus and Her Family. The index case (Li. D.) is a black woman, born in 1949, who attended clinic for obstetrical

Abbreviation: Hb, hemoglobin.

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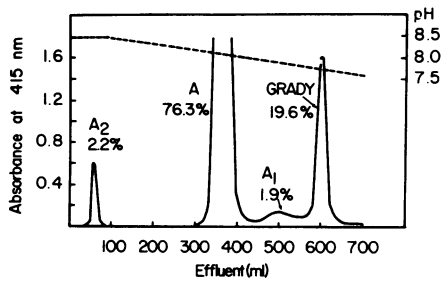

FIG. 1. Chromatographic separation of hemoglobins from propositus Li. D. A column (0.9 × 50 cm) of DEAE-cellulose was used with 50 mM Tris·HCl buffers, pH 8.5–7.0, as developers for 45 mg of protein. The *broken line* gives the pH values of the effluent.

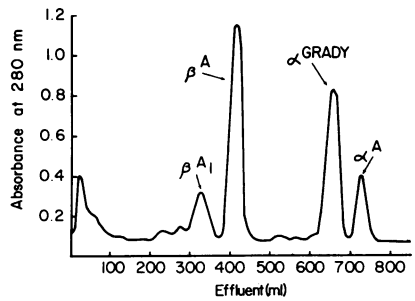

FIG. 2. The separation of the chains from globin of 300 mg of a mixture of Hb-Grady and Hb-A_1 on a 1.7 × 15-cm column of carboxymethylcellulose in 8 M urea. β^{A_1} refers to the β chain of Hb-A_1 in which the chain is condensed with a hexose residue at the amino terminus.

reasons. She and her father (Le. D.), a 55-year-old alcoholic, have an electrophoretically fast-moving hemoglobin variant that is readily detectable by cellulose acetate electrophoresis and by starch gel electrophoresis. Her daughter, brother, mother, and paternal grandmother have normal hemoglobin. Propositus and her father have been studied repeatedly since 1971, and hematological data have remained essentially normal for Li. D. and perhaps also for Le. D. considering his condition (Table 1).

The Quantity of Hb-Grady in the Heterozygote. Chromatography on columns of DEAE-cellulose or DEAE-Sephadex did result in an incomplete separation of Hb-Grady and Hb-A_1 (Fig. 1). Consequently, the data listed in Table 1 give the sum of these components. The mean value for Li. D. is 21.3% and for Le. D. a surprisingly low 11.7%. Considering that the average quantity of Hb-A_1 in a freshly prepared red cell hemolysate is about $1/20$ of that of Hb-A_0 (5), the Hb-Grady levels appear to be about 17–18% in Li. D. and 7–8% in Le. D.

Structural Analyses. The separation of the α chain of Hb-Grady and the α^A chain by carboxymethylcellulose chromatography is depicted in Fig. 2. Material isolated from the appropriate zone and from similar zones of additional columns was used for further analyses. All expected soluble tryptic peptides (T-1 through T-11, T-13, and T-14) were recovered, and the amino-acid composition of all peptides was the same as that of corresponding fragments of the α chain of Hb-A. Thirteen peptides were isolated from the chymotryptic digests of oxidized core material; nearly all fragments were observed in all three digests that were analyzed. Data from amino-acid analyses are given in Table 2. Ten fragments (numbered 1–5, 8, 10–13) are identical in composition to peptides of the normal α chains as listed in Fig. 3. Fragment 7 was recovered in relatively high yield from the 40-hr digest, and phenylthiohydantoin degradation of this tripeptide established its sequence as Thr-Glu-Phe. Peptides 6 and 8 were recovered in relatively low yields from the 8- and 16-hr digests. Fragment 6 is a combination of fragments 5 and 7; the amino-terminal sequence Ala-Ala-His indicates that fragment 7 is attached at the carboxyl-terminal end of fragment 5. Similarly, fragment 9 is a combination of peptides 7, 8, and 11. Phenylthiohydantoin degradation gave a Thr-Glu-amino-terminal sequence, indicating that fragment 7 is attached to the amino-terminal end of fragment 8. The specificity of chymotrypsin is such that fragment 7 could have been produced from the hydrolysis of an α chain only if these three additional residues are present in the indicated positions. Consequently, the primary sequence of the core segment (residues 100–139) is that of Fig. 3, and the abnormality of the α chain of Hb-Grady involves the insertion of three residues.

TABLE 1. *Hematological and hemoglobin composition data*

	Li. D.[a]			Le. D.[b]	
	11-8-71	5-28-73	1-7-74	11-8-71	1-7-74
Hb (g/100 ml)	12.2	13.4	13.3	12.5	13.1
PCV (%)	37	41	40	39	42
RBC (10^6/mm^3)	4.31	4.53	4.89	3.70	4.19
MCV (μm^3)	85	88	82	103	99
MCH (pg)	28	29	28	34	32
MCHC (%)	32	33	33	32	31
Retics (%)	4.0	—	1.4	1.6	0.8
Hb-F_{AD} (%)	—	—	0.4	—	0.3
Hb-A_2 (%)[c]	2.0	2.2	2.2; 2.2	2.5	2.4; 2.5
Hb-A_1 + Hb-Grady (%)[c]	21.7	23.1	18.7; 21.5	10.8	10.2; 14.2

PCV, packed cell volume; *RBC*, erythrocytes; *MCV*, mean corpuscular volume; *MCH*, mean corpuscular Hb; *MCHC*, mean corpuscular Hb concentration; *Retics*, reticulocytes.

[a] Peripheral blood smears showed an occasional target cell.

[b] Peripheral blood smears showed stomatocytes, macrocytes, and target cells. Blood folate level 53 μg/ml (normal range: 19–68 μg/ml).

[c] Hemoglobin samples were analyzed within 7 days after collection.

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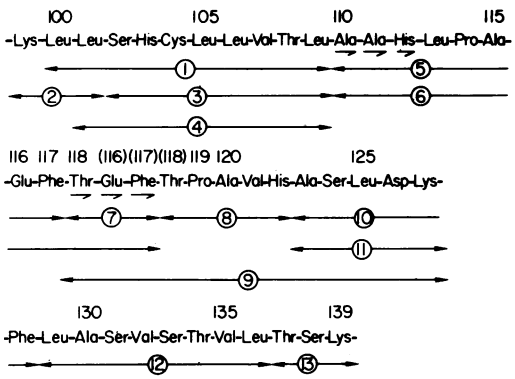

FIG. 3. The proposed sequence of the core section of the α chain of Hb-Grady. *Numbers above residues* refer to positions in chains, and the *numbers in circles* to the fragments listed in Table 2.

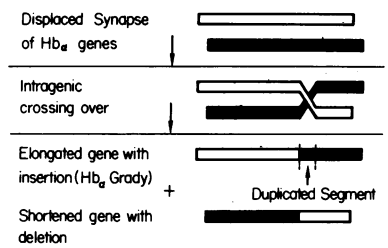

FIG. 4. Possible origin of the Hb$_\alpha$Grady gene carrying a duplicated segment, and of a Hb$_\alpha$ gene with a deletion.

DISCUSSION

The chemical evidence clearly shows that the α chain of Hb-Grady is elongated to 144 residues through the insertion of three residues. The extra glutamyl residue in the inserted segment Glu-Phe-Thr is responsible for the observed electrophoretic and chromatographic properties. Although chains elongated at the carboxyl terminus have been described (14–17), Hb-Grady is the first example of elongation by insertion of residues somewhere between the termini. Seven variants are known in which one to five residues are deleted (18–23). Such a deletion might arise by a simple loss of base pairs from a chromosome or chromatid or it might arise by intragenic unequal crossing-over involving two chromatids. This event in the case of the α chain of Hb-Grady is shown in Fig. 4, where it is assumed that one single Hb$_\alpha$ locus is present on each chromosome. On this basis it appears that the amino-terminal segment (residues 1–118, inclusive) of the elongated Hb$_\alpha$ gene originates from the Hb$_\alpha$ locus of one chromosome and the carboxyl-terminal segment (residues 116–141 with the duplication of residues 116–118) from the Hb$_\alpha$ locus of the other. If the chromosome carries two tandemly repeated loci, as some have suggested (24–26), and these are in direct tandem repetition, then the elongated Hb$_\alpha$ locus of Hb-Grady could have arisen by mismatched intragenic crossing-over that occurred between unequally paired Hb$_\alpha$ loci of such a repetition. This process could lead either to a chromosome with only one Hb$_\alpha$ locus (Hb-Grady type) or it could lead to a triplication of the Hb$_\alpha$ locus; the middle locus would be Hb$_\alpha$ Grady and the two outer loci would be normal Hb$_\alpha$ loci. Although it is not possible to decide among any of these possible mechanisms for the origin of the Hb$_\alpha$ Grady gene, the triplication possibility is of interest because heterozygotes for the triplication would have five Hb$_\alpha$ loci, one of which would be the Hb$_\alpha$ Grady type. This could explain the low yield of Hb-Grady chains in such heterozygotes.

The insertion can be considered to have occurred between residues 115 and 116 or between residues 118 and 119. Three-dimensionally, residue 118 (threonine) is the amino-terminal residue of helix H (see refs. 27, 28). Residue 117 (phenylalanine) as well as 114 (proline) are involved in the $\alpha_1\beta_1$ contact (27, 28). Other residues in the vicinity are also participating in the $\alpha_1\beta_1$ contact. Wherever the point of insertion is considered to be, it does not appear to produce undue stress on the molecule, as shown by the absence of ill effects in the heterozygotes and by the lack of gross abnormalities in stability and function of the molecule.

The difference between the relative amounts of Hb-Grady in the two known heterozygotes is indeed puzzling. It is also possible that the clinical condition of the father decreases the activity of the Hb$_\alpha$ Grady genome similarly as a megalo-

TABLE 2. *Amino-acid composition of peptides from chymotryptic digests of the oxidized core of the α chain from Hb-Grady*[a]

Amino acid	1[b]	2	3	4	5	6	7	8	9	10	11	12	13
Lysine		1.11							1.00	1.01	1.10		1.19
Histidine	1.00		0.80	0.89	1.16	0.90		1.02	1.04				
Cysteic acid	0.98		1.15	0.79									
Aspartic acid									0.92	1.07	1.02		
Threonine	0.95		1.06	0.95		1.03	1.07	0.89	2.16			1.06	0.89
Serine	1.05		0.97	1.28					1.04	0.93	0.91	1.94	0.92
Glutamic acid					0.99	1.97	0.97		1.08				
Proline					0.87	1.01		1.13	1.06				
Alanine					2.86	3.05		0.98	2.00	1.00	0.97	1.06	
Valine	0.98		1.00	1.03			0.20	0.96	1.02			2.12	
Leucine	4.84	1.89	3.03	3.97	1.05	0.93	0.16		0.96	1.05	1.00	1.80	
Phenylalanine					1.04	2.12	0.97		0.92	0.94			
Yield (%)[c]	9	12	6	8	18	8	18	25	6	18	10	18	15

[a] Data are given in residues/peptide and are for peptides isolated from three separate digests. Residues present in less than 0.1 residue molecules are omitted.

[b] Numbers refer to peptides indicated in Fig. 3.

[c] Calculation is based on μmoles of α chains available for the initial tryptic digestion, and on the recovery after Dowex 50 chromatography; a 100% hydrolysis at the appropriate cleavage points during the tryptic digestion of the α chain and the subsequent chymotryptic digestion of the oxidized core is assumed.

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blastic anemia and an iron deficiency anemia will decrease the activity of the Hb_δ gene and the $Hb_\beta S$ gene in the Hb-S heterozygote (29, 30).

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