Am J Hum Genet 27:81–90, 1975

Hemoglobin Koya Dora: High Frequency of a Chain Termination Mutant

W. W. De Jong,[1, 2] P. Meera Khan,[1] and L. F. Bernini[1]

In the course of a population genetical study of two tribal populations from Andhra Pradesh (India), the Koya Dora and the Konda Reddi, several abnormal hemoglobins were observed. Apart from the major hemoglobin variants Hb S and Hb Rampa [1], a minor hemoglobin abnormality was detected in many individuals of the Koya Dora tribe. This minor variant, designated Hb Koya Dora (or Hb KD), showed many remarkable characteristics and is the subject of this report. Some preliminary data were previously reported [2].

MATERIALS AND METHODS

Blood samples were obtained from 200 members of the Koya Dora tribe. The 10-ml samples were collected in Vacutainers containing EDTA and were stored and shipped on ice to Leiden, arriving within 2–7 days after collection. Hemolysates were prepared immediately on arrival and used for starch gel electrophoresis in Tris-EDTA-borate buffer, pH 8.6 [3]. The remainder of each hemolysate was dialyzed against 0.003 M Tris-phosphate buffer, pH 8.6, and stored at −20° C for later characterization of possible hemoglobin variants.

Quantitative determinations of major hemoglobin variants were performed after electrophoresis on cellulose-acetate strips (Cellogel, Chemetron, Milano) in Tris-EDTA-borate buffer, pH 9.1 [4]. The separated hemoglobin bands were cut out, extracted in 5 ml Drabkin's solution, and read at 418 nm. All determinations were done in duplicate and showed a reproducibility within 2% of total hemoglobin.

The abnormal minor hemoglobin was isolated by column chromatography on diethylaminoethyl (DEAE) cellulose (Whatman DE52) using a linear gradient from 0.003 M to 0.010 M Tris-phosphate, 0.10 M NaCl; all buffers were pH 8.6 and contained 100 mg KCN per liter. Globin preparation, chain separation, aminoethylation, and tryptic digestion were carried out according to Clegg et al. [5]. Peptide maps were made as described earlier [1]. Amino acid analyses of globin chains and of peptides eluted from fingerprints were carried out on a Beckman Unichrom amino acid analyzer. Analytical separation of globin chains was performed on Cellogel strips in 8 M urea (L.F. Bernini, manuscript in preparation).

Received June 12, 1974; revised August 5, 1974.

This work is part of the Human Adaptability Section of the International Biological Program and was supported by grants from the Italian National Research Council and the Royal Dutch Academy of Sciences.

[1] Department of Human Genetics, State University of Leiden, Leiden, the Netherlands.

[2] Present address: Department of Biochemistry, University of Nijmegen, Nijmegen, the Netherlands.

RESULTS

The results of electrophoretic analysis of the 200 hemolysates are reported separately (P. Meera Khan and L. F. Bernini, in preparation). In 20 samples a slow moving minor variant, Hb Koya Dora (Hb KD), was observed. The variant was usually found as two components, KD 1 and KD 2, both migrating slower than Hb A_2 (fig. 1). The amount of the slower moving component (Hb KD 2)

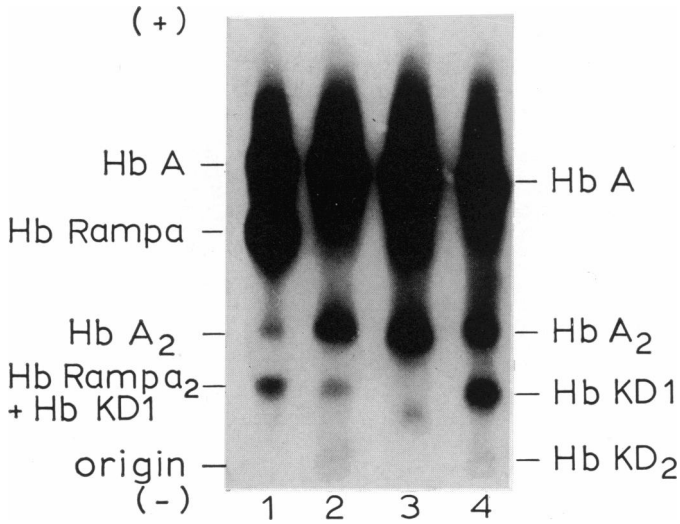

FIG. 1.—Starch gel electrophoresis at pH 8.6. *1*, Carrier of Hb Rampa and Hb KD; *2*, carrier of low level of Hb KD; *3*, carrier of a very weak slow moving hemoglobin variant (not further dealt with in this paper); *4*, carrier of elevated level of Hb KD.

seemed to increase on storage of the hemolysate. As a result of storage, a third fraction appeared, Hb KD 3, in a position between Hb A_2 and Hb A. The total amount of the Hb KD components was estimated by visual comparison with Hb A_2 to vary usually between 0.5% and 2% of total hemoglobin, although in four cases considerably more Hb KD was observed (up to 10%).

Biochemical Characteristics of Hb KD

Since only a limited quantity of blood from each individual was available, structural studies of Hb KD were performed on pooled hemolysates from two brothers (I-2 and I-4 in fig. 5). Both carried a high level of Hb KD. The results of the hemoglobin separation on DEAE-cellulose columns were surprising: starch gel electrophoresis of the different peak fractions showed that, apart from Hb A and Hb A_2, at least seven different hemoglobin components could be distinguished (fig. 2). Since most of these components were not detectable in fresh hemolysates, it must be concluded that under certain conditions the original components Hb KD 1 and 2 are converted to derivatives which behave differently. The causes and nature of these

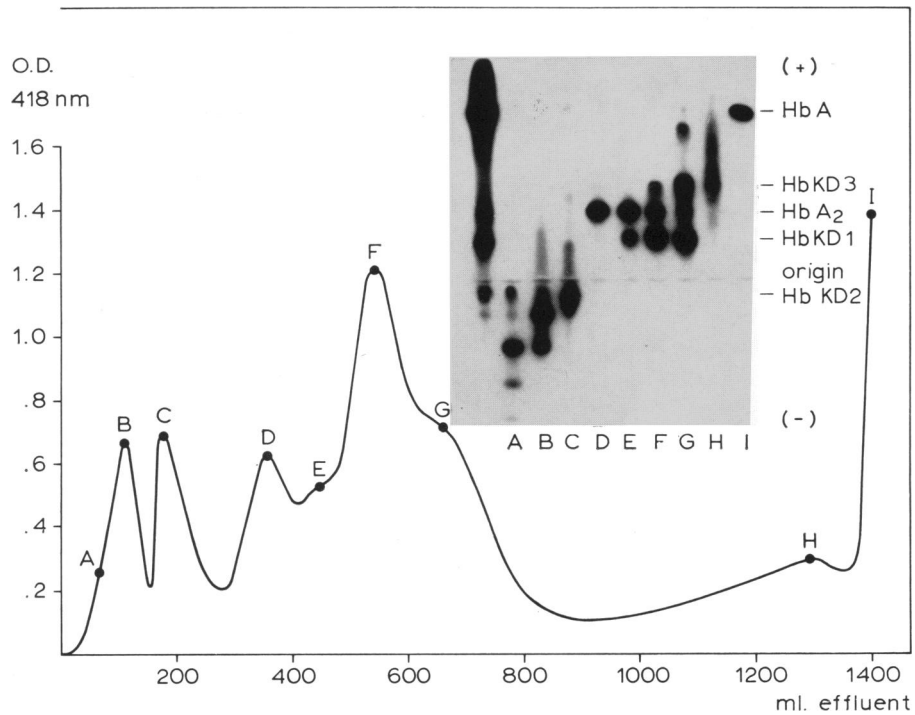

FIG. 2.—DEAE-cellulose chromatography of total hemoglobin from two presumed homozygotes for Hb KD. The inset shows the starch gel electrophoretic pattern of the starting material (*left*) and of the different eluted fractions.

conversions are as yet unclear. The main peak (*F-G* in fig. 2) containing Hb KD 1, Hb KD 3, and some Hb A_2 was converted to globin. The chain separation of this globin is shown in figure 3.

Peptide mapping of the separated and aminoethylated globin chains showed the presence of apparently normal β and, in smaller amount, δ chains. Two α-like fractions were present. The first fraction was eluted in the position of normal α^A chain and should contain the α chain of Hb A_2 together with a not further identified α-like chain from the Hb KD components. The second, most basic fraction only contained the α-like chain of Hb KD 1, as judged by cellulose-acetate gel electrophoresis in the presence of 8 M urea. This chain, designated as $\alpha^{KD\,1}$, deviated considerably in its amino acid composition from normal α chain and seemed to contain more than the normal 141 residues. The tryptic peptide map showed all the normal soluble α chain peptides and two additional spots (fig. 4). The trypsin-resistant core of $\alpha^{KD\,1}$ was digested with chymotrypsin and revealed a fingerprint pattern indistinguishable from the normal α chain core. The additional peptides of the tryptic peptide map of $\alpha^{KD\,1}$ were analyzed for amino acid composition. It appears from table 1 that the residues present in these peptides account for most of the differences between the α^A and $\alpha^{KD\,1}$ composition. It can be concluded

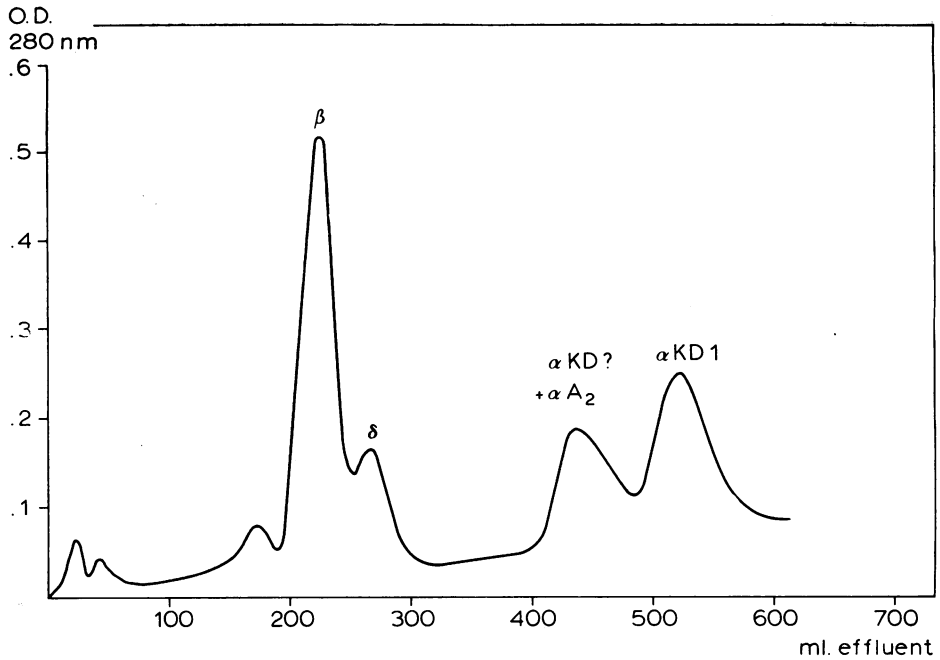

FIG. 3.—Carboxymethyl-cellulose chromatography [5] of 55 mg globin prepared from fractions F and G of fig. 2. The peak fraction containing $\alpha^{KD\ 1}$ was used for further characterization. The normal α chain of HB A_2 was eluted together with an α-like chain ($\alpha^{KD\ ?}$) from the Hb KD components, which was not further identified.

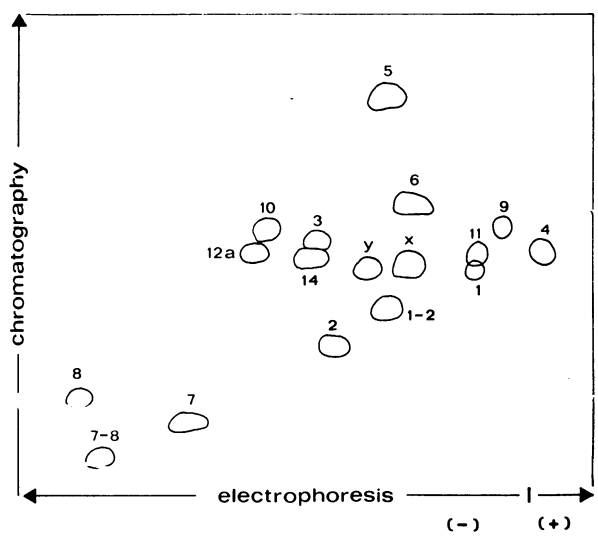

FIG. 4.—Peptide map of tryptic digest of the aminoethylated $\alpha^{KD\ 1}$ chain. Electrophoresis at pH 6.5 was followed by descending chromatography [1]. The numbers indicate peptides which correspond to normal α chain peptides; X and Y are the additional peptides present in the $\alpha^{KD\ 1}$ digest, of which the compositions are given in table 1.

TABLE 1

AMINO ACID COMPOSITION OF $\alpha^{KD\,1}$, α^{A}, AND ADDITIONAL TRYPTIC PEPTIDES X AND Y OF $\alpha^{KD\,1}$

AMINO ACID	$\alpha^{KD\,1}$	α^{A}	DIFFERENCE $\alpha^{KD\,1} - \alpha^{A}$	$\alpha^{KD\,1}$ PEPTIDES X	$\alpha^{KD\,1}$ PEPTIDES Y
Lysine	11.2 (11)	11.3 (11)	...	...	...
Histidine	9.8 (10)	9.9 (10)	...	...	...
Arginine	5.0 (5)	2.8 (3)	2	0.9 (1)	1.0 (1)
Aspartic	11.2 (11)	12.1 (12)	−1	...	...
Threonine*	8.1 (9)	8.5 (9)	...	...	...
Serine*	12.2 (14)	9.6 (11)	3	1.6 (2)	0.8 (1)
Glutamic	6.1 (6)	5.0 (5)	1	...	0.9 (1)
Proline	8.9 (9)	7.1 (7)	2	2.0 (2)	...
Glycine	7.6 (8)	7.1 (7)	1	1.1 (1)	...
Alanine	26.0 (26)	21.3 (21)	5	4.1 (4)	1.1 (1)
Cysteine	+ (1)	+ (1)	...	...	...
Valine	14.5 (15)	12.9 (13)	2	2.2 (2)	...
Methionine	1.7 (2)	1.8 (2)	...	...	...
Leucine	18.8 (19)	17.8 (18)	1	...	...
Tyrosine	2.7 (3)	2.9 (3)	...	...	...
Phenylalanine	7.3 (7)	6.9 (7)	...	...	...
Tryptophan	+ (2)†	+ (1)	...	...	(1)†
Total	158	141	...	12	5

NOTE.—Numbers in parentheses are the presumed integral number of residues in the polypeptide chain or peptide.

* Not corrected for hydrolytic destruction.

† The presence of tryptophan in peptide Y was not demonstrated but is likely on the basis of arguments given in the text.

that the abnormality in Hb KD resides in its α chain and consists of the presence of at least 16 additional residues.

Family Studies of Hb KD

Given the high frequency of the Hb KD trait among the Koya Dora, several homozygotes might be expected in the investigated sample. The carriers of high levels of Hb KD might indeed represent the homozygous state, and the pedigree in which three of them occur is in agreement with such a concept (fig. 5). The hemolysate of two presumed homozygotes contains, apart from the KD components, Hb A and Hb A_2. The α^{A} chains of the latter two have the expected amino acid composition. Interesting is the pedigree shown in figure 6, where both I-12 and II-4 were found to possess Hb KD in addition to the α chain variant Hb Rampa and normal Hb A (as judged by peptide mapping). This demonstrates that the α^{KD} gene can occur in one individual together with α^{A} and α^{Rampa}, thus proving the existence of at least two α chain loci in this family.

DISCUSSION

Hb KD resembles in many respects Hb Constant Spring (Hb CoSp), which has C-terminally elongated α chains [6, 7]. Both are minor variants, comprising in

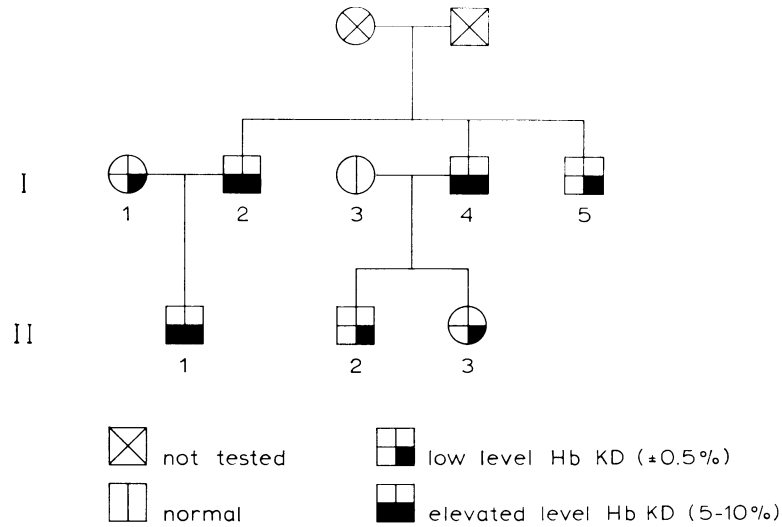

FIG. 5.—Pedigree in which three presumed homozygotes for Hb KD occur (I-2, I-4, and II-1)

the heterozygous state less than 2% of the total hemoglobin and showing the same slow mobility in starch gel electrophoresis at pH 8.6. Like Hb KD, Hb CoSp is usually present as two components, whereas on storage the relative proportion of these components changes and additional fractions appear. The homozygous state for Hb CoSp has been described by Lie-Injo et al. [8] in a Malay boy and compares with our observations in the carriers of high levels of Hb KD.

Hb CoSp has 31 additional residues located at the C-terminal end of the α chain. The two additional tryptic peptides found in $\alpha^{KD\,1}$ correspond in composition to residues 142–158 of the α^{CoSp} chain, apart from the apparent replacement of glutamine residue 142 in α^{CoSp} by a serine residue in $\alpha^{KD\,1}$ (fig. 7). The final proof of this substitution should be given by the sequence determination of peptide X of

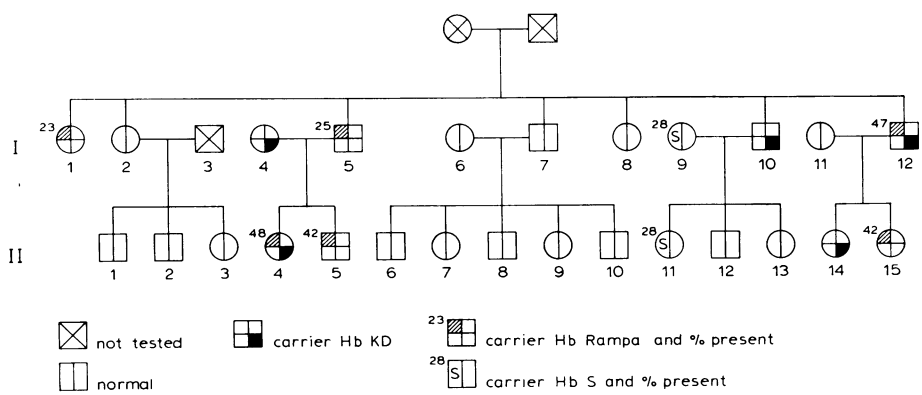

FIG. 6.—Pedigree demonstrating the multiplicity of the α chain locus

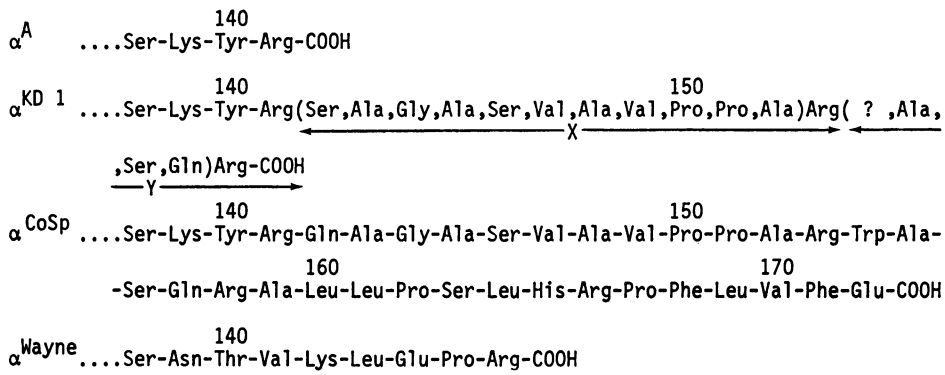

FIG. 7.—C-terminal sequences of the α chains of Hb A, Hb KD 1, Hb Constant Spring [7, 10], and Hb Wayne [9]. The X and Y indicate the two additional tryptic peptides on the peptide map of $\alpha^{KD\ 1}$.

$\alpha^{KD\ 1}$. The small amount of material available made such a determination as yet impossible. We could not detect the tryptophan residue corresponding to residue 154 of the α^{CoSp} chain, due to hydrolytic destruction before the amino acid analysis. However, the position of peptide Y on the fingerprint of $\alpha^{KD\ 1}$ was identical with that of the homologous α^{CoSp} peptide [7], thus making likely the presence of tryptophan.

The α^{CoSp} gene is supposed to have arisen from a mutation of the terminating codon of an α^{A} gene to a glutamine codon [7]. Analysis of the frame-shift mutant Hb Wayne [9] shows that the terminating codon of the normal α^{A} chain gene is most probably UAA ("ochre"). In Hb CoSp this codon then may have changed to CAA, giving glutamine in position 142. Similarly, a mutation from UAA to UCA, coding for serine, might explain the origin of $\alpha^{KD\ 1}$. Confirmation of this finding would considerably strengthen the hypothesis that a mutation of the chain-termination codon may lead to variants with elongated polypeptide chains.

The $\alpha^{KD\ 1}$ chain seems to be shorter than the α^{CoSp} chain. The tryptic peptides corresponding to residues 159–166 and 167–172 of the α^{CoSp} chain could not be found on the peptide map of $\alpha^{KD\ 1}$. Moreover the amino acid composition of $\alpha^{KD\ 1}$ does not indicate the presence of more than about 16 extra residues. However, the possibility that some additional residues are lost from the $\alpha^{KD\ 1}$ chain during the successive isolation procedures cannot be entirely excluded. The extra C-terminal sequence in α^{CoSp} seems to be quite prone to proteolytic attack, resulting in a chain which lacks the terminal residues Val-Phe-Glu [7]. Additional cleavages, for example at the susceptible Leu-Leu bond, 160–161, might in principle result in even shorter chains, corresponding in length to the $\alpha^{KD\ 1}$ chain. Further investigations are clearly needed to fully characterize the $\alpha^{KD\ 1}$ chain and its relation to the α^{CoSp} chain.

If the $\alpha^{KD\ 1}$ chain in fact turns out to be shorter than the α^{CoSp} chain, this might be the result of a terminating codon in the $\alpha^{KD\ 1}$ gene replacing, for example, the codon for leucine in position 160 of the α^{CoSp} chain. A one-step mutation could

account for such a phenomenon. The presence of a second difference between $\alpha^{KD\ 1}$ and α^{CoSp}, apart from the putative Gln → Ser replacement, would not be too unlikely. The genetic material in the α chain gene, made visible by the mutation of the terminating codon in Hb CoSp and Hb KD 1, is not normally translated and therefore under less stringent selective control than those parts of the gene coding for the α polypeptide chain. As a result, base substitutions may be quite abundant in this extracistronic material.

The heterozygous state for Hb CoSp is virtually symptomless. When inherited together with a severe α thalassemia gene (α thal 1), it gives rise to the clinical picture of hemoglobin H disease [6, 7, 10]. The homozygous case of Hb CoSp showed hepatosplenomegaly and mild hematological changes [8]. All findings indicate that the α^{CoSp} gene has the same effect as the mild α thal 2 gene. The hematological and clinical data collected during the present survey of the Koya Dora were too incomplete to allow the diagnosis of Hb H disease or α thalassemia. However, in a previous field study (unpublished results) Hb H was found to be present in several of the individuals who have been shown to possess Hb KD. An α thal 2–like expression of the $\alpha^{KD\ 1}$ gene could also explain the remarkable differences in the levels of Hb Rampa in the carriers of this variant (fig. 6). The levels of 23% and 25% Hb Rampa in individuals I-1 and I-5 correspond to the usually observed values for α chain variants in heterozygotes. The levels of 47% and 48% Hb Rampa in individuals I-12 and II-4 might result from the interaction with the α^{KD} gene. However, a normal α thal 2 (or α thal 1) gene has to be invoked in this family to explain the levels of 42% Hb Rampa in subjects II-5 and II-15.

Hb CoSp has been found and characterized in Chinese, Thais, Greeks, and Malays [6, 10, 11]. The frequency of Hb CoSp in Malaysia was found to be 2.2% in Malays, 0.7% in Chinese, 0.2% in Indians, and up to 3.2% in Malayan aborigines [12, 13]. In the last two racial groups, the minor variant was not structurally characterized and is therefore not necessarily identical with Hb CoSp. Since Hb CoSp and Hb KD are probably difficult to distinguish on starch gel electrophoresis, we cannot be sure that all the Koya Dora samples showing the minor hemoglobin components do indeed contain Hb KD. We can, however, conclude that the frequency of elongated α chain variants among the Koya Dora is very high, 10%, which makes this tribe an interesting object of future studies.

The pedigree in figure 6 shows that among the Koya Dora there are at least two α chain loci. This finding further extends the evidence that the human α chain locus is duplicated [14–17], although in some populations only one locus may be expressed [18, 19].

SUMMARY

Approximately 10% of the members of the Koya Dora tribe from Andhra Pradesh (India) carry an α chain hemoglobin variant, Hb Koya Dora (Hb KD), usually in amounts of 0.5%–2% of total hemoglobin. In four presumed homozygotes for Hb KD, up to 10% of the abnormal hemoglobin was present. The α chain of Hb KD was found to be elongated by at least 16 residues, possibly as a result of

a mutation of the normal α chain termination codon UAA to UCA, coding for serine. A pedigree in which two individuals possess Hb KD as well as the α chain variant Hb Rampa and normal Hb A proves the existence of two α chain loci in this population. Hb KD resembles the previously described Hb Constant Spring [6, 7] in many aspects, probably also in its α thalassemia–like expression.

ACKNOWLEDGMENTS

We thank Dr. Marcello Siniscalco for continuous support and interest in this work. The help of Mrs. Prabha Khan during the collection of the material and the valuable technical assistance of Miss M. Vervloet and Mr. P. Giordano is gratefully acknowledged.

REFERENCES

1. De Jong WW, Bernini LF, Meera Khan P: Haemoglobin Rampa: α95 Pro → Ser. *Biochim Biophys Acta* 236:197–200, 1971
2. Bernini LF, De Jong WW, Meera Khan P: Varianti emoglobiniche nella popolazione tribale dell'Andhra Pradesh. Molteplicità del locus α^{Hb} nell'uomo. *Atti Assoc Genet Ital* 15:191–194, 1970
3. Smithies O: Characterization of genetic variants of blood proteins. *Vox Sang* 10:359–362, 1965
4. Sheena AH, Fox FA, Bayha M, Stevens KM: A simple microtechnique for screening abnormal hemoglobins and for quantitation of A_2 hemoglobin by electrophoresis on cellulose acetate. *Am J Clin Pathol* 50:142–145, 1968
5. Clegg JB, Naughton MA, Weatherall DJ: Abnormal human haemoglobins. Separation and characterization of the α and β chains by chromatography, and the determination of two new variants, Hb Chesapeake and Hb J (Bangkok). *J Mol Biol* 19:91–108, 1966
6. Milner PF, Clegg JB, Weatherall DJ: Haemoglobin-H disease due to a unique haemoglobin variant with an elongated α-chain. *Lancet* 1:729–732, 1971
7. Clegg JB, Weatherall DJ, Milner PF: Haemoglobin Constant Spring—a chain termination mutant? *Nature* (Lond) 234:337–340, 1971
8. Lie-Injo LE, Ganesan J, Clegg JB, Weatherall DJ: Homozygous state for Hb Constant Spring (slow-moving Hb X components). *Blood* 43:251–259, 1974
9. Seid-Akhavan M, Winter WP, Abramson RK, Rucknagel DL: Hemoglobin Wayne: a frameshift variant occurring in two distinct forms. *Blood* 41:927, 1972
10. Efremov GD, Wrightstone RN, Huisman THJ, Schroeder WA, Hyman C, Ortega J, Williams K: An unusual hemoglobin anomaly and its relation to α-thalassemia and hemoglobin-H disease. *J Clin Invest* 50:1628–1636, 1971
11. Fessas P, Lie-Injo LE, Na-Nakorn S, Todd D, Clegg JB, Weatherall DJ: Identification of slow-moving haemoglobins in haemoglobin H disease from different racial groups. *Lancet* 1:1308–1310, 1972
12. Lie-Injo LE, Duraisamy G: The slow-moving haemoglobin X components in Malaysians. *Hum Hered* 22:118–123, 1972
13. Lie-Injo LE, Baer A, Lewis AN, Welch QB: Hemoglobin Constant Spring (slow-moving hemoglobin X components) and hemoglobin E in Malayan aborigines. *Am J Hum Genet* 25:382–387, 1973
14. Lehmann H, Carrell RW: Differences between α- and β-chain mutants of human haemoglobin and between α- and β-thalassemia. Possible duplication of the α-chain gene. *Br Med J* 4:748–750, 1968
15. Hollán SR, Szelenyi JG, Brimhall B, Duerst M, Jones RT, Koler RD, Stocklen Z: Multiple alpha chain loci for human haemoglobins: Hb J-Buda and Hb G-Pest. *Nature* (Lond) 235:47–50, 1972

16. OSTERTAG W, VON EHRENSTEIN G, CHARACHE S: Duplicated α-chain genes in Hopkins-2 haemoglobin of man and evidence for unequal crossing over between them. *Nature* (Lond) 237:90–94, 1972
17. WASI P: Is the human globin α-chain locus duplicated? *Br J Haematol* 24:267–273, 1973
18. ABRAMSON RK, RUCKNAGEL DL, SHREFFLER DC, SAAVE JJ: Homozygous Hb J Tongariki: evidence for only one alpha chain structural locus in Melanesians. *Science* 169:194–196, 1970
19. BEAVEN GH, HORNABROOK RW, FOX RH, HUEHNS ER: Occurrence of heterozygotes and homozygotes for the α-chain haemoglobin variant Hb-J (Tongariki) in New Guinea. *Nature* (Lond) 235:46–47, 1972