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Heterogeneity in the Molecular Basis of Three Types of Hereditary Persistence of Fetal Hemoglobin and the Relative Synthesis of the $^{G}\gamma$ and $^{A}\gamma$ Types of γ Chain

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Restriction endonuclease analyses of DNA from one Black $^{G}\gamma^{A}\gamma$-HPFH homozygote and four Black and one Indian $^{G}\gamma^{A}\gamma$-HPFH heterozygotes have identified three different HPFH types which are the result of large deletions including the δ and β genes. Two of the types are comparable to those characterized previously, but the third, which is present in the Indian heterozygote, shows a distinct difference in the size of the deletion. The 5′ end point of the deletion in this type III $^{G}\gamma^{A}\gamma$-HPFH extends 0.5–1.0 kb beyond the 5′ end point of one of the Black types of HPFH (type I). Each of the three types is associated with a distinct ratio between the $^{G}\gamma$ and the $^{A}\gamma$ chains, an observation supported by family data. The highest ratio is found in the heterozygote with the Indian type III $^{G}\gamma^{A}\gamma$-HPFH, with 69.3% $^{G}\gamma$ chains, while the averages for the other types were 50.7% $^{G}\gamma$ (type I) and 32.3% $^{G}\gamma$ (type II).

KEY WORDS: hereditary persistence of fetal hemoglobin; different types of HPFH; $^{G}\gamma$:$^{A}\gamma$ ratio; restriction endonucleases; DNA; *in vitro* chain synthesis; family data.

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INTRODUCTION

In normal newborn infants human fetal hemoglobin (Hb F) is readily replaced by the adult hemoglobins A and A_2, and at the age of 6 months only a small percentage of Hb F is detectable. Several genetic disorders have been described where the heterozygote retains some 3 to 30% Hb F in circulation even during adult life. Some of these are known as HPFH, or the hereditary persistence of fetal hemoglobin, and are not associated with any significant hematological or clinical abnormality (reviewed by Schroeder and Huisman, 1980). Different forms have been recognized. The differentiation is based primarily on the types of γ chain present in the Hb F, the level of Hb F in the heterozygote, the presence or absence of β- and δ-chain synthesis *in cis* to the HPFH determinant and, most recently, on the presence and type of deletion of a large segment of DNA from the 5′-ϵ-$^{G}\gamma$-$^{A}\gamma$-$\psi\beta$1-δ-β-3′ gene cluster on chromosome 11 (reviewed by Mears, 1981).

The studies to be described here concern the types of HPFH with the following characteristics: (a) observed among Blacks and Indians; (b) no hematological abnormalities in the heterozygote with a mild erythrocytosis in the homozygote (Huisman, 1981); (c) only Hb F present in the homozygote, while compound heterozygotes for HPFH and a β-chain (Hb S, Hb C, or Hb E) or a δ-chain (Hb A_2') variant do not produce Hb A or Hb A_2, thereby indicating an absence of β- and δ-chain production *in cis;* (d) the quantity of Hb F (Hb F_{AD} or alkali-resistant hemoglobin) in the heterozygote varies between 20 and 30%; and (e) the Hb F contains both types of γ chain, i.e., the $^{G}\gamma$ chain with glycine in position 136 and the $^{A}\gamma$ chain with alanine in that position, being the products of the $^{G}\gamma$ and $^{A}\gamma$ genes, respectively (Huisman *et al.,* 1969; 1971).

In the past (Huisman *et al.,* 1969, 1974; Schroeder *et al.,* 1973) we observed three different types of HPFH which were distinguished on the basis of the ratios of $^{G}\gamma$ and $^{A}\gamma$ chains in the Hb F of the heterozygote. The two major types in Blacks had $^{G}\gamma$:$^{A}\gamma$ ratios averaging about 30:70 and 45:55 (these two types are referred to as $^{G}\gamma^{A}\gamma$-HPFH types II and I, respectively), while in Indian HPFH heterozygotes the ratio was about 65:35 (Schroeder *et al.,* 1973; $^{G}\gamma^{A}\gamma$-HPFH type III). Gene mapping also identified two types among Black heterozygotes which are characterized by large deletions involving the δ and β genes. The 5′ end points of the deletions are different: one occurs about 3 kilobases (kb) 5′ to the δ gene and the other 9 kb 5′ to the δ gene (Mears *et al.,* 1978; Bernards and Flavell, 1980; Tuan *et al.,* 1980; Mears, 1981; Jagadeeswaran *et al.,* 1982; Ottolenghi *et al.,* 1982). The 3′ end points are unknown.

The present study was undertaken to characterize further the HPFH conditions in Blacks and Indians through a detailed analysis with restriction endonucleases of the DNAs from four Black heterozygotes (two with a low $^{G}\gamma$:$^{A}\gamma$ ratio and two with a higher ratio), one Indian heterozygote, and one

Black homozygote. The main objectives were to compare further the differences in the 5′ end points of the deletions, to attempt a characterization of the 3′ end points, and to evaluate a possible relationship between the occurrence of different types of deletions and the relative ratios of the $^{G}\gamma$ and $^{A}\gamma$ chains of the fetal hemoglobin.

MATERIALS AND METHODS

Blood Samples. Ninety-seven Black adults and children of 62 families as well as one Indian adult participated in this study, which was initiated some 4 years ago. Venous blood (5–10 ml) was collected in vacutainer tubes with EDTA as anticoagulant through the activities of the Comprehensive Sickle Cell Center in Augusta, Georgia, and of the local health departments of the various health districts in Georgia and South Carolina. Informed consent was obtained. Four families involving 18 heterozygous members were studied in detail; chain synthesis analyses were made in 11 members of two of these families as well as in nine unrelated HPFH heterozygotes. One Black adult with a HPFH homozygosity (Huisman, 1981), and four Black adults and one Indian adult with a HPFH heterozygosity, each belonging to a different family, were studied in greater detail. Some 20–40 ml of blood was collected from each of these six individuals and used for the isolation of genomic DNA from the white cells. The Indian adult is subject BPC discussed in a previous communication (Schroeder *et al.*, 1973).

Hematological Procedures. Hematological values were obtained with a Coulter counter Model S cell counter. The initial hemoglobin identification was made by starch gel electrophoresis at *p*H 8.9 and by citrate agar gel electrophoresis at *p*H 6.1 (Huisman and Jonxis, 1977). All samples containing abnormal hemoglobin types in addition to Hb A, Hb F, and Hb A_2 (mainly Hb S or Hb C) were excluded. Hb F was quantitated by an alkali denaturation procedure (Betke *et al.*, 1959), and Hb A_2 by DEAE-cellulose chromatography (Abraham *et al.*, 1976). *In vitro* chain synthesis was determined for 20 HPFH heterozygotes and the one HPFH homozygote using the procedure reviewed in detail elsewhere (Huisman and Jonxis, 1977). Quantitation of the $^{G}\gamma$ and $^{A}\gamma$ types of γ chains in the Hb F was made with a previously published HPLC procedure (Huisman and Wilson, 1980; Huisman and Altay, 1981; Huisman *et al.*, 1981).

Restriction Endonuclease Analysis. Genomic DNA was isolated from the white cells of 20–40 ml blood using a modification of the method described by Poncz *et al.* (1982). For mapping of the α-chain genes, 6 to 10 μg of DNA was digested with the restriction endonucleases *Xba*I and *Bgl*II under the recommended conditions. The fragments were separated by electrophoresis in

0.8% agarose gels. Transfer to nitrocellulose membranes followed the procedure of Southern (1975). Hybridization was accomplished using the recombinant plasmid cDNA JW-101 (Wilson *et al.*, 1978) after radiolabeling with [α-^{32}P]dCTP by nick translation using a standard kit. Fragments which hybridized to the α-chain sequences were identified by autoradiography using XAR-5 Kodak X-ray film and duPont Cronex lightning plus intensifying screens.

Maps of restriction endonuclease sites in and around the $^{G}\gamma$, $^{A}\gamma$, and δ genes in the DNA were determined for the Black HPFH homozygote, four Black HPFH heterozygotes (the selection of these individuals is discussed below), and the one Indian HPFH heterozygote. In general, the techniques involving digestion of DNA with selected enzymes (these are listed in Table II), electrophoretic separation of the fragments on agarose gels, transfer, hybridization, and identification of the fragments were the same as those used for the mapping of the α genes. The probes used in hybridization were the $^{G}\gamma$IVS-II and βIVS-II DNA fragments, which specifically hybridize with the IVS-II sequences of the $^{G}\gamma$ or $^{A}\gamma$ genes and of the β gene, respectively, and the $\gamma\delta$1.6BX probe, which is a 1.6-kb fragment of DNA located between the $^{A}\gamma$ and the δ genes (the location of the three probes is detailed in Fig. 7; the three probes were kindly provided by Dr. Oliver Smithies and associates, Madison, Wisconsin). The area of the deletions 3′ to the β gene was investigated in the Black HPFH homozygote and one Black heterozygote using as a probe the 0.8-kb *Bgl*II fragment that lies about 16 kb 3′ to the β gene. This probe, known as pRK28, was provided by Dr. Russell Kaufman (Duke University). The probes were isolated from the plasmids by selected enzyme cleavage, followed by polyacrylamide gel electrophoresis as described by Maxam and Gilbert (1980). A commercial kit was used for the labeling of these probes with [α-^{32}P]dCTP by nick translation.

The experiments with recombinant DNA were performed in a P1 facility following the guidelines given by the National Institutes of Health.

RESULTS AND DISCUSSION

The Percentages of $^{G}\gamma$ Chain in the Hb F of HPFH Heterozygotes. The Black adult HPFH heterozygotes fell into two categories (Fig. 1). Fifty-six (56) members of 35 families had $^{G}\gamma$ chain values between 20 and 40%, with an average of 32.3 ± 4.8%. This type of HPFH has been designated type II. Forty members of 27 additional families had considerably higher $^{G}\gamma$ values, which varied from 43 to 59%, with an average of 50.7 ± 4.3% (HPFH type I). None of the relatives of heterozygotes for either HPFH type I or HPFH type II had $^{G}\gamma$ values which placed them in another category. The only HPFH homozygote had a $^{G}\gamma$ value of 44%. The average $^{G}\gamma$ value in the Hb F of his four

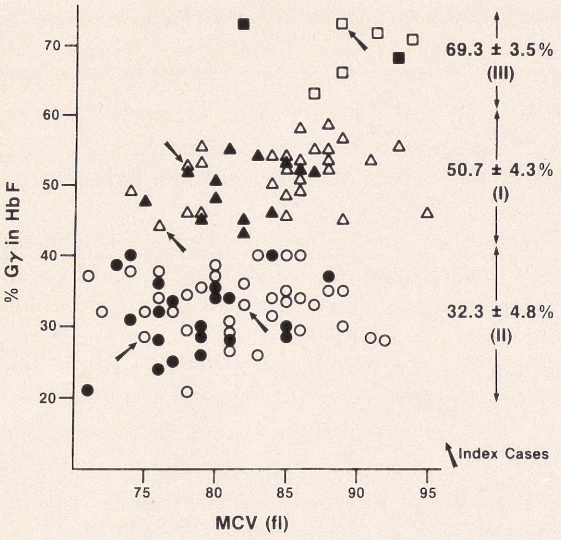

Fig. 1. The relationship between the MCV values and the percentages of $^{G}\gamma$ chain in the Hb F of 96 Black and seven Indian $^{G}\gamma^{A}\gamma$-HPFH heterozygotes. Nearly all data were obtained by HPLC. Each of the open circles represents one member of 35 different families with a $^{G}\gamma^{A}\gamma$-HPFH (type II) heterozygosity. The 21 filled circles refer to data for relatives. Similarly, the 40 open triangles and 13 filled triangles refer to $^{G}\gamma^{A}\gamma$-HPFH heterozygotes considered to be of type I, while the seven squares represent seven Indian heterozygotes (of five families) with a simple $^{G}\gamma^{A}\gamma$-HPFH (type III) heterozygosity.

heterozygous children (28.9%; range, 24.2–35.0%) placed this family in the HPFH type II category. The two subclasses are similar to those discussed in earlier publications (Huisman *et al.*, 1969, 1974). The HPLC procedure probably provides more accurate results than those obtained with the older chemical procedure (Schroeder *et al.*, 1968), eliminating an overlap between the two types.

The data for six of the seven Indian HPFH heterozygotes were published earlier (Schroeder *et al.*, 1973; Efremov *et al.*, 1982). The $^{G}\gamma$ values obtained with the chemical procedure and the HPLC method were nearly identical. The average $^{G}\gamma$ value for the seven HPFH type III heterozygotes was 69.3 ± 3.5%.

Table I compares average values for the mean red cell volume and the total level of Hb F with the percentage of $^{G}\gamma$ chain in the Hb F of the three HPFH types. No significant difference was present except for a slightly larger red cell volume in the Indian HPFH heterozygotes.

Five subjects with a HPFH heterozygosity as well as the HPFH

Table I. Average Values for MCV, the Level of HbF, and the Percentage $^{G}\gamma$ Chain in the Three Types of HPFH

HPFH type	Number of cases	MCV (fl)	HbF_{AD} (%)	$^{G}\gamma$ (%)
Type I (Black)	40	83.7 ± 4.8[a]	24.8 ± 3.1[a]	50.7 ± 4.3[a]
Type II (Black)	56	80.7 ± 5.0	24.4 ± 2.8	32.3 ± 4.8
Type III (Indian)	7	90.1 ± 1.9	22.6 ± 1.0	69.3 ± 3.5

[a]Standard deviation.

homozygote (P.B.) were selected for DNA analyses. These index cases (M.I. and K.C. of type II, Q.B. and D.E. of type I, B.C. of type III) are indicated by arrows in Fig. 1.

Family Studies. Figure 2 presents the abbreviated pedigrees of four Black families mainly to emphasize again the familial nature of the low or higher $^{G}\gamma$ values. Several members of families I and C have the $^{G}\gamma^{A}\gamma$-HPFH type II heterozygosity. The $^{G}\gamma$ values in the Hb F of the two index cases were

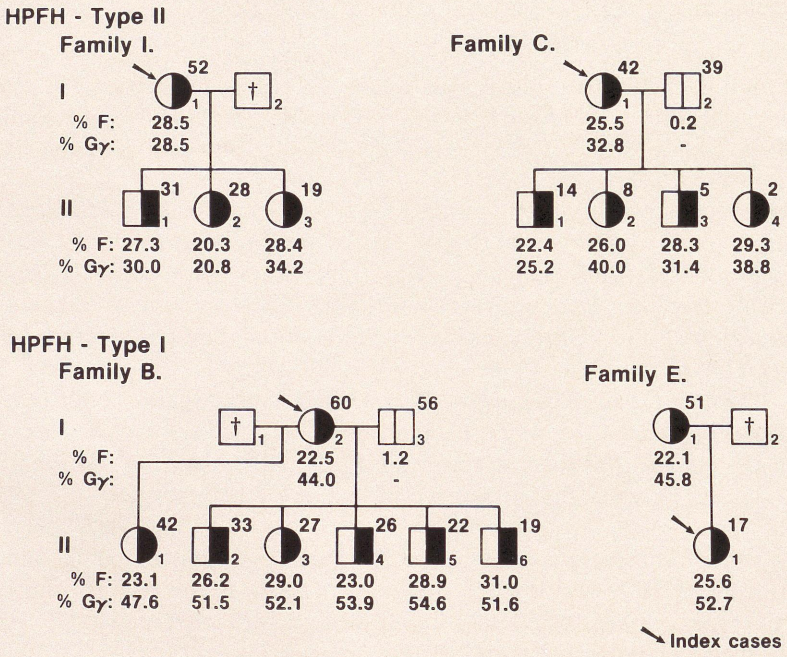

Fig. 2. Abbreviated pedigrees of four Black families with two different types of $^{G}\gamma^{A}\gamma$-HPFH.

28.5 and 32.8%, respectively, and the values in their seven heterozygous children varied between 20.8 and 40.0% (average value for the nine adults, 31.3 ± 5.8%). The Hb F averaged 26.2 ± 2.9%. In contrast, the $^{G}\gamma$ values in the Hb F of the two index cases of the two families (Family B, Family E) with the $^{G}\gamma^{A}\gamma$-HPFH type I heterozygosity were 44.0 and 52.7%, respectively. The values in seven heterozygous relatives ranged from 45.8 to 54.6% (average value for the nine adults, 50.4 ± 3.5%). The Hb F averaged 25.7 ± 3.1%.

Restriction endonuclease analyses with the JW-101 probe allowed the determination of the number of α genes in several members of these families. Moreover, a successful *in vitro* chain synthesis analysis was possible for 12 HPFH (type II) heterozygotes and eight HPFH (type I) heterozygotes. These data are shown in Fig. 3. Most individuals showed a (nearly) balanced chain synthesis. The data collected for family B were most complete and showed a rather insignificant difference between the HPFH heterozygotes with either four α genes ($\alpha\alpha/\alpha\alpha$) or an α-thal-2 heterozygosity ($\alpha\alpha^{0}/\alpha\alpha$). High α/non-α ratios were seen in the four HPFH (type II) heterozygotes of family I. This high ratio was not the result of an excessive number of α genes, as two of the four subjects had a complement of four α genes ($\alpha\alpha/\alpha\alpha$).

Only limited data are available regarding the *in vitro* chain synthesis in subjects with a heterozygosity for $^{G}\gamma^{A}\gamma$-HPFH type III. Results published by Ringelhann *et al.* (1977) suggest a balanced synthesis.

DNA Analysis by Restriction Endonucleases. Several studies (Bernards and Flavell, 1980; Tuan *et al.*, 1980; Mears, 1981; Jagadeeswaran *et al.*, 1982; Ottolenghi *et al.*, 1982) have identified two types of $^{G}\gamma^{A}\gamma$-HPFH which differ in the extent of the deletion 5′ to the δ-chain gene. Information on the size of

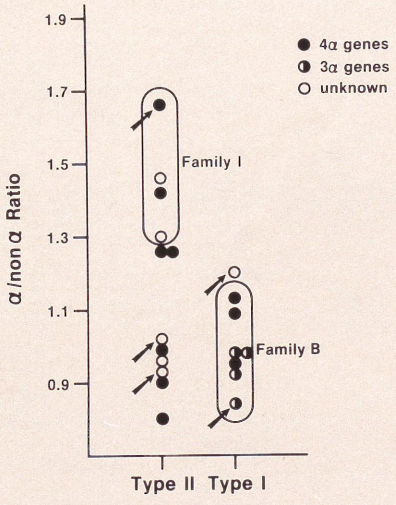

Fig. 3. The α/non-α *in vitro* chain synthesis ratios in 12 $^{G}\gamma^{A}\gamma$-HPFH type II and eight $^{G}\gamma^{A}\gamma$-HPFH type I heterozygotes. For further discussion, see text.

the deletion 3′ to the β-chain gene is lacking for both types. The 5′ end point of the deletion in $^{G}\gamma^{A}\gamma$-HPFH-type I is about 3 kb 5′ to the δ gene, while that in $^{G}\gamma^{A}\gamma$-HPFH type II is about 9 kb 5′ to this gene. All reports state that the $^{G}\gamma^{A}\gamma$-HPFH heterozygotes are phenotypically identical despite the significant difference in the 5′ end point of the deletion.

To examine the extent of the 5′ end of the deletion in the three types of $^{G}\gamma^{A}\gamma$-HPFH, defined in Fig. 1, DNAs from white cells of the one homozygote and of five heterozygotes (identified in Fig. 1 and in the pedigrees of Fig. 2) were digested with various restriction enzymes and analyzed with the $^{G}\gamma$IVS-II, γδ1.6BX (=ψβ1), and βIVS-II probes. The quantity of DNA available for

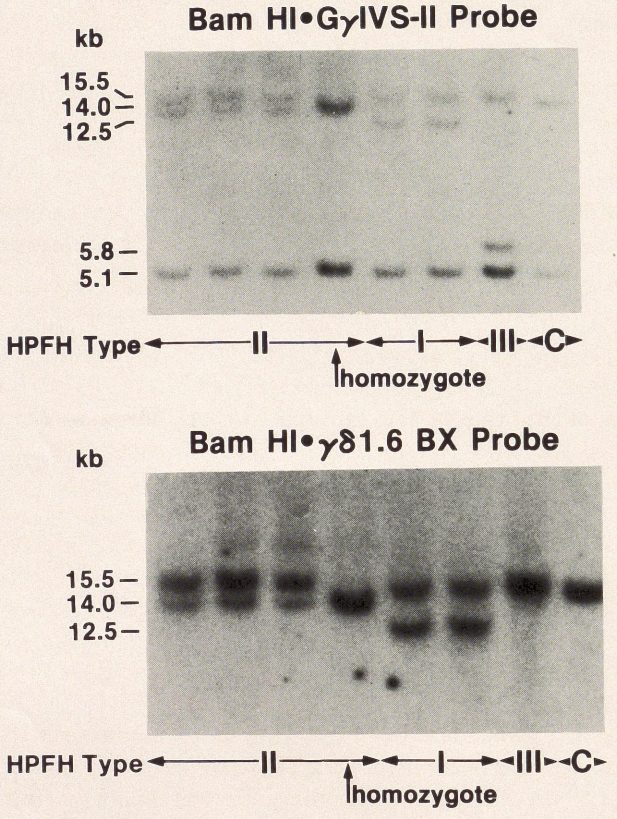

Fig. 4. Analysis of DNA restriction endonuclease fragments with the $^{G}\gamma$IVS-II and γδ1.6BX probes. DNA from three HPFH (type II) heterozygotes, one HPFH (type II) homozygote, two HPFH (type I) heterozygotes, one HPFH (type III) heterozygote, and one control were digested with *Bam*HI. The same membrane was used for both hybridizations.

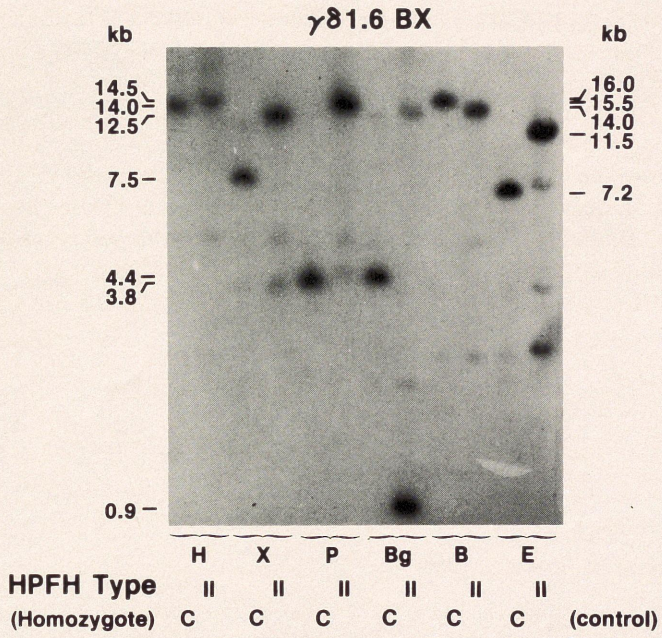

Fig. 5. Analysis of restriction endonuclease fragments of DNA from the HPFH type II homozygote and from a control using the γδ1.6BX probe. The enzymes used were *Hpa*I (H), *Xba*I (X), *Pst*I (P), *Bgl*II (Bg), *Bam*HI (B), and *Eco*RI (E).

B.C. with a heterozygosity for $^{G}\gamma^{A}\gamma$-HPFH type III was relatively small, which limited the analyses. Figures 4, 5, and 6 show autoradiographs of digests of DNA samples from persons with the different types of $^{G}\gamma^{A}\gamma$-HPFH. Figure 4 illustrates the sizes of the fragments in the *Bam*HI digests after hybridization with the $^{G}\gamma$IVS-II and $\psi\beta 1$ probes, clearly identifying unique fragments in the digests of each of the three $^{G}\gamma^{A}\gamma$-HPFH types. Figure 5 compares the sizes of the fragments in six enzyme digests of DNA from the $^{G}\gamma^{A}\gamma$-HPFH type II homozygote and of a control DNA after hybridization with the $\psi\beta 1$ probe, while data for comparable analysis, but with the $^{G}\gamma$IVS-II probe, are shown in the two autoradiographs in Fig. 6.

All data are summarized in Table II, which lists the sizes (as kb) of the various fragments that were identified. These results show consistent patterns for the homozygote P.B. and the heterozygotes M.I. and K.C., indicating that the $^{G}\gamma^{A}\gamma$ type of HPFH in these persons is the same. Similarly, the results for subjects Q.B. and D.E. were identical to one another but distinctly different from those obtained for P.B., M.I., and K.C. Finally, the data for subject B.C. are unique and different from those of the other five patients, thus identifying a third type of HPFH.

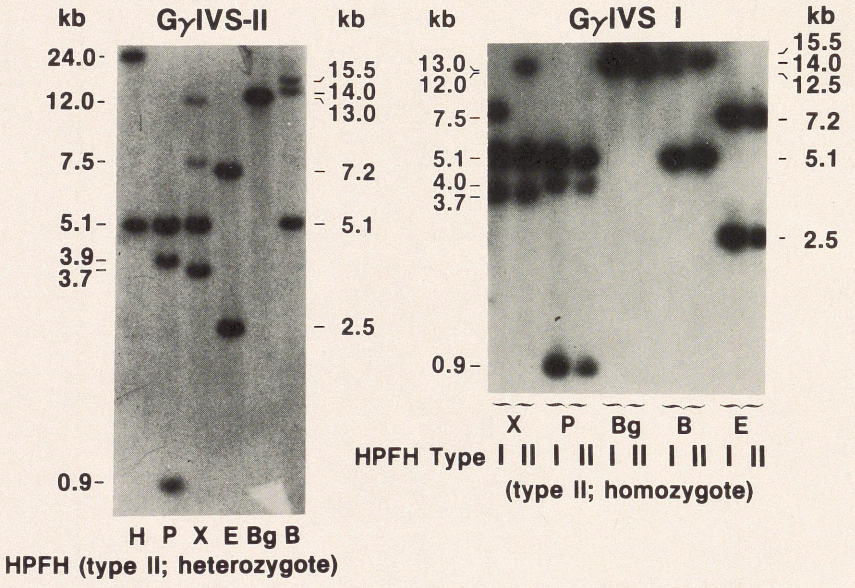

Fig. 6. Analyses of restriction endonuclease fragments of DNA from HPFH heterozygotes and the HPFH homozygote using the $^{G}\gamma$IVS-II probe and the enzymes *Hpa*I (H), *Pst*I (P), *Xba*I (X), *Eco*RI (E), *Bgl*II (Bg), and *Bam*HI (B).

$^{G}\gamma^{A}\gamma$-HPFH Type II. Two abnormal fragments were identified in the hybridizations to the $^{G}\gamma$IVS-II probe. The normally occurring 15.5-kb *Bam*HI fragment was missing and replaced by one of 14 kb. Similarly, the 7.5-kb *Xba*I fragment was replaced by a fragment of 12 kb. This indicates a deletion which extends 5′ beyond the *Bam*HI restriction site located in the δ gene and the *Xba*I site, located about 8 kb at the 5′ side of the δ gene. Only abnormal-sized fragments were seen in the seven different digests after hybridization with the $\gamma\delta$1.6BX ($\psi\beta$1) probe, which indicates that the 5′ end of the deletion extends into the $\psi\beta$1 gene and is located between the *Bgl*II and the *Xba*I restriction sites, which are the 5′ and 3′ ends of the $\psi\beta$1 gene, respectively. As expected, hybridization to the βIVS-II probe did not occur. These data identify our $^{G}\gamma^{A}\gamma$-HPFH type II as being the same as that described by others as Type II (Mears *et al.*, 1978; Bernards and Flavell, 1980; Ottolenghi *et al.*, 1982; Jagadeeswaran *et al.*, 1982).

$^{G}\gamma^{A}\gamma$-HPFH Type I. DNA digested with various enzymes hybridized to the $^{G}\gamma$IVS-II and $\gamma\delta$1.6BX probes, giving fragments with normal sizes only except for the *Bam*HI digest. This digest produced an abnormal 12.5-kb fragment which hybridized to both types of probes. This observation, together with the appearance of only normal fragments in the *Eco*RI and *Bcl*I digests

Table II. Restriction Endonuclease Fragments Identified in Digests of DNA from Six Persons with Different Types of HPFH Using Three Specific Probes

Probe	Enzyme	HPFH type II ($^{G}\gamma$ = 32.8%) Homozygote (P.B.)	HPFH type II ($^{G}\gamma$ = 32.8%) Heterozygote (M.I., K.C.)	HPFH type I ($^{G}\gamma$ = 50.7%) Heterozygote (Q.B., D.E.)	HPFH type III ($^{G}\gamma$ = 69.3%) Heterozygote (B.C.)	Control
$^{G}\gamma$IVS-II	*Eco*RI	7.2; 2.5	7.2; 2.5	7.2; 2.5	—	7.2; 2.5
	*Bam*HI	14.0; 5.1	15.5; 14.0; 5.1	15.5; 12.5; 5.1	15.5; 5.8; 5.2	15.5; 5.1
	*Pst*I	5.1; 3.9; 0.9	5.1; 3.9; 0.9	5.1; 3.9; 0.9	—	5.1; 4.0; 0.9
	*Xba*I	12.0; 5.1; 3.9	12.0; 7.5; 5.1; 3.7	7.5; 5.1; 3.7	11.5; 7.5; 5.1; 3.7	7.5; 5.1; 3.7
	*Hpa*I	24.0; 5.1	24.0; 5.1	—	—	24.0; 5.1
	*Bgl*II	13.0	13.0	13.0	13.0	13.0
γδ1.6BX	*Eco*RI	11.5	11.5; 7.2	7.2	7.2	7.2
	*Bam*HI	14.0	15.5; 14.0	15.5; 12.5	15.5	15.5
	*Pst*I	16.0	—	—	—	3.8
	*Xba*I	12.5	—	—	—	7.5
	*Hpa*I	14.5	14.0	14.0	14.0	14.0
	*Bgl*II	0.9	4.4; 0.9	4.4	4.4	4.4
	*Bcl*I	6.2	7.5; 6.2	7.5	—	7.5
βIVS-II	*Eco*RI	Absent	5.2	—	—	5.2
	*Bam*HI	Absent	8.3	8.3	—	8.3
	*Pst*I	Absent	—	4.4	—	4.4
	*Xba*I	Absent	10.8	—	—	10.8
	*Hpa*I	Absent	—	7.8 (13.6; 7.8)[a]	—	13.6 (7.8; 7.0)[a]
	*Bgl*II	Absent	5.2	—	—	5.2

[a] Polymorphic sites.

when hybridized to the γδ1.6BX probe, places the 5′ end of the deletion between the *Bcl*I restriction site located about 2.5 kb 5′ to the δ gene and the *Hpa*I restriction site located about 1 kb 5′ to the δ gene. Most likely, our type I HPFH is similar to that described by others (Tuan *et al.*, 1980).

$^{G}\gamma^{A}\gamma$-HPFH Type III. This form of HPFH, observed in five Indian families, has some unique features. No abnormal fragments were observed in any of the four digests after hybridization to the γδ1.6BX probe, indicating that the ψβ1 gene is part of the deletion. Of the three digests used in hybridization experiments with the $^{G}\gamma$IVS-II probe, two contained unique fragments different from those seen in digests of the other types of HPFH, while only a 13-kb fragment was observed in the *Bgl*II digest. These results indicate that the 5′ end of the deletion in this type of HPFH is slightly different from that of type II and is probably located at, or in the close vicinity of, the *Bgl*II restriction site which is at the 5′ end of the ψβ1 gene.

To examine the 3′ end of the deletions, DNA from the Black type II

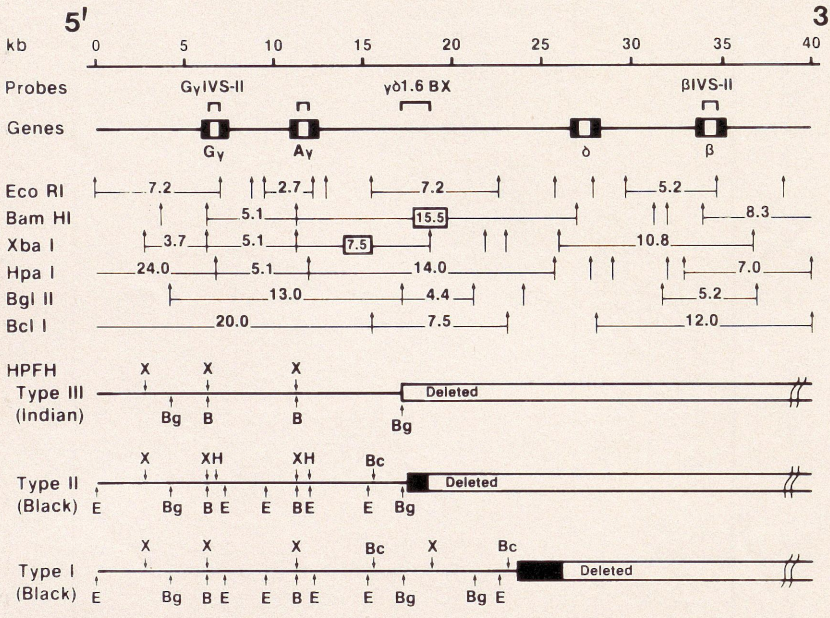

Fig. 7. Restriction enzyme map of the $^{G}\gamma$-$^{A}\gamma$-ψβ1-δ-β-gene cluster in the DNA of a normal person and in that of subjects with the three types of $^{G}\gamma^{A}\gamma$-HPFH. The positions of the probes are indicated, as are the sizes of the fragments that were identified in the DNA digests of the normal control. The *Bam*HI and *Xba*I fragments which hybridize with both the $^{G}\gamma$IVS-II and the γδ1.6BX probes are placed in boxes. The 5′ end points of each type of deletion are indicated as accurately as the data permit; shaded areas indicate uncertainties about the location of the end points. E, *Eco*RI; B, *Bam*HI; X, *Xba*I; H, *Hpa*I; Bg, *Bgl*II; Bc, *Bcl*I.

homozygote and a Black type I heterozygote was digested with the restriction enzymes *Eco*RI, *Bam*HI, and *Bgl*II and hybridized with the pRK28 nick translated probe. The homozygote showed no hybridization at all, while the heterozygote showed only normal-sized fragments which may be assumed to have come from the normal chromosome. Thus, the 3′ end point of the deletion extends beyond a point approximately 17 kb 3′ to the β gene.

The results of the restriction endonuclease analyses are summarized in Fig. 7. The data for the Black $^{G}\gamma^{A}\gamma$-HPFH types I and II are similar to those previously described (Mears *et al.*, 1978; Bernards and Flavell, 1980; Tuan *et al.*, 1982; Mears, 1981; Jagadeeswaran *et al.*, 1982; Ottolenghi *et al.*, 1982). The deletion as characterized includes the δ and β genes, and extends beyond the 3′ side of the β gene for at least 17 kb, but has differing 5′ end points. In type I the end point is located between the *Bcl*I and the *Hpa*I restriction sites, or 1.0–3.5 kb on the 5′ site of the δ chain, and between the *Bgl*II and the *Xba*I restriction sites, or within the $\psi\beta 1$ gene, which is 8–9 kb on the 5′ site of the δ chain for type II. Our data also demonstrate the existence of a third type of $^{G}\gamma^{A}\gamma$-HPFH, an Indian type III which is distinctly different from the Black type II. The data listed in Table II indicate that the deletion includes the entire $\psi\beta 1$ gene with a 5′ end point at or in the close vicinity of the *Bgl*II restriction site, which is at the 5′ site of this pseudogene. Thus, the deletion in type III may extend 0.5–1.0 kb beyond the 5′ end point observed for type II.

The survey data presented in Fig. 1 and the pedigree information in Fig. 2 leave no doubt that the three types of $^{G}\gamma^{A}\gamma$-HPFH are characterized by specific ratios between the $^{G}\gamma$ and the $^{A}\gamma$ chains, while the total quantity of γ chains produced in heterozygotes appears to be the same. It probably is futile to speculate about a possible relationship between the locations of the 5′ breakpoints of the deletions and the $^{G}\gamma$:$^{A}\gamma$ ratios. It is indeed most surprising to observe only a small difference of some 0.5–1.0 kb between two types with the vastly different $^{G}\gamma$ values of 32.3% (type II) and 69.3% (type III). More definitive results describing the sizes of the deletions, including the 3′ end points, in the three types of $^{G}\gamma^{A}\gamma$-HPFH as well as additional data on other types of HPFH will be required.

In vitro chain synthesis analyses of reticulocytes from some HPFH heterozygotes have provided results suggesting an imbalance somewhat comparable to that seen in a mild β-thalassemia (Charache *et al.*, 1976). Such a chain deficiency was also observed among members of one of our families (Fig. 3). However, results on additional families are not consistent with the view that such an imbalance is characteristic for one specific type of $^{G}\gamma^{A}\gamma$-HPFH or that an α-thalassemia-2 heterozygosity (i.e., deletion of one of the four α genes or the $\alpha\alpha^{0}/\alpha\alpha$ genotype) is the cause of this imbalance. This possibility has been mentioned in a previous publication (Headlee *et al.*, 1983) when less extensive data were available.

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