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Prenatal diagnosis of sickle cell anemia by restriction endonuclease analysis: *Hin*dIII polymorphisms in γ-globin genes extend test applicability

(restriction endonuclease site polymorphisms)

JOHN A. PHILLIPS III*, SUSAN R. PANNY*†‡, HAIG H. KAZAZIAN, JR.*, CORINNE D. BOEHM*, ALAN F. SCOTT†‡, AND KIRBY D. SMITH†‡

Departments of *Pediatrics and †Medicine, Johns Hopkins University School of Medicine; and ‡The Howard Hughes Medical Institute, Baltimore, Maryland 21205

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ABSTRACT Polymorphism for a *Hpa* I restriction endonuclease site associated with about 60% of β^S genes in American Blacks allows exact prenatal diagnosis of sickle cell anemia by amniocentesis in 36% of couples at risk. In three families in whom exact diagnosis by *Hpa* I sites was impossible, we found analysis for the presence of polymorphic *Hin*dIII sites in the $^G\gamma$ and $^A\gamma$ intervening sequences would allow an exact prenatal diagnosis of sickle cell status in all three. In one of these families, the presence of an $^A\gamma$ *Hin*dIII site in amniocyte DNA confirmed the diagnosis (sickle cell trait) made by synthetic studies using fetal erythrocytes obtained at fetoscopy. Studies of other Black families and individuals provide evidence for linkage disequilibrium in the $^G\gamma$-$^A\gamma$-δ-β gene complex involving the four sites, $^G\gamma$ *Hin*dIII, $^A\gamma$ *Hin*dIII, β^S, and *Hpa* I, which span 33 kilobases (kb). Ten of 14 chromosomes bearing a β^S gene in a 7.6-kb *Hpa* I fragment contained a $^G\gamma$ but not an $^A\gamma$ *Hin*dIII site, whereas 16 of 16 chromosomes bearing a β^S gene in a 13-kb *Hpa* I fragment lacked both the $^G\gamma$ and $^A\gamma$ *Hin*dIII sites. Two-thirds of β^A-bearing chromosomes lacked both $^G\gamma$ and $^A\gamma$ sites, whereas one-third contained either the $^G\gamma$ or both $^G\gamma$ and $^A\gamma$ sites. These data demonstrate that combined analysis of both *Hpa* I and *Hin*dIII polymorphisms and verification of their linkage phase should increase the fraction of couples for whom amniocentesis can provide an exact diagnosis of sickle cell status from 36% to greater than 80%.

Kan and Dozy (1) reported polymorphism for a *Hpa* I restriction endonuclease site located about 5000 base pairs [5 kilobases (kb)] 3′ to the β globin gene in American Blacks. These workers found that 87% of β^S-containing chromosomes lacked this *Hpa* I restriction endonuclease site, and that, when these DNAs were digested with *Hpa* I, the β^S gene was present in a 13-kb fragment. In contrast, 97% of β^A-bearing chromosomes had the β^A gene in either a 7.6-kb or, rarely, a 7.0-kb DNA fragment after *Hpa* I digestion. These findings allow prenatal diagnosis of sickle cell anemia by amniocentesis in certain at-risk couples (2). Previously, sickle cell anemia had been diagnosed prenatally by globin or hemoglobin synthesis studies on fetal erythrocytes obtained by fetoscopy or placental aspiration (3, 4). However, both fetoscopy and placental aspiration carry a mortality risk to the fetus (5–9%) (4, 5) that is perhaps 10 times greater than that of amniocentesis (<0.5%) (6, 7). For this reason, restriction endonuclease analysis of DNA from fetal amniocytes, when applicable, should become the preferred method for prenatal detection of sickle cell anemia (2, 8).

However, in American Blacks residing in the eastern United States, about 60% of β^S chromosomes lack the *Hpa* I site, while 8% of β^A chromosomes also lack this site (9, §). Thus, exact prenatal diagnosis by this linked polymorphism can be accomplished in only 36% of pregnancies at risk (AS × AS), and sickle cell anemia can be excluded in another 24% of at-risk pregnancies (about half of pregnancies in which only one parent has the β^S gene in a 13-kb fragment). Because of this limitation, investigators have sought other polymorphisms in the $^G\gamma$-$^A\gamma$-δ-β^S gene complex that could be applied to this diagnosis.

Recently, polymorphisms of *Hin*dIII sites in the large intervening sequences of both $^G\gamma$ and $^A\gamma$ genes have been reported (10, 11). These *Hin*dIII sites were present in 23% of $^A\gamma$ genes and in roughly 40% of $^G\gamma$ genes in 50 Caucasians (10). We have now determined the presence or absence of these *Hin*dIII sites in DNA from families undergoing prenatal diagnosis of sickle cell anemia. Among eight couples at risk, exact fetal genotype, *vis à vis* the β globin genes ($\beta^A\beta^A$, $\beta^A\beta^S$, or $\beta^S\beta^S$), could be predicted in only five by the *Hpa* I polymorphism. However, when the *Hin*dIII and *Hpa* I polymorphism data were combined in analysis of the remaining three families, exact prenatal diagnoses of sickle cell status became possible in all eight families.

These data led us to study the association of the *Hin*dIII and *Hpa* I polymorphisms with the β^S mutation in other Black families and individuals. Two common $^G\gamma$-$^A\gamma$-δ-β^S gene complexes were found, suggesting linkage disequilibrium of a large portion of this gene complex in Blacks.

METHODS

A probe for β globin gene sequences was made from an *Mbo* II plus *Hin*dIII fragment (1.2 kb) of the recombinant plasmid JW 102 (12, 13). A 1.1-kb fragment containing γ sequences was obtained by digestion of the recombinant plasmid JW 151 with *Taq* I (12). The β- and γ-containing fragments were radiolabeled with [^{32}P]dCTP and [^{32}P]dTTP by the nick translation function of *Escherichia coli* DNA polymerase I (14). Ten to 20 ml of whole blood was obtained from all subjects, and DNA was isolated from leukocytes (15). Ten micrograms of DNA was digested with either *Hpa* I or *Hin*dIII under conditions recommended by the commercial supplier. The DNA was then subjected to electrophoresis in 0.8% agarose gels, transferred to nitrocellulose filters, and hybridized with either the β globin probe (*Hpa* I digests) or the γ globin probe (*Hin*dIII digests). DNA transfer, prehybridization of filters, hybridization with

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Abbreviations: kb, kilobase(s); AA, hemoglobin A (normal); AS, hemoglobins A and S (sickle cell trait); SS, hemoglobin S (sickle cell disease).

§ Panny, S. R., Scott, A. F., Phillips, J. A., Smith, K. D., Kazazian, H. H., Charache, S. & Talbot, C. C. (1979) *Program and Abstracts of the American Society of Human Genetics 30th Annual Meeting*, Abstr. 168, p. 58A.

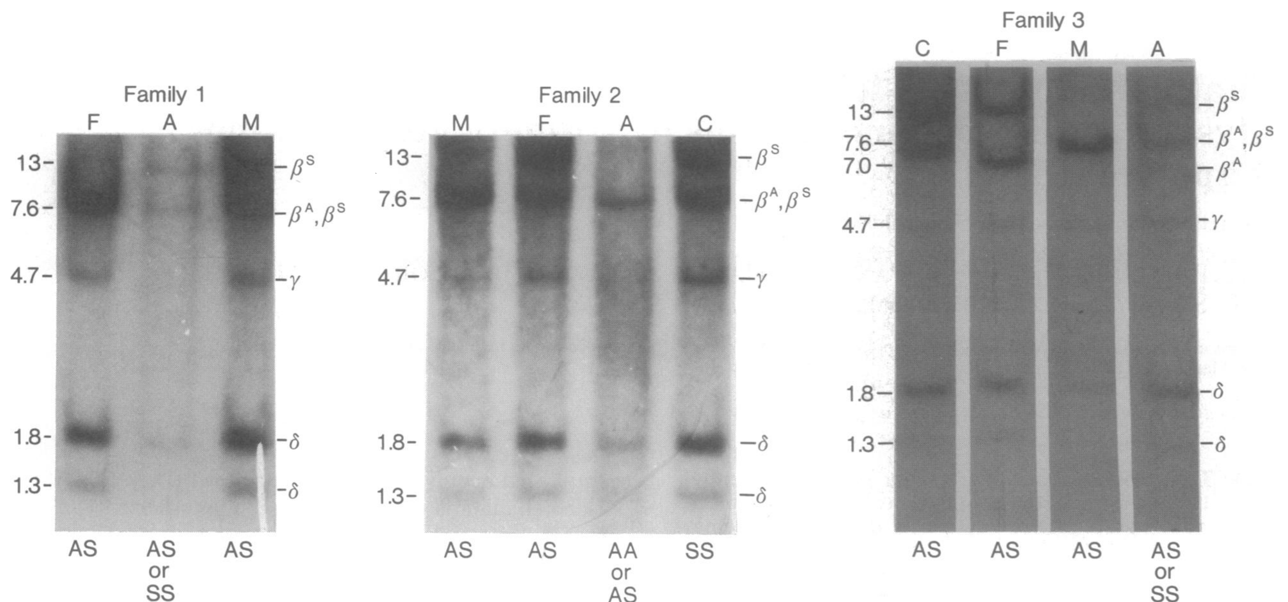

FIG. 1. Autoradiogram patterns of DNA from families 1–3 after restriction endonuclease digestion with *Hpa* I and hybridization with a ^{32}P-labeled β probe. Numbers on the left of each autoradiogram refer to sizes in kb of fragments containing the globin gene sequences denoted on the right. Lanes: F, father; M, mother; C, child; A, amniocytes. The hemoglobin phenotype is given at the bottom of each lane.

probe, washing of filters, and autoradiography were carried out as described (13, 16, 17). Experiments involving recombinant DNA were conducted at P2–EK2 containment in accordance with the National Institutes of Health Guidelines.

RESULTS

Analysis of β Genes by Linked *Hpa* I Polymorphism Alone. In five of eight couples requesting prenatal diagnosis of sickle cell anemia, each member had his/her β^S gene associated with a 13-kb *Hpa* I fragment, and an exact diagnosis of sickle cell status in each fetus could be made. Analysis of one or more previous children was used to confirm linkage of the β^S gene to the 13-kb fragment in these couples. In the other three couples, exact diagnosis was not possible because of the absence of the *Hpa* I marker in one member of each couple. In couples 1 and 2 (Fig. 1), one member had a 13-kb/7.6-kb β gene pattern whereas the other member with sickle cell trait had a 7.6-kb/7.6-kb pattern. Each of these couples had an affected child whose DNA contained a 13-kb/7.6-kb pattern, proving that the 13-kb fragment contained a β^S gene. In couple 3, the AS father had a 13-kb/7.0-kb pattern and the AS mother had a 7.6-kb/7.6-kb pattern. Their AS child had a 13-kb/7.6-kb pattern, again indicating that the 13-kb fragment contained the β^S gene (Fig. 1).¶ Thus, in these three couples, an exact diagnosis of sickle cell status could not be obtained using the *Hpa* I polymorphism alone.

In the pregnancy of couple 2, DNA from amniotic fluid cells showed a 7.6-kb/7.6-kb pattern consistent with an AA or AS phenotype, but inconsistent with an SS phenotype. In two pregnancies (couples 1 and 3), amniotic fluid cell DNA had a

¶ By pooling other data (1, 9) with those of Panny *et al.*§, we obtained a likelihood estimate of 120:1 that the father's 13-kb and 7.0-kb fragments contained β^S and β^A genes, respectively. This estimate is less than the 8% probability that a 13-kb fragment contains a β^A gene due to the rarity of 7.0-kb β^S-containing fragments.

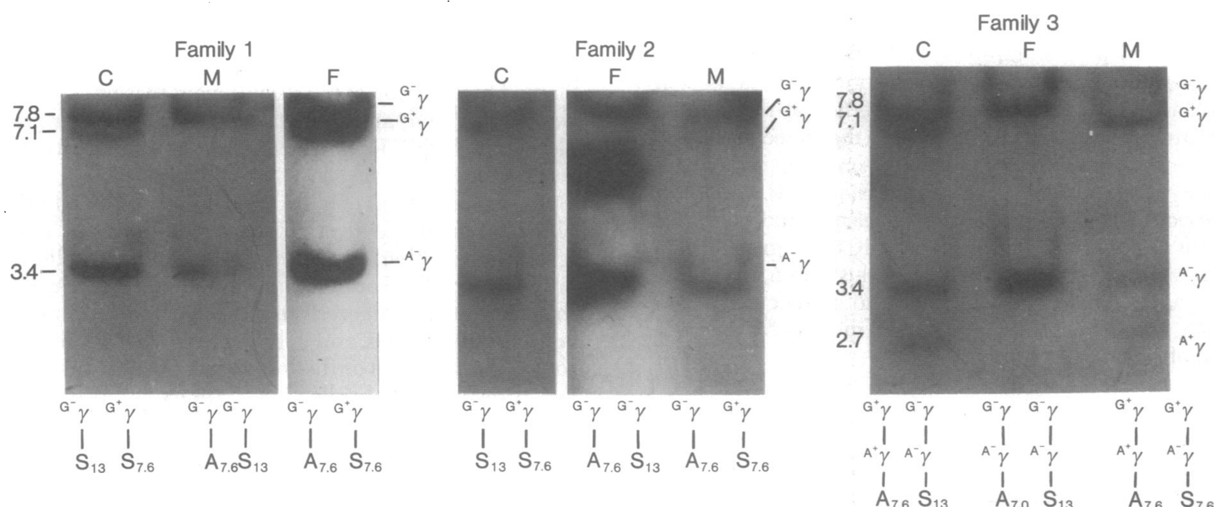

FIG. 2. Autoradiogram patterns of DNA from families 1–3 after restriction endonuclease digestion with *Hin*dIII and hybridization with ^{32}P-labeled γ probe. Linkages of $^G\gamma$ and $^A\gamma$ *Hin*dIII sites, the β^S gene, and the *Hpa* I site were deduced from a combination of these data and those of Fig. 1 and are shown below each lane. The 0.7-kb fragments found in association with $^G\gamma$ and $^A\gamma$ *Hin*dIII sites were seen on other autoradiograms, but were not seen on the autoradiograms presented because these fragments were run off the gels during electrophoresis.

13-kb/7.6-kb pattern, indicating that the fetus had a 50% risk of sickle cell anemia and a 50% chance of sickle cell trait. One of these couples elected to terminate pregnancy without further studies, while the other (couple 3) chose fetoscopy and hemoglobin synthetic studies of fetal blood (see below).

Analysis of β Genes by a Combination of *Hpa* I and *Hin*dIII Polymorphisms. Genomic DNAs of family members were also analyzed for polymorphisms in γ genes after digestion with *Hin*dIII and hybridization with a ^{32}P-labeled γ probe (Fig. 2). Jeffreys (10) and Tuan *et al.* (11) found that the polymorphic *Hin*dIII site in the large intervening sequence of the $^{G}\gamma$ gene reduces the size of the normal $^{G}\gamma$-containing fragment by 0.7 kb, whereas a similar polymorphic site in the large intervening sequence of the $^{A}\gamma$ gene produces fragments of 2.7 and 0.7 kb instead of the more frequent 3.4-kb fragment containing the $^{A}\gamma$ gene.

Analysis of our families for both *Hin*dIII and *Hpa* I polymorphisms indicates that one member of each couple has a β^{S} chromosome that contains both the *Hpa* I and $^{G}\gamma$ polymorphic

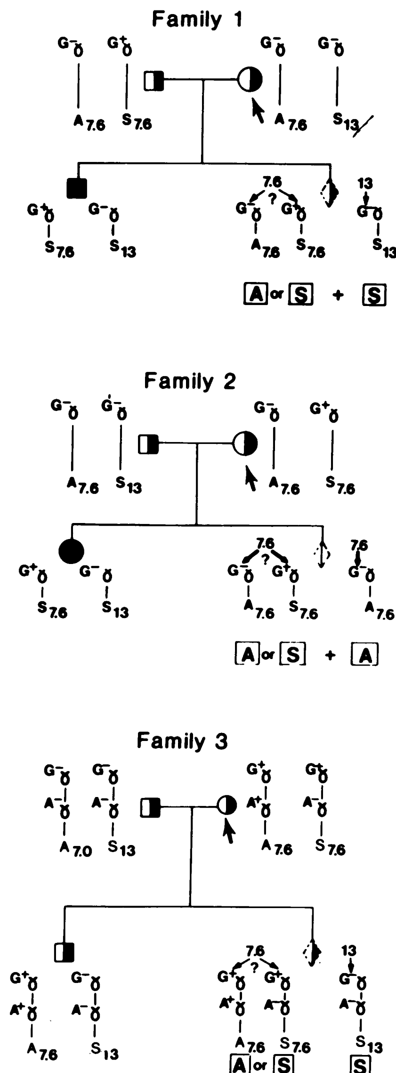

FIG. 3. Pedigrees of families 1–3. Linkage of $^{G}\gamma$ and $^{A}\gamma$ *Hin*dIII sites, β genes, and *Hpa* I sites are presented for each individual. Possible genotypes are given for each fetus. In family 3, analysis of amniocyte DNA showed that the mother donated her $^{G+}\gamma$-$^{A+}\gamma$-$\beta^{A}_{7.6}$ chromosome to her fetus. Arrows indicate consultands. ◨ and ◑, AS; ■ and ●, SS. Sizes of *Hpa* I fragments containing β^{A} or β^{S} genes are 7.6, 7.0, or 13 kb, as indicated. $^{G+}\gamma$ and $^{A+}\gamma$, *Hin*dIII polymorphism present; $^{G-}\gamma$ and $^{A-}\gamma$, *Hin*dIII polymorphism absent.

restriction sites, but lacks the $^{A}\gamma$ site (Fig. 3). We designate this chromosome $^{G+}\gamma$-$^{A-}\gamma$-$\beta^{S}_{7.6}$. On the other hand, the other member of each couple has a β^{S} chromosome that lacks the *Hpa* I, $^{G}\gamma$, and $^{A}\gamma$ polymorphic restriction sites. We designate this chromosome $^{G-}\gamma$-$^{A-}\gamma$-β^{S}_{13}. Linkage of the four sites was established by examining DNA patterns of offspring of the couples. In couples 1 and 2, the member who had a 7.6-kb/7.6-kb *Hpa* I pattern was $^{G+}\gamma/^{G-}\gamma$ with respect to the *Hin*dIII polymorphism (7.1-kb and 7.8-kb fragments, respectively). The SS children of these individuals, who received the β^{S} chromosome with the 7.6-kb *Hpa* I pattern, have simultaneously acquired the linked $^{G}\gamma$ *Hin*dIII site ($^{G+}\gamma$). The lack of the $^{G}\gamma$ *Hin*dIII site in the other member of couples 1 and 2 allows assignment of the linkage of $^{G+}\gamma$ to $\beta^{S}_{7.6}$.

In contrast, diagnosis in couple 3 relies on the presence of the $^{A}\gamma$ *Hin*dIII site in the mother (Fig. 2). She had hemoglobins A and S, both β genes in 7.6-kb fragments after *Hpa* I digestion of DNA, both $^{G}\gamma$ genes with the *Hin*dIII polymorphic restriction sites (7.1-kb fragment), and heterozygosity for the $^{A}\gamma$ *Hin*dIII site (3.4-kb and 2.7-kb fragments). The linkage phase of her β genes, with respect to the *Hpa* I and *Hin*dIII sites, could be established because her mate lacked both $^{G}\gamma$ and $^{A}\gamma$ *Hin*dIII polymorphic sites and his β^{S} gene was associated with a 13-kb *Hpa* I fragment (Fig. 3). Inspection of the *Hpa* I and *Hin*dIII patterns of the AS child (7.6-kb/13-kb *Hpa* I pattern) revealed that his β^{A} chromosome (inherited from his mother) has markers in the following linkage phase: $^{G+}\gamma$-$^{A+}\gamma$-$\beta^{A}_{7.6}$. The mother's β^{S} chromosome, by exclusion, must then be $^{G+}\gamma$-$^{A-}\gamma$-$\beta^{S}_{7.6}$. Because the father's β^{S}-bearing chromosome is $^{G-}\gamma$-$^{A-}\gamma$-β^{S}_{13}, one can diagnose those offspring who have a 13-kb/7.6-kb *Hpa* I pattern by the presence or absence of the $^{A}\gamma$ *Hin*dIII polymorphic restriction site (Fig. 3). Offspring that are $^{A+}\gamma/^{A-}\gamma$ must have received the β^{A} gene from the mother, whereas $^{A-}\gamma/^{A-}\gamma$ offspring have acquired the mother's β^{S} gene.

While fetal amniocytes were being cultured to complete the DNA studies, the mother in family 3 underwent fetoscopy and a diagnosis was made by globin synthetic studies of fetal erythrocytes. We purposely did not learn the nature of that diagnosis until after our DNA studies were completed. After cell culture, DNA of fetal amniocytes was digested and a $^{G+}\gamma$-$^{A+}\gamma/^{G-}\gamma$-$^{A-}\gamma$ *Hin*dIII pattern [identical to the previous AS child (Fig. 2)] was found. Thus, we independently diagnosed sickle cell trait in this fetus, confirming the result obtained by using synthetic studies in fetal erythrocytes.

Linkage Disequilibrium in the $^{G}\gamma$-$^{A}\gamma$-δ-β^{S} Gene Complex. Data on these families led us to study further the association between *Hin*dIII and *Hpa* I sites and β^{S} genes in other Black families and individuals (Table 1). Sixteen of 16 chromosomes bearing a β^{S} gene in a 13-kb *Hpa* I fragment lacked both $^{G}\gamma$ and $^{A}\gamma$ *Hin*dIII sites ($^{G-}\gamma$-$^{A-}\gamma$-β^{S}_{13}), whereas 10 of 14 chromosomes bearing a β^{S} gene in a 7.6-kb *Hpa* I fragment contained a $^{G}\gamma$, but not an $^{A}\gamma$, *Hin*dIII site ($^{G+}\gamma$-$^{A-}\gamma$-$\beta^{S}_{7.6}$). Chromosomes bearing a β^{A} gene in a 7.6-kb *Hpa* I fragment fell into three groups: 67% lacked both $^{G}\gamma$ and $^{A}\gamma$ *Hin*dIII sites, 21% contained both $^{G}\gamma$ and $^{A}\gamma$ sites, and 12% contained the $^{G}\gamma$ site only. The linkage phase of all but the eight $\beta^{A}_{7.6}$ chromosomes in parentheses in Table 1 was proven by family studies or homozygosity.

DISCUSSION

In the present study, we have found two common $^{G}\gamma$-$^{A}\gamma$-δ-β^{S} gene complexes in American Blacks, $^{G-}\gamma$-$^{A-}\gamma$-β^{S}_{13} and $^{G+}\gamma$-$^{A-}\gamma$-$\beta^{S}_{7.6}$. These findings provide a basis for extending the application of restriction endonuclease techniques to the prenatal diagnosis of sickle cell anemia by amniocentesis. They also

Table 1. Associations of *Hin*dIII sites in γ genes with β^A- and β^S-bearing chromosomes in American Blacks

Hpa I genotype	*Hin*dIII genotype			Total
	$^{G-}\gamma^{A-}\gamma$	$^{G+}\gamma^{A-}\gamma$	$^{G+}\gamma^{A+}\gamma$	
$\beta^A{}_{7.6}$	28	6	6	40
	(4)		(4)	(8)
$\beta^S{}_{7.6}$	3	10	1	14
$\beta^S{}_{13}$	16	0	0	16

A $\beta^S{}_{13}$ chromosome has the β^S gene in a 13-kb *Hpa* I fragment, whereas a $\beta^S{}_{7.6}$ gene is in a 7.6-kb *Hpa* I fragment. Numbers in parentheses refer to chromosomes found in four doubly heterozygous individuals in whom linkage studies were not done. The genotypes $^{G-}\gamma^{A-}\gamma$ and $^{G+}\gamma^{A+}\gamma$ were assigned because the $^{G-}\gamma^{A+}\gamma$ genotype has not been found by Jeffreys (10) or us. χ^2 analysis of the data indicates that: (*i*) *Hin*dIII genotypes for $\beta^A{}_{7.6}$ chromosomes have a different distribution from those of $\beta^S{}_{7.6}$ chromosomes ($\chi^2 = 15.9, P < 0.001$); (*ii*) *Hin*dIII genotypes for $\beta^S{}_{7.6}$ chromosomes have a different distribution from those of $\beta^S{}_{13}$ chromosomes ($\chi^2 = 19.8, P < 0.001$); and (*iii*) all three distributions differ from each other ($\chi^2 = 28.9, P < 0.001$).

suggest linkage disequilibrium of a large portion of the $^G\gamma$-$^A\gamma$-δ-β^S gene complex in Blacks.

For prenatal diagnosis of sickle cell anemia, we suggest the following routine in the work-up of consulting couples: (*i*) Determine the *Hpa* I pattern in the DNA of each member of the couple and one or more informative children. (*ii*) If *Hpa* I patterns in the couple do not allow exact diagnosis in the fetus, determine the *Hin*dIII pattern with a γ probe in the same individuals. (*iii*) If both *Hpa* I and *Hin*dIII patterns are necessary for diagnosis, culture amniotic fluid cells for analysis. Cultured cells may be required because the yield of DNA (usually less than 10 μg) from cells in 15–20 ml of amniotic fluid may be insufficient for multiple analyses. The degree of linkage disequilibrium observed in our sample (Table 1) suggests that a combination of *Hin*dIII and *Hpa* I polymorphism data and verification of linkage phase should increase the fraction of couples for whom amniocentesis can provide an exact diagnosis of sickle cell status from 36% to greater than 80%.

Other restriction endonuclease polymorphisms in the $^G\gamma$-$^A\gamma$-δ-β gene complex will probably be found, and some of these may be useful in linkage analysis of β^S, β^C, and $\beta^{thalassemia}$ genes. However, diagnosis by linkage analysis of these markers may soon be superceded by direct detection of alterations in restriction sites due to the β^S or $\beta^{thalassemia}$ mutations themselves.

The two polymorphic restriction endonuclease sites, $^G\gamma$ *Hin*dIII and *Hpa* I, associated with the β^S gene are separated by 33 kb (18). Thus, linkage disequilibrium may occur throughout the β^S gene complex. Whether such disequilibrium arose through recurrent mutation to β^S or through recombination with a single β^S gene is uncertain. Clues to the origin and evolution of the $^G\gamma$-$^A\gamma$-δ-β^S gene complex should come from detailed analysis of this gene complex in the population described here as well as in Saudi Arabians with β^S genes in 7.6-kb *Hpa* I fragments and American Blacks with β^A genes in 13-kb *Hpa* I fragments.

Note Added in Proof. Since submission of this manuscript another four AS × AS couples have been studied. The exact fetal genotype CAA, AS, or SS) could be determined in two couples by the presence of the polymorphic *Hpa* I restriction site and in one additional couple by a combination of data on the polymorphic *Hin*dIII and *Hpa* I sites. Thus, in 11 of 12 couples (7 by *Hpa* I and 4 by *Hin*dIII and *Hpa* I data) exact fetal diagnosis was possible.

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