Heterogeneity of DNA Fragments
Associated with the Sickle-Globin Gene

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A B S T R A C T We have examined the genetic poly-
morphism previously reported to be associated with the
sickle-cell (β^s) gene. The polymorphism involves an
alteration of the DNA sequence 3′ to the β-globin gene
as detected with the restriction endonuclease, Hpa I. In
normal individuals, the β-globin gene is contained
within a DNA fragment of 7.6 kilobases (kb), whereas
87% of individuals with sickle-cell anemia have been
reported to have the β^s-gene associated with a 13.0-kb
Hpa I fragment. We have studied this polymorphism
in 31 New York Black individuals homozygous for
sickle-cell anemia to ascertain its genetic and bio-
chemical significance and to evaluate its potential use
in the prenatal diagnosis of sickle-cell disease. Our
results show only a 58% association of the β^s-gene and
the 13.0-kb Hpa I fragment, as well as the presence
of additional variants involving the Hpa I site. In
addition, the 13.0-kb fragment is also found associated
with the β^c- and β^A-genes. Thus, the Hpa I poly-
morphism probably represents a change in DNA not
specifically associated with the β^s-gene, and appears
to antedate the β^s- and β^c-mutations.

INTRODUCTION

Until recently, genetic polymorphisms have been
studied by analyzing and defining precise structural
differences in normal and variant gene products by
serological and biochemical methods. Newer techniques,
including the use of restriction endonucleases, now
permit an analysis of such polymorphisms at the gene
level by detecting changes in DNA sequence
organization (1). Restriction endonuclease mapping
with the Southern "blotting" technique has provided
detailed information on the organization of the human
δ- and β-globin structural genes (2–5). This procedure
involves the cleavage of cellular DNA with restriction

enzymes, separation of the DNA fragments by size with
agarose gel electrophoresis, transfer of the DNA to
nitrocellulose filters, and subsequent hybridization to
specific radioactive globin complementary DNA
(cDNA)[1] probes.

With restriction endonuclease mapping, Kan and
Dozy (1) recently reported a polymorphism in the DNA
sequence ≅5 kilobases (kb) from the 3′ end of the
β-globin gene in individuals with the sickle-cell (β^s)
gene. In normal individuals, Hpa I generates one
β-globin fragment, 7.6 kb in size. In association with
87% of β^s-genes, there is a change in the sickle DNA
sequence at a site which is cleaved by Hpa I that
results in a 13.0-kb fragment.

To determine the potential usefulness of this finding
in the prenatal diagnosis of sickle-cell anemia and
its significance in association with the sickle gene, we
have examined this region of the DNA in 70 individuals
of African and Caucasian origin. We find that the
frequency with which the 13.0-kb fragment is associated
with the β^s-gene is considerably less than previously
reported. Approximately 42% of the time, the β^s-gene
is present with a normal 7.6-kb Hpa I fragment, which
reduces its use as a unique genetic marker for the
β^s-gene in prenatal diagnosis. In addition, two other
Hpa I fragments, 7.0 kb and 5.6 kb, are detected with
the β^s-gene, which suggests significant heterogeneity
of the DNA sequence at the Hpa I site. The 13.0-kb
fragment is also found in Black individuals with the
β^c- and β^A-globin genes, which suggests that it is most
likely a result of a change in DNA structure which
preceded the β^c and β^s-mutations.

METHODS

High molecular weight DNA was isolated from peripheral
blood leukocytes and cultured lymphocyte cell lines (6). 20 μg
of cellular DNA was digested in a 150-μl reaction mixture

TABLE I
Hpa I β-Globin DNA Fragments

	7.6/7.6	13.0/7.6	13.0/13.0	Total
Black				
SS*	5	14	10	29
AA	11	2	0	13
AS	6	3	1	10
AC	0	1	0	1
SC	0	3‡	2	5
CC	0	1	0	1
S-β-Thal.	0	3	0	3
Caucasian				
AA	2	0	0	2
β-Thal.	3	0	0	3
HbH	1	0	0	1

β-Thal., β-Thalassemia.

* One SS individual had 13.0- and 5.6-kb fragments, and one had 13.0- and 7.0-fragments.

‡ One individual was an SC-Harlem variant.

for 3 h at 37°C with 5 U of Hpa I (New England Biolabs, Beverly, Mass.) in a buffer that contained 10 mM Tris-HCl (pH 7.4), 10 mM MgCl₂, 20 mM KCl, 1 mM dithiothreitol, and 100 μg/ml bovine serum albumin. The DNA was reisolated, and fragments were separated on 0.9% vertical agarose gels in a buffer (pH 7.9) consisting of 40 mM Tris-acetate, 20 mM Na acetate, 20 mM EDTA, and 0.5 μg/ml ethidium bromide. Gels were electrophoresed at 25–35 V for 18 h at room temperature with the buffer recirculating. The gels were soaked in 1 M KOH for 0.5 h, neutralized in 1 M Tris-HCl (pH 7.0) for 1 h, and equilibrated with 6 × SSC (1 × SSC = 0.15 M NaCl plus 15 mM trisodium citrate, pH 6.8) for 0.5 h. Separated DNA fragments were then transferred from the agarose gels to nitrocellulose filters (12 × 3 cm) over 24 h. After transfer, the filters were rinsed in 2 × SSC and dried under vacuum at 80°C for 4 h. Pure β-globin cDNA was obtained with plasmid JW102 (kindly supplied by Dr. John Wilson), a PMB9 plasmid containing a double-stranded β-cDNA insert (7). Double-stranded β-cDNA was isolated from PMB9 plasmid DNA after restriction with Hha I and purification of the 1.6-kb fragment by neutral sucrose gradient centrifugation. The cDNA probe was nick-translated, as described, to a 2–4 × 10⁸ cpm/μg sp act (8). The hydbridization, washing of filters, and autoradiography were performed as described (4, 5). A Cot value of 0.001 was attained after hybridization at 68°C for 24 h (4, 5).

RESULTS

64 Black individuals were studied including 31 with sickle-cell disease (SS, $\beta^s\beta^s$), 10 with sickle-cell trait (AS, $\beta^A\beta^s$), 13 with normal hemoglobin (AA, $\beta^A\beta^A$), four with SC ($\beta^s\beta^c$), one with $\beta^s\beta^c$-Harlem, one with CC ($\beta^c\beta^c$), one with AC ($\beta^A\beta^c$), and three β^s-β-thalassemia heterozygotes. Six Caucasian DNA were also analyzed. The genotypes of the SS homozygotes were established by measuring the biosynthesis of α- and β-globin in peripheral blood reticulocytes (9). The β^s:α ratio

was ≅1.0 in all individuals with SS studied. 10 of the 31 SS homozygotes showed only the 13.0-kb variant indicating that the Hpa I sites 3′ to both β^s-genes had been altered (Table I, Fig. 1). 14 individuals had both 13.0-kb and 7.6-kb bands, and five had only the 7.6-kb fragments (Table I). Our data indicate that the frequency of the 13.0-kb variant in association with the sickle-cell gene is ≅58% (Table I). In addition, 13.0/7.0 and 13.0/5.6 sickle-cell variants were detected (Fig. 2).

Several families were studied to confirm the genetic basis of this polymorphism and evaluate its usefulness as an antenatal diagnostic tool. The Hpa I globin gene fragments are inherited in a simple Mendelian fashion in six families studied. We found one family in which both AS parents were 7.6/13.0, and in which the Hpa I polymorphism could be used for a definitive prenatal diagnosis. In another family, both parents with AS had only 7.6-kb fragments, thus preventing a distinction between the β^s- and β^A-genes. In four of the six families studied, one AS parent was 13.0/7.6, and the other was 7.6/7.6 (Fig. 3).

We also examined DNA from normal individuals, AS heterozygotes, and patients with other hemoglobin variants. In these experiments, we detected the 13.0-kb variant with the β^c-gene in three of six opportunities for analysis (Table I). The β^c-gene codes for a single amino acid change at position 6 (glu → lys) of the normal β^A-globin chain. One individual with only hemoglobin C (CC) had a 13.0- and 7.6-kb fragment, whereas two individuals doubly heterozygous for sickle and C hemoglobin (SC) had only the 13.0-kb variant (Fig. 4). One subject heterozygous for the β^c- and β^A-genes (AC) had a 13.0- and 7.6-kb fragment (Table I, Fig. 4). We have also found the 13.0-kb Hpa I fragment in association with the β^A-gene in the Black population studied (Table I).
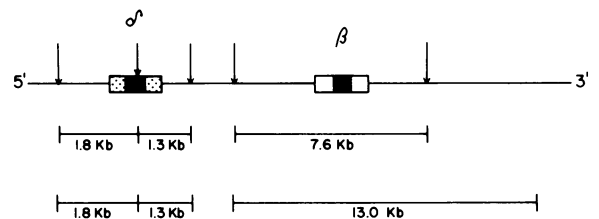

FIGURE 1 Restriction map of Hpa I sites within and surrounding the δ- and β-globin genes. These sites, originally described by Kan and Dozy (1), were confirmed by digests of normal, sickle cell, and Lepore DNA with Hpa I and Eco RI, alone and in combination. Hpa I generates one fragment, 7.6 kb in size, that contains the entire β-globin gene. In some sickle-cell individuals, variants other than 7.6 kb are found. Hpa I cleaves the intervening sequence of the δ-globin gene to yield two fragments: 1.8 kb containing the 5′ end of the δ-gene and most of the δ-intervening sequence and 1.3 kb comprising the 3′ end of the δ-gene.
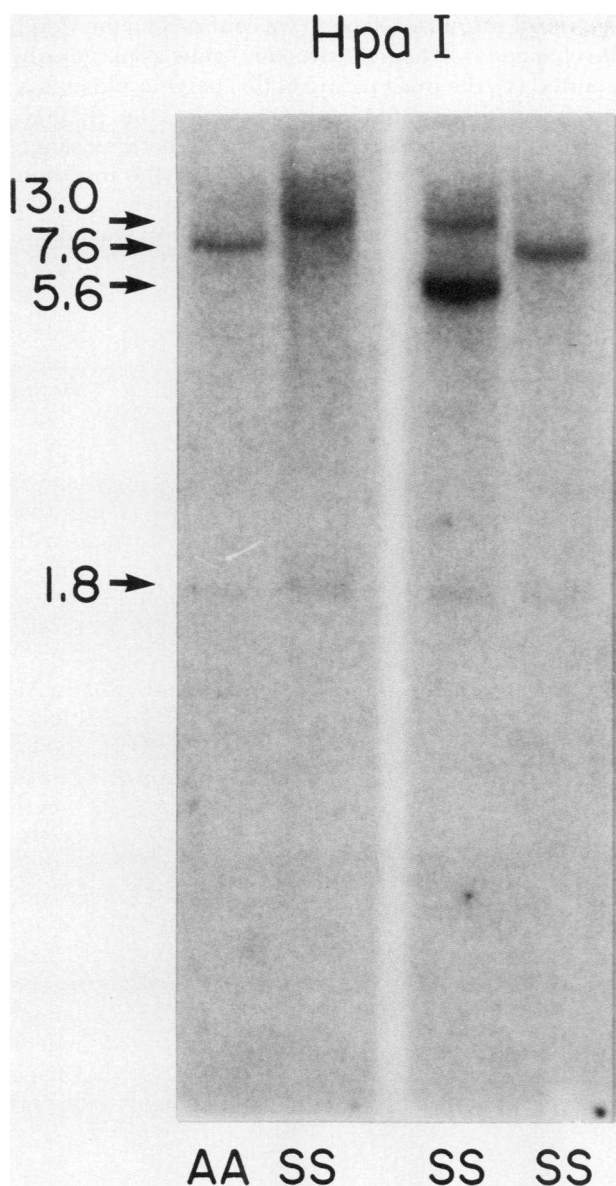
FIGURE 2 Hpa I restriction fragments containing β- and δ-globin sequences from homozygotes with hemoglobin A (AA) or sickle hemoglobin (SS). Sizes on the left are in kilobases of DNA. 20 μg of each DNA were electrophoresed in a 0.9% neutral agarose gel, transferred to nitrocellulose filters, and hybridized to ³²P-labeled β-globin cDNA from plasmid JW102. Globin fragment sizes were estimated by comparison with a Hind III digest of phage λ-DNA run in a parallel channel. Column 1 shows DNA from an AA individual with only a 7.6-kb β-band. Columns 2, 3, and 4 show DNA from SS individuals. Column 2, only the 13.0-kb β-fragment; Column 3, 13.0-kb and 5.6-kb β-fragments; and Column 4, only the 7.6-kb band. The 1.3-kb δ-fragment only weakly hybridizes to the probe and may be detected in long exposures (>7 d) of film. The weak hybridization may reflect poor homology beteen the 3′ end of the δ-gene and the β-cDNA probe.

We have detected other polymorphisms in patients with sickle-cell anemia. One individual had only 13.0- and 7.0-kb Hpa I fragments. Kan and Dozy (1) reported the presence of a 7.0-kb variant with the βᴬ-gene. Our results indicate that this variant is also found with the βˢ-gene, although the 7.0-kb fragments seen in these two cases may not be identical. Another SS patient showed two Hpa I β-globin fragments (13.0 and 5.6 kb) (Fig. 2). The 5.6-kb band hybridized with consistently greater intensity than other β-globin fragments in several separate experiments. This is not a result of better transfer because of its small size since other comparably sized fragments are not nearly as intense. In this case, the 5.6-kb fragment may either be associated with the βˢ-gene, or the patient may be homozygous
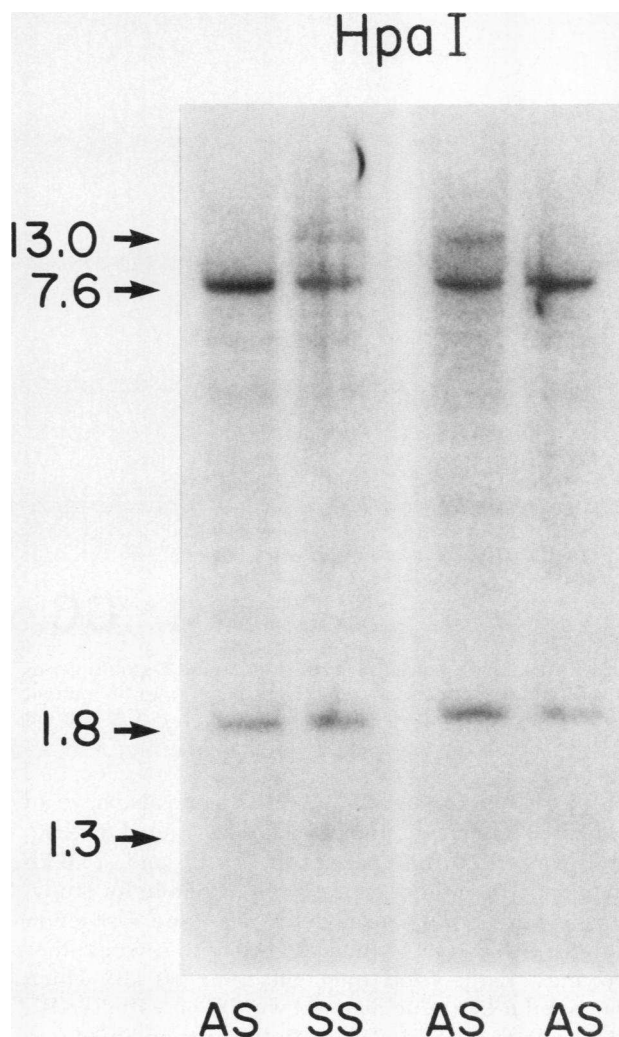

FIGURE 3 Hpa I restriction pattern of β- and δ-globin genes in a family. The procedure is described in Methods and Fig. 2. From left to right: AS son (7.6/7.6); SS daughter (13.0/7.6); AS father (13.0/7.6); and AS mother (7.6/7.6).
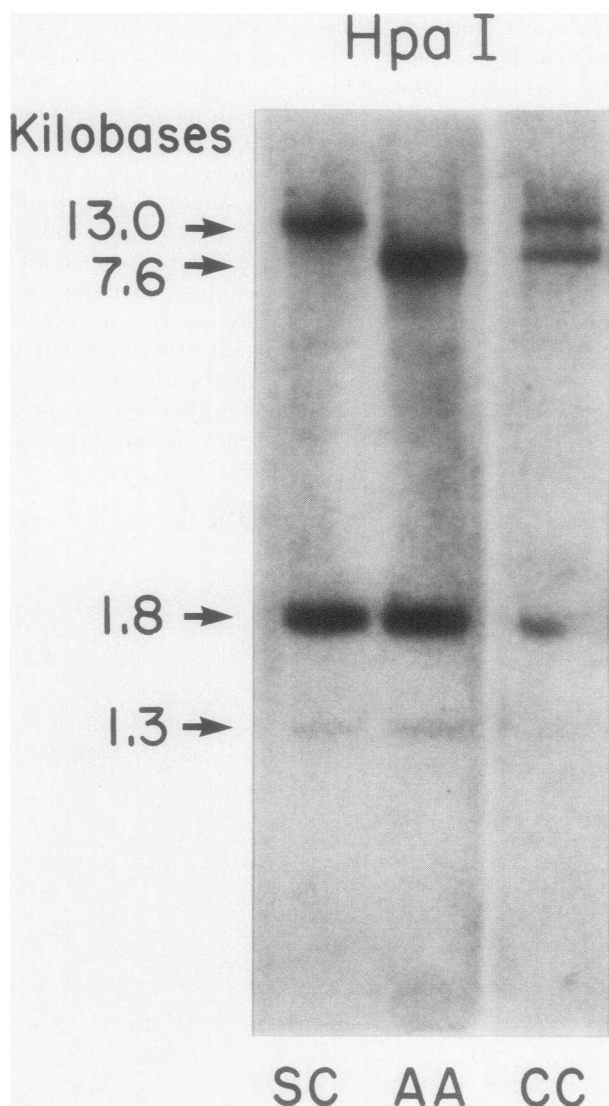
Hpa I

Kilobases

13.0 →
7.6 →

1.8 →

1.3 →

SC   AA   CC

FIGURE 4   Hpa I restriction pattern of β- and δ-globin genes in individuals with the β^s-gene. Left to right: an SC patient (13.0/13.0); AA homozygote (7.6/7.6); and a CC patient (13.0/7.6).

for the 13.0-kb variant (i.e., 13.0/13.0), and also have an extra 5.6-kb fragment unrelated to a functional β^s-gene. One parent of this patient had 7.6- and 13.0-kb fragments; the other has not been available for study. This patient's DNA also yields a unique restriction pattern with β-cDNA when cleaved with several other enzymes. Large-sized fragments (one 60 kb) which contained β-like gene material were seen with Eco RI, which suggests a partial gene duplication not involving the intragenic Eco RI site (Fig. 5).

DISCUSSION

Our results indicate the considerable heterogeneity in the restriction fragments detected with Hpa I in

sickle-cell patients. Although we confirm that the Hpa I site changed is 3' to the β-structural gene as previously reported (1), the exact nature of this polymorphism has not been determined. For example, the 13.0-kb fragment could result from a single nucleotide change in the recognition sequence of Hpa I or the insertion of a DNA fragment. The frequency of the 13.0-kb variant with the β^s-gene in our study was 58%,
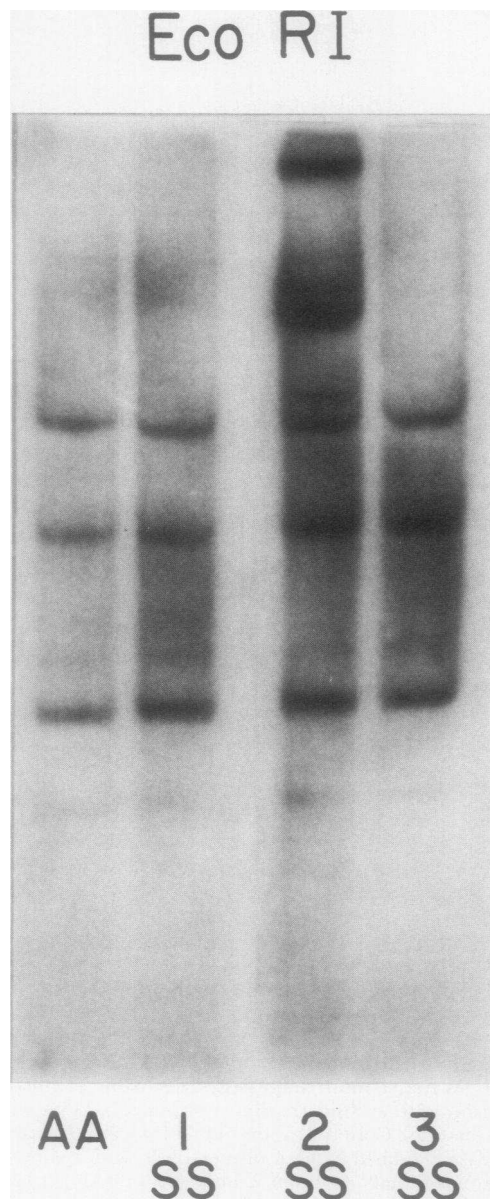

Eco RI

AA   1   2   3
     SS   SS   SS

FIGURE 5   Eco RI restriction pattern of β- and δ-globin genes in one AA and three SS patients. DNA from the patient with the intensely hybridizing 5.6-kb fragment in Fig. 2 is SS2. The AA patient and patients SS1 and SS3 have the normal Eco RI fragments previously reported (1, 4, 5). The SS2 DNA has at least two larger DNA fragments which contain β-like genes not seen in the other samples.

substantially less than the 87% frequency previously reported (1). However, it will require analysis of a much larger population study to determine the true incidence of the 7.6- and 13.0-kb fragments with the β^s-gene. We detected no correlation of the 13.0-kb variant with the clinical severity of sickle-cell disease.

Surprisingly, we also found the 13.0-kb variant commonly associated with the β^c-gene. In addition, three of the β^A-genes were linked to the 13.0-kb fragments. The finding that the 13.0-kb variant occurs with the β^c- and β^A-genes suggests that the 13.0-kb fragment is not specifically linked to the β^s-gene and probably occurred in a subpopulation of β^A-Blacks who subsequently developed the β^s- and β^c-genes. Kurnit (10) recently suggested that the β^s-genes associated with 7.6- and 13.0-kb Hpa I fragments arose independently. Our data are consistent with this interpretation, and would further suggest that the β^c-genes also arose independently in separate 7.6- and 13.0-kb Hpa I-containing populations. The New York Black population studied is of African origin, and migrated to New York from the southern United States and the West Indies. In this regard, we have recently found a high incidence of the 13.0-kb fragment in Blacks of West African origin (Nigeria and Ghana). Large-scale studies of defined Black populations should clarify the true frequency and origins of the fragments in different populations. The specific significance of the linkage of the β^s-gene with the 13.0-kb fragment, if any, remains to be elucidated.

Our results also indicate that the application of this polymorphism in the prenatal diagnosis of sickle-cell disease is less useful than previously suggested (11). Clearly, DNA analysis with amniotic fluid cells is more desirable than fetoscopy because of the reduced risk to mother and fetus and a relatively easier technical procedure. However, the Hpa I polymorphism will be most useful in cases where both parents possess the 13.0/7.6 genotype. Only 30% (3 of 10) of our sickle-trait subjects carried the 13.0/7.6-kb genotype. The Hpa I polymorphism will be less useful in families in which one $\beta^A\beta^s$-parent has 13.0/7.6 genotype and the other $\beta^A\beta^s$-parent has 7.6/7.6. If amniocentesis DNA reveals a 7.6/7.6 genotype, the $\beta^s\beta^s$-genotype is most unlikely. If, however, amniocentesis reveals a 13.0/7.6 genotype, sickle-cell anemia and sickle-cell trait in the fetus cannot be distinguished. This situation poses special problems in genetic counselling.

In the past, polymorphisms have proven exceedingly valuable in many types of genetic analyses. The data presented here and previously (1) indicate that they may become particularly useful at the gene level in the diagnosis and prevention of hereditary defects. It will become increasingly important, however, to define if a specific relationship exists between the polymorphism and the defective gene or whether the association is a result of unrelated genes occurring in the same population. In general, large population studies will be required for an accurate assessment of this kind.

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