Use of restriction endonucleases for mapping the allele for β^S -globin

(sickle cell/prenatal diagnosis)

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ABSTRACT We have reported the direct analysis of the allele for β^S -globin by using restriction endonuclease *Dde* I coupled with blot-hybridization analysis. In that report we predicted that a major use of our analysis could be for the prenatal diagnosis of sickle cell anemia. Here we present such an analysis. In addition, this report also describes the use of a new enzyme *Mst* II, which also can distinguish the β^S allele from the normal β -globin allele. Blot-hybridization analysis with restriction endonuclease *Mst* II shows the 5' end of the normal β -globin gene to reside on a fragment of ≈ 1.14 kilobases, whereas the 5' end of the β^S -globin gene resides on a fragment of ≈ 1.34 kilobases. Because the fragment sizes generated by *Mst* II are significantly larger than those generated by *Dde* I, one can easily perform a prenatal diagnosis for sickle cell by standard blot hybridizations onto nitrocellulose filters.

During the past several years, considerable research interest has been concerned with the prenatal diagnosis of hemoglobinopathies. Using fibroblast DNA obtained from fetal amniotic fluid cells, researchers initially used molecular hybridization analysis in solution for the prenatal diagnosis of hemoglobinopathies that were the result of gene deletions (1). This research then was extended to the use of restriction endonucleases for mapping the genes of deletion types of hemoglobinopathies (2) and a point-mutation variation (3). The finding of polymorphic sequences associated with the human β -globin-like gene and their segregation with the allele for β^S -globin allowed the use of restriction endonucleases for the prenatal diagnosis of sickle cell anemia. Initially Kan and Dozy reported (4) the finding of a *Hpa* I polymorphic site 3' to the β -globin gene. They showed that the β -globin gene resided on a DNA fragment of 7.6 kilobases (kb) after digestion of genomic DNA with restriction endonuclease *Hpa* I. Digestion of DNA isolated from the Black population often showed two patterns. In some cases the β^S allele resided on the 7.6-kb fragment, whereas in others that gene was found on a 13.0-kb fragment. The 13.0-kb fragment was shown to have an 87% association with the β^S allele. Therefore, they predicted that that polymorphism could be used for accurate prenatal diagnosis of the sickle cell phenotype in 70% of the fetuses at risk (4). Their work was extended by Phillips *et al.* (5) by using a *Hind*III polymorphism which was found in the human γ -globin genes (6, 7). By coupling the *Hind*III analysis to the *Hpa* I analysis, Phillips *et al.* (5) predicted that they could use blot hybridizations for an accurate prenatal diagnosis of sickle cell anemia in 80% of the fetuses at risk.

We have reported a direct analysis of the β^S allele with restriction endonuclease *Dde* I (8). This restriction endonuclease

recognizes the DNA base sequence C-T-N-A-G, which is present in normal DNA at codons 5 and 6 but not in the β^S allele due to the single point mutation. Blot-hybridization analysis with DNA isolated from peripheral blood showed normal individuals (genotype A/A) had a 201-base pair (bp) and a 175-bp fragment complementary to the 5' end of the β -globin gene, whereas the β^S -globin gene DNA had a loss of the 175-bp fragment and an appearance of a new fragment of 376 bp. Sickle cell trait (genotype A/S) individuals showed the 175-bp, 201-bp, and 376-bp fragments being present. Autoradiograms of the 376-bp fragment associated with the A/S genotype generally show that band to be less intense than the corresponding band associated with the S/S genotype. This data is consistent with the hypothesis that the appearance of the 376-bp band is a result of the loss of the *Dde* I recognition site in the β^S allele and is supported by the known DNA base sequence data on the β -globin gene (9).

MATERIALS AND METHODS

Patients and Blood Collection. This study utilized DNA isolated from peripheral blood samples taken from individuals with A/A, A/S, or S/S or S/ β^0 *thalassaemia* genotype. Generally, 10- to 20-ml blood samples were obtained, with EDTA as an anticoagulant. Informed consent was obtained for each sample. The amniotic fluid cell analysis was performed with 10 μ g of DNA isolated directly from cells obtained by amniocentesis on a pregnant woman whose fetus was at risk for sickle cell anemia.

For DNA isolation, peripheral blood samples were centrifuged at $2,000 \times g$, and the plasma fraction was removed by aspiration. Residual plasma was removed from the cell pellet, washed with 2 cell-pellet volumes of 0.9% NaCl solution, and centrifuged at $2,000 \times g$. Reticulocytes and older erythrocytes then were hemolyzed by freeze-thawing the cell pellet, followed by the addition of 2 vol of sterile H_2O . Unlysed cells, mostly lymphocytes, were pelleted and collected by centrifugation at $5,000 \times g$ for 15 min. Lymphocyte pellets were then washed twice with 10 ml of sterile H_2O to remove additional traces of hemoglobin protein. Amniotic fluid cells were isolated from 40 ml of amniotic fluid obtained from a woman at gestation week 17. Cells were pelleted directly from the fluid by centrifugation at $2,000 \times g$ for 15 min. Residual amniotic fluid was removed from the cell pellet by washing three times with 2 ml of 0.9% NaCl solution.

DNA of high molecular weight was isolated from peripheral blood cell lymphocytes and amniotic fluid cells by slight mod-

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Abbreviations: bp, base pair(s); kb, kilobase(s); A/A, normal hemoglobin phenotype; A/S, sickle cell trait (hemoglobins A and S); S/S, sickle cell anemia; NaCl/Cit, sodium chloride/sodium citrate; DBM, diazobenzoyloxymethyl.

ification to the method of Blin and Stafford (10). Briefly, a volume equal to the original sample volume of cell lysing solution (0.1 M NaCl/0.05 M Tris-HCl, pH 7.5/0.01 M EDTA/0.5% NaDodSO₄) was added, and the cells were incubated at 37°C for 10 min. For DNA isolation from amniotic fluid cells, 15 ml of lysing solution was added to the cell pellet. After the initial incubation, 100 µg of proteinase K (Beckman) per ml of lysing solution was added, and the lysed cells were allowed to incubate at 37°C for an additional 48 hr. The solution was then deproteinized by repeated extraction with chloroform/phenol, 1:3 (vol/vol), and the supernatant was dialyzed against 50 mM Tris-HCl, pH 8/10 mM EDTA/10 mM NaCl overnight at 5°C. Nucleic acids were precipitated by the addition of 2.5 vol of 100% ethanol, incubated at -70°C for 20 min, and collected by centrifugation at 11,000 × g for 15 min. The pellet was resuspended in 10 ml of 10 mM Tris-HCl, pH 7.5/1 mM EDTA. RNA was digested by the addition of 50 µg of heat-treated RNase (RNase A; Sigma) per ml, followed by incubation at 37°C for 45 min and chloroform/phenol extraction. DNA was pelleted by ethanol precipitation, centrifuged, and redissolved in sterile 0.5 mM Tris (pH 7.5) at 0.2 mg/ml.

Isolation and ³²P-Labeling of the Probe. An *Alu* I digestion of pBR322βPst (4.4 kb) produces a 737-bp DNA fragment specific for the 5' end of the human β-globin gene. This fragment has been isolated by gel electrophoresis and inserted into pBR322 to produce a recombinant DNA molecule cloned into *E. coli* and has been designated strain pSS737. Plasmid DNA, isolated from this strain was used in the substrate for DNA polymerase (New England BioLabs) in a nick-translation reaction as described by Rigby *et al.* (11) with [α-³²P]dCTP and [α-³²P]TTP (500 mCi/mmol; 1 Ci = 3.7 × 10¹⁰ becquerels) purchased from New England Nuclear. Only DNA fragments labeled to an estimated specific activity of 2.0 × 10⁸ cpm or greater were used as probes in the hybridization reaction.

DNA Transfer, Hybridization, and Detection of Genomic Gene Sequences. Studies on identifying the human β^S-globin gene in human genomic DNA with restriction endonuclease *Dde* I were performed as described (8). Briefly, 10–25 µg of genomic DNA was digested with a 4-fold excess of restriction endonuclease *Dde* I (1 hr/unit) under conditions as described by the supplier (New England BioLabs). Digested DNA samples were extracted with phenol, precipitated with ethanol, and redissolved in buffer A (40 mM Tris-HCl, pH 7.8/2 mM EDTA/20 mM sodium acetate). Restriction endonuclease fragments were then separated by gel electrophoresis on 4.5% (wt/vol) polyacrylamide/buffer A gels at 90 V for 18 hr, and the resulting DNA pattern was transferred to diazobenzoyloxymethyl (DBM) paper. Preparation of DBM paper was as described by Levy *et al.* (12), and electroelution of DNA fragments from the gel to the DBM paper was performed as described by Bittner *et al.* (13).

Studies on identifying the human β^S-globin gene within genomic DNA using restriction endonuclease *Mst* II were performed as follows. Total genomic DNA was digested overnight with a 4-fold excess of restriction endonuclease *Mst* II (New England BioLabs) under conditions as recommended by the supplier. The resulting fragments were separated by gel electrophoresis on 0.8% agarose/buffer A gels and transferred to nitrocellulose paper (Schleicher & Schuell) by the method of Southern (14).

Hybridization of the ³²P-labeled nick-translated probe to genomic DNA bound to filter paper supports were as described (8) except that the denatured salmon sperm DNA in both the prewash and hybridization solution was at a final concentration of 200 µg/ml. The filters were allowed to hybridize with the probe at 65°C for 48 hr. After the hybridization reaction, the

filters were washed initially in 0.90 M NaCl/0.090 M sodium citrate, pH 7.5, at room temperature for 10 min, followed by two successive washes in that NaCl/citrate buffer at full, half, and one-sixth strength at 65°C for 30 min. All NaCl/citrate solutions contained 0.5% NaDodSO₄. The filter papers were then dried and exposed to Kodak XRP-5 x-ray film for 2–8 days with Dupont Lightning-Plus intensifying screens. All experiments using recombinant DNA molecules were performed at P1-Ek1 conditions.

RESULTS

Identification of the Human β^S-Globin Gene in Amniotic Fluid Cells. Our original report describing the use of restriction endonuclease *Dde* I for the identification of the β^S allele (8) predicted that a possible use of the analysis could be for the prenatal diagnosis of sickle cell anemia. To date, seven different attempts have been made to identify a fetus with genotype A/S or S/S. Six of these studies showed the fetus to be normal. In the remaining case, DNA was isolated from the amniotic fluid cells taken from a pregnant woman carrying a child at risk for sickle cell anemia. The amniocentesis was performed for chromosomal analysis because of maternal age. Additional amniotic fluid was taken for the *Hpa* polymorphism analysis because the fetus was at risk. At the same time, peripheral blood samples were taken from the parents for the *Hpa* I analysis. *Hpa* I analysis on the parental DNAs failed to show the 13.0-kb band in association with the β^S allele. Therefore, the fetal cells were frozen until the direct *Dde* I analysis was available. From these fetal cells, 10 µg of DNA was obtained and digested with restriction endonuclease *Dde* I. Both the mother (Fig. 1, lane 1) and father (Fig. 1, lane 2) were shown to be sickle cell trait through the presence of the 175-bp and 376-bp bands, whereas the fetus at risk (Fig. 1, lane 3) had a single 376-bp band of greater intensity than the 376-bp band associated with the mother and father. After birth, both the child and father were seen at the Medical College of Georgia Comprehensive Sickle Cell Center. The child at age 10 months had 68.55% Hb S, 29.3% Hb F, and 2.05% Hb A₂. No Hb A was present. The father was shown to have 40.35% Hb S, 56.30% Hb A, and 3.35% Hb A₂, confirming sickle cell trait. This data confirmed our prediction of a sickle cell anemia infant based on DNA analysis of amniotic fluid cells.

Analysis of β^S/β^{thalassemia} Individuals. Because our analysis of the β^S allele is a direct genotype analysis and not a phenotype analysis, individuals with β^S allele on one chromosome and a nondeletion type of β-thalassemia gene on the other chromosome would be expected to show a restriction endonuclease pattern identical with that of an individual with sickle cell trait (genotype A/S). This pattern would be expected regardless of clinical or hemoglobin findings. To demonstrate this, a comparative analysis was performed with DNA from normal (genotype A/A), a sickle cell/β⁺-thalassemic heterozygote (β^S/β⁺), and sickle cell (β^S/β^S) individuals. Blot hybridizations were performed with 25 µg of DNA isolated from peripheral blood cells that was digested with *Dde* I and hybridized to the ³²P-labeled pSS737 probe (Fig. 2). The normal individual showed 201-bp and 175-bp fragments, whereas the sickle cell individual showed the 201-bp (δ-globin gene) and 376-bp fragments. The β^S/β⁺ individual showed the expected pattern of 175-, 201-, and 376-bp fragments. Other fragments were present in this analysis because the 737-bp probe was isolated as described in our previous report and used rather than a pure probe obtained from pSS737.

Analysis of the β^S-Globin Gene with Restriction Endonuclease *Mst* II. The DNA base sequence found in codons 5, 6,

FIG. 1. Autoradiograms of genomic DNAs obtained from peripheral blood samples of the mother and father (lanes 1 and 2) and of DNA isolated from amniotic fluid cells (lane 3; S/S, sickle cell anemia). For this experiment, 10 μ g of total genomic DNA was digested with restriction endonuclease *Dde* I, and the resulting fragments were separated on 4.5% polyacrylamide gels. Blot hybridizations used gel-purified pSS737 probe. The autoradiogram was a 4-day exposure. The 201-bp band found in lane 3 represents the δ -globin contribution as described (8).

and 7 for the normal human β -globin gene are CCT, GAG, and GAG, with codon 6 being GTG in the β^S allele. This single point mutation eliminates a *Dde* I recognition sequence (C-T-N-A-G, in which N is any base) in the β^S allele, resulting in a new fragment of 376-bp (8) as compared with 201-bp and 175-bp fragments found in normal individuals. Furthermore, this single point mutation also eliminates the recognition sequence for restriction endonuclease *Mnl* I (G-A-G-G). We have reported an analysis for detecting the β^S allele by using restriction endonuclease *Dde* I (8). However, another enzyme, *Mst* II, is available which is valuable for the sickle cell analysis. This enzyme recognized the nucleotide base sequence C-C-T-N-A-G-G, which contains the *Dde* I recognition sequence and also is present in codons 5, 6, and 7 of the normal β -globin gene but not of the β^S allele. Because *Mst* II enzyme recognizes seven DNA bases as compared to five DNA bases for enzyme *Dde* I and four DNA bases for enzyme *Mnl* I, it is reasonable to assume that the resulting fragments from *Mst* II would be somewhat larger than that from *Dde* I or *Mnl* I.

A survey of the known DNA base sequences in and around the sickle cell mutation sequence shows that all *Dde* I sites are also *Mst* II sites. However, in our *Dde* I sickle cell analysis, the DNA base sequence around the *Dde* I recognition site that is

FIG. 2. Autoradiograms of *Dde* I-digested genomic DNA obtained from normal individuals (N) and individuals with sickle cell/ β^+ -thalassemia heterozygote (β^S/β^+_{thal}) or sickle cell anemia (β^S/β^S). For this experiment, 25 μ g of DNA obtained from peripheral blood was used for blot hybridizations as described. Exposure was for 8 days.

5' to the β -globin gene is not known. Cleavage with *Dde* I at this site and at codon 6 in normal DNA produces the 175-bp fragment found in the *Dde* I analysis. If this *Dde* I site is not a *Mst* II site, then one would expect to find a fragment larger than 176 bp. Furthermore, if that fragment is found to be considerably larger than the 175-bp fragment, then it is conceivable that a sickle cell analysis can be performed with *Mst* II on nitrocellulose paper rather than on DBM paper.

To test this hypothesis, we digested 25 μ g of genomic DNA from normal (A/A), sickle cell trait (A/S), and sickle cell (S/S) individuals with restriction endonuclease *Mst* II, separated the resulting fragments on 0.8% agarose gels, and transferred the DNA to nitrocellulose filters as described by Southern (14). Blot hybridization analysis (Fig. 3) with the pSS737 probe showed that normal individuals had a single band present of ≈ 1.14 kb, whereas the sickle cell individual had a single band of ≈ 1.34 kb. Sickle cell trait individuals showed both the 1.14- and 1.34-kb bands.

We interpret these results as showing that the *Dde* I site 5' to the β -globin gene is not a *Mst* II site and that the next *Mst* II site is ≈ 1 kb 5' to that *Dde* I site. Therefore, the normal β -globin gene resides on an *Mst* II fragment of ≈ 1.14 kb, and the β^S allele resides on a fragment that is the summation of the 1.14-kb and the 201-bp fragments. The 201-bp band found in the normal β -globin gene would not be retained and, therefore, visualized with our transfer and washing conditions.

FIG. 3. *Mst* II analysis of the β^S -globin gene. A 2-day exposure of a blot hybridization with a *Mst* II digestion of 25 μ g of blood cell DNA. M_r controls: lane 1, *Hind*III-digested λ phage DNA (5 ng); lane 8, *Hpa* I-digested ϕ X174 phage DNA (5 ng). N, normal.

DISCUSSION

Our original report on the *Dde* I analysis for identifying the β^S -globin gene predicted that a major application of this technique could be for the prenatal diagnosis of sickle cell anemia. Chang and Kan (15) have reported the use of our procedure for identifying the genotype of a fetus at risk for sickle cell anemia. In their study, they used DNA isolated from cultured amniotic fluid cells and showed that the fetus was in fact normal and did not carry the β^S allele. We also have worked with cultured cells, but here we report the use of a procedure using DNA isolated directly from the cells present in the amniotic fluid that predicted a fetus was in fact the β^S/β^S genotype. Our diagnosis was confirmed by a hemoglobin analysis on the child at age 10 months. The *Dde* I analysis requires about 10 μ g of genomic DNA, which often cannot be obtained from amniotic fluid cells. In such cases it would be necessary to use cultured cells as a DNA source.

It has been suggested (15) that a disadvantage of using the *Dde* I analysis is that the small fragments generated by *Dde* I give weak hybridization signals as compared to the polymorphic analysis (4) in which the β -globin gene resides on either a 7.6-kb or 13.0-kb *Hpa* I fragment. However, the strength of the hybridization signal is directly proportional to the size of complementary sequences available for hybridization, the specific activity of the labeled probe, and perhaps the membrane support. The size of the restriction fragment (in which the gene resides) produced by endonuclease digestion should not be a major contributing factor, providing enough complementary base sequences are present to form a stable hybrid. Perhaps the weak signal found is a result of the use of nitrocellulose filter paper as the membrane support rather than DBM paper as used by this laboratory. It has been suggested (14) that small DNA fragments (<1 kb) do not bind efficiently to nitrocellulose, whereas DBM paper (13) can efficiently bind DNA fragments >100 bp. However, the problems with the use of the *Dde* I analysis with nitrocellulose filters may be eliminated by use of the *Mst* II analysis as described in this report.

Here we present an additional analysis for the β^S -globin gene

with restriction endonuclease *Mst* II. Another common hemoglobin variant is Hb C, which is also a point mutation in codon 6 of the β -globin gene. The point mutation found in the β^C -allele is in the first position of codon 6, whereas the point mutation in the β^S -allele is in the second position of codon 6. The first position of codon 6 corresponds to the N position of the restriction endonuclease recognition sequence for both *Dde* I (C-T-N-A-G) and *Mst* II (C-C-T-N-A-G-G). Therefore, neither *Dde* I or *Mst* II will distinguish the β^C allele from the β^A allele. This means that the genotype *S/C* will not be distinguished from the genotypes *A/S*, *S/\beta^0_{thalassemia}*, or nondeletion *S/\beta^0_{thalassemia}* in prenatal diagnosis. However, the *S/C* and *S/\beta^0_{thalassemia}* genotypes are compatible with normal development and relatively asymptomatic existence with a good life span. *S/\beta^0_{thalassemia}* is not as severe a condition as that characterized by genotype *S/S* (16); therefore, the inability to distinguish it from genotype *A/S* by direct analysis is not a major drawback. Within the human hemoglobin gene system and its associated hemoglobinopathies, the β^S allele is probably the most important. The techniques described here directly identify the important *S/S* genotype with complete certainty.

An advantage of using *Mst* II restriction endonuclease rather than *Dde* I would be the ease of using nitrocellulose filters rather than DBM paper for the blot hybridization analysis. Chang's and Kan's work (15) applied the use of nitrocellulose filters for the *Dde* I analysis. As mentioned, such a procedure may be a factor for weak hybridization signals. This problem may be overcome by using *Mst* II or a more sensitive probe.

Perhaps a major clinical interest in the *Mst* II analysis is that now two analyses (*Mst* II and *Dde* I) are available for corroborating each other for a most reliable prenatal diagnosis of fetuses at risk for sickle cell anemia.

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