Direct identification of sickle cell anemia by blot hybridization
(prenatal diagnosis)

ROBERT F. GEEVER*, LOIS B. WILSON†, FERZ S. Nallaseth*, PAUL F. MILNER‡, MICHAEL BITTNER§, AND JOHN T. WILSON*

*Department of Cell and Molecular Biology, and †Department of Pathology and Medicine and Comprehensive Sickle Cell Center, Medical College of Georgia, Augusta, Georgia 30912; and ‡Department of Molecular and Population Genetics, University of Georgia, Athens, Georgia 30602

Communicated by Norman H. Giles, May 11, 1981

ABSTRACT Several reports have been published on the use of polymorphisms found in the human hemoglobin genes as a means for prenatal diagnosis of sickle cell anemia. The disadvantages of this approach reside in its limited application and the need for family analysis. Here we report that, by use of restriction endonuclease Dde I and diazobenzoxymethyl-paper transfer procedures, a direct analysis can be made. Individuals with normal hemoglobin (AA) show two bands (175 and 201 base pairs) complementary to a $'g$-specific $\beta$-globin gene probe. Sickle cell trait individuals (AS) exhibit an additional band (376 base pairs). Individuals with sickle cell anemia (SS) show the band at 376 base pairs with a concomitant loss of the 175-base pair band. We interpret these changes in banding pattern to be the result of the elimination of a restriction site for Dde I in the altered codon associated with the sickle cell allele. Because an analysis can be performed on as little as 20 mg of cellular DNA, the application to prenatal diagnosis of sickle cell anemia should be possible.

Recombinant DNA techniques coupled with blot hybridization analysis have proven to be valuable tools for studying the molecular basis of hemoglobinopathies. Various researchers have used blot hybridization to confirm that $\delta$-$\beta$-thalassemia (1-5) and hereditary persistence of fetal hemoglobin (1-6) are the result of gene deletions, whereas $\alpha$-thalassemia (7-9) and $\beta$-thalassemia (10-12) are due to both gene deletions and point mutations. One study has shown that at least one case of $\delta$-thalassemia is probably due to a base mutation (13). These studies have also been extended to the clinical setting as methods for prenatal diagnosis of various genetic hematological conditions (14-19). Kan and Dozy have reported (20) the finding of a polymorphism for a Hpa I restriction endonuclease site in American Blacks to the $\beta$-globin gene, which was shown to have a 60% association with the sickle cell allele. From their studies, they estimated that blot hybridization using this polymorphism alone could be successfully used for prenatal diagnosis of a sickle cell anemia in 36% of couples at risk. Phillips et al. (21) have combined the Hpa I analysis with a second polymorphism found in the $\gamma$-globin genes (22). In so doing, they have reported (21) an extension of blot hybridization for prenatal diagnosis of sickle cell anemia to over 90% of the couples at risk. However, both require family studies in order to establish the association of the polymorphic sites with the sickle cell allele. This limited application is a major disadvantage of these procedures.

A direct analysis of the sickle cell anemia should be possible by use of a restriction enzyme whose recognition sequence is created or eliminated by the sickle cell mutation. This approach would not require family studies and should be useful for all couples at risk.

Nienhuis has proposed such a direct analysis with restriction endonuclease Mnl I (23). However, efforts in various laboratories have failed to attain the sensitivity requisite for the resolution of the small [60- to 80-base pair (bp)] fragments generated by this enzyme. With the discovery of the restriction enzyme Dde I (24), we report that direct analysis is now feasible.

MATERIALS AND METHODS

Patients and Blood Collection. Patients with sickle cell anemia (SS), sickle cell trait (AS), and control individuals with no known hematological disorder (AA) are the subjects of this study. Approximately 10-20 ml of blood was collected in Vacutainers with EDTA as anticoagulant. The samples were placed in ice and transported to the laboratory for immediate processing. Informed consent was obtained.

DNA Isolation. High molecular weight DNA was isolated from peripheral blood lymphocytes by the method of Blin and Stafford (25). Briefly, 10- to 20-ml blood samples were centrifuged at 2000 X g and the plasma fraction was removed by aspiration. Residual amounts of plasma were extracted by repeated washes of the cell pellet with 0.9% NaCl. The reticulocytes and older erythrocytes were hemolyzed by the addition of two cell pellet volumes of sterile H2O, and the lymphocytes were collected by centrifugation at 2000 X g for 15 min. The lymphocytes were lysed by the addition of 20 ml of lysing solution [0.05 M Tris-HCl, pH 7.5/0.5% NaDodSO4/0.1 M NaCl/ 0.001 M EDTA/100 mug of proteinase K (Beckman) per ml] and incubated at 37°C for 48 hr. After lysis, the solution was deproteinized by repeated chloroform/phenol (1:3, vol/vol) extractions and dialyzed overnight at 4°C against 50 mM Tris-HCl, pH 8/10 mM EDTA/10 mM NaCl. Nucleic acids were isolated by the addition of 2.5 vol of ethanol followed by centrifugation at 11,000 X g for 15 min. The pellet was resuspended in 10 mM Tris-HCl, pH 7.5/1 mM EDTA, incubated at 37°C for 30 min with heat-treated RNase (RNase A, Sigma) at 50 mug/ml, and extracted with phenol/chloroform. DNA was collected by ethanol precipitation followed by centrifugation as above and was redissolved in sterile H2O at a concentration of about 0.2 mg/ml.

Determination of Fragment Lengths. A DNA fragment 1.8 kilobases (kb) long was resolved by digestion of recombinant DNA clone pBR322!Pst (4.4 kb) with BamHI and electrophoresis through 0.8% agarose in TEA buffer (40 mM Tris-HCl, pH 7.8/2 mM EDTA/20 mM sodium acetate). Recovery was attained by electroelution into dialysis tubing (Spectrapor 4) immersed in $1/2$ X TEA buffer, partial volume reduction by ly-
Ophoillumination, phenol/chloroform extraction, and ethanol precipitation. Subsequent restriction enzyme digestions were performed with 2 units of enzyme per μg of DNA and digested for 4 hr (1 unit will digest 1 μg of a DNA in 1 hr) and resolved on polyacrylamide gels containing 4% (wt/vol) acrylamide and 0.2% N,N'-methylenebisacrylamide. Fragments generated by Hae III digestion of φX174 replicative form DNA were used as standards for approximate fragment length determinations.

Isolation and \(^{32}\)P-Labeling of the Probe. Alu I digestion of pBR322/BstI (4.4 kb) produces a 737-bp, 5′-specific β-globin fragment. This fragment was resolved on and isolated from 4% polyacrylamide gels. On the average, 40% recovery was obtained after the electroelution process described above. Because preparations were contaminated with residual amounts of polyacrylamide, the quantity used as substrate for DNA polymerase I (New England Biolabs) was based on an estimated average recovery. Nick-translations were performed with [α-\(^{32}\)P]dCTP and [α-\(^{32}\)P]dATP (500 mCi/mmol, 1 Ci = 3.7 \(\times\) 10\(^{10}\) becquerels) purchased from New England Nuclear. Reaction conditions were those of Rigby et al. (26), but with the buffer reported by Maniatis et al. (27). DNA fragments were labeled to an estimated specific activity of 1.0 \(\times\) 10\(^{8}\) cpm/μg of DNA. After nick-translation, an aliquot from each reaction was analyzed by electrophoresis on 4% polyacrylamide gels under denaturing conditions and autoradiographed. Nick-translations suitable for subsequent hybridization exhibited a near even distribution of fragment lengths between 100 and 700 nucleotides.

Diolobenzoxymethyl (DBM) Transfer, Hybridization, and Detection of Sequences in Cellular DNAs. Details for preparing DBM-paper and transferring DNA are as given by Bittner et al. (28), with the exception that aminobenzoxymethyl-DBM paper was made according to Levy et al. (29). For these studies 20 μg of cellular DNA was digested with 4-fold excess Dde I restriction endonuclease (1-hr units) overnight under conditions suggested by the supplier (New England Biolabs). Samples were extracted with phenol, precipitated with ethanol, and resuspended in TEA buffer before electrophoresis on 5% polyacrylamide/TEA gels at 90 V for 18 hr at room temperature. The DNA was denatured at 4°C for 20 min in 0.2 M NaOH and 0.06 M NaCl, then equilibrated for 1 hr with several changes of transfer buffer (25 mM sodium phosphate buffer, pH 6.5). Electrophoretic transfer to DBM-paper was conducted at a current flux of 2 A for 90 min.

Conditions for pretreatment and hybridization to DBM-paper are given by Wahl et al. (30). However, we omitted formamide, increased the incubation temperature to 55°C, and allowed the probe to hybridize for 48 hr. After hybridization the DBM-paper was washed once in 6× NaCl/Cit (1× NaCl/Cit is 0.15 M NaCl/0.015 M sodium citrate) containing 0.5% NaDodSO\(_4\) at room temperature, followed by successive washing in 6×, 3×, 1×, and 0.3× NaCl/Cit. Each of the latter steps was performed twice at 55°C for 30 min and all solutions contained 0.3% NaDodSO\(_4\). The DBM-paper was dried and exposed to Kodak XRP-5-x-ray film, using Du Pont Lightning-Plus intensifying screens for 2–5 days. Experiments involving recombinant DNA were conducted at P2–E1K1 containment in accordance with National Institutes of Health guidelines.

RESULTS

Rationale of the Analysis. Hb S has been shown (31) to be the result of a single nucleotide base mutation in the β-globin gene that converts the glutamic acid codon (GAG) at amino acid position 6 to one for valine (GTC). As shown in Table 1, the A to T transversion within the β-globin gene sequence affects a restriction endonuclease recognition site for both enzymes Mnl I (G-A-G-G) and Dde I (C-T-N-A-G, in which N = any nucleotide).

<table>
<thead>
<tr>
<th>Type of Hb</th>
<th>Amino acid sequence (codons 5, 6, and 7)</th>
<th>Corresponding nucleotide sequence</th>
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<table>
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<tr>
<th>A</th>
<th>Pro - Glu - Glu</th>
<th>CCT GAG GAG</th>
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<tbody>
<tr>
<td>S</td>
<td>Pro - Val - Glu</td>
<td>CCT GTG GAG</td>
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The Dde I recognition site is underlined.

Restriction Endonuclease Digestion of pBR322/BstI. To determine the feasibility of using Dde I, we first analyzed the known β-globin sequence (33). Dde I cleavage sites within the 5′ end of the β-globin gene up to the BamHI site (Fig. 1). This analysis showed Dde I cleavage sites 33, 122, 210, and 411 bp from the BamHI reference site, with the 411-bp site being affected by the sickle cell mutation. Our analysis of the known intergenic region, 103 bp 5′ to the β-globin gene, failed to indicate any Dde I cleavage site. Therefore, digestion of the normal β-globin gene with Dde I, followed by blot hybridization with the 5′-specific probe, should reveal two fragments over 100 bp. One fragment of 201 bp represents the DNA restriction fragment 3′ from the sickle cell site and another fragment more than 170 bp (103 bp intergenic, 53 bp untranslated, and 14 bp translated region) represents the region 5′ to the sickle cell mutation site. For the purpose of discussion, these fragments will be referred to as the 3′ fragment and the 5′ fragment.

![Fig. 1. Restriction map from the part of the β-globin region complementary to the Alu I (737-bp) probe. The representation of restriction sites within and around the human β-globin gene was reproduced in part from Lawn et al. (33). For consistency, we have maintained their method for numbering restriction sites from the intragenic reference point (R) for BamHI cleavage. The protein-encoding regions (filled areas), intervening sequences (open areas), noncoding regions (hatched areas), and the sequenced portion of the 5′ intergenic region (wavy line) are indicated. The approximate location of additional restriction sites is so indicated (=). Fragment lengths generated by Dde I cleavage of β and δβ alleles within the region of probe complementarity are given below in the expanded portion of the restriction map. Numbers represent bp fragment lengths.](image-url)
The identification of the 5' fragment was made by digestion of the 1.8-kb BamHI fragment (see Materials and Methods) with Dde I and Dde I + Hae III. A digest of the 1.8-kb BamHI fragment with Dde I produced fragments with lengths of 460, 285, 210, 201, 175, 170, 105, 88, and 89 bp (Fig. 2). The 201-, 88-, and 89-bp fragments were expected from the gene sequence and were known not to be the 5' fragment. Because the minimal fragment size expected was greater than 170 bp, the 105-bp Dde I fragment was also eliminated. Base sequence analysis (33) also showed that within the 5' fragment there is a Hae III site 143 bp from the Dde I site. Therefore, the 5' gene fragment was identified by redigestion with Hae III and the subsequent assay for the fragment that was converted to 143 bp. A double digest (Dde I + Hae III, Fig. 2) showed that the 460-, 210-, and 170-bp fragments were not cut by Hae III and that the 285-bp fragment was converted to a 265-bp fragment. Therefore, the 175-bp fragment that was cut by Hae III to 143 bp must include the 5' end of the β-globin gene. Consequently, Dde I analysis of the normal β-globin gene would be expected to show two fragments, 201 and 175 bp, complementary to the specific probe. Digestion of homozygous Hb S DNA should show a single fragment of 376 bp complementary to the specific β-globin probe.

Characterization of the Probe. Digestion of pBR322Bst (4.4 kb) with Alu I is represented in Fig. 3, lane A. A fragment approximately 855 bp long, specific to the 5' region of the β-globin gene, was expected from the restriction map presented according to Lawn et al. (33). When subsequent redigestions with Hae III were performed, only fragment d gave the predicted pattern (Fig. 1; Fig. 3, lane B). However, a slight discrepancy in the length of one Hae III fragment was observed (denoted by an arrow), which indicates the existence of an additional Alu I site in the 5' intergenic region. This may be due to the creation of a new Alu I site during the construction of this clone. As a consequence, however, the actual length for the Alu I probe is 737 bp, rather than the anticipated 855 bp. Furthermore, blot hybridizations using 32P-labeled Alu I (737-bp) fragment hybridized to Alu I, Hae III, and Dde I digests of cloned β-globin gene have also given the expected banding patterns (data not shown).

Blot Hybridization Analysis of Hb S Gene. After our preliminary studies, blot hybridization analyses were performed on Dde I-digested lymphocyte DNAs obtained from normal, sickle cell, and sickle cell trait individuals. Autoradiograms are presented in Fig. 4. As can be seen, DNA from control individuals (AA) shows both the 175- and 201-bp bands, whereas sickle cell DNA (SS) shows the 376-bp band. DNA from a sickle cell trait individual (AS) shows the expected combination of 175-, 201-, and 376-bp bands. The presence of larger additional bands may be the result of a slight contamination of our probe with other DNA fragments. Such contamination is most probably due to the difficulty in isolating the 5'-specific probe free from adjacent DNA fragments. Repeated experiments with DNA obtained from different individuals have shown these larger bands to be consistent for AA and SS individuals. These bands are also present in blot hybridizations from Dde I digests of the pBR322Bst plasmid (data not shown). Purification of the 5'-specific probe by recombinant DNA techniques (see Discussion) should eliminate these bands.

**DISCUSSION**

Our analysis of Dde I-digested normal, sickle cell, and sickle cell trait DNAs are consistent with our expected results. A potential complication in this analysis would result from cross-hy-
bridization of the probe with other β-globin-like genes. It has been shown (34) that under stringent conditions cross-hybridization of γ-globin genes does not occur. However, due to the close similarity of the nucleotide sequence of the δ- and β-globin DNAs, cross-hybridization of the probe with the δ-globin gene is detectable, even under stringent conditions (34). A survey of the known δ-globin sequence (35) shows that the 3’ δ-globin gene fragment would not be 175 bp, but a minimum of 180 bp. Even though we have not yet identified the length of this fragment, it does not seem to interfere with our analysis and should be present in both normal and sickle cell DNA. The known δ-globin gene sequence (35) also shows a 199-bp fragment similar to that of the 3’ β-globin gene fragment. Therefore, the 201-bp band in our analysis probably represents a contribution of restriction fragments from both the δ- and β-globin gene in the normal DNA, but only the δ-globin gene in sickle cell DNA. Because the 201-bp band could represent only the δ-globin gene in sickle cell DNA, it would be expected to bind only 1/2 as much radioactivity in those individuals compared to normal individuals. To date our analysis has been performed on DNAs isolated from three unrelated sickle cell individuals and two unrelated control individuals. In all cases, the 376-bp band has been present in association with the sickle cell allele. However, when autoradiograms from the same individual and autoradiograms from different individuals are compared, the 201-bp fragment in sickle cell DNA is often not present, or, when present, it is very faint. This probably reflects the 12–16% (36) divergence of the δ-globin and β-globin nucleotide sequence in that region of the DNA complementary to our probe. Because the 201-bp fragment is sometimes present in sickle cell DNA, the essence for diagnosis of sickle cell anemia is the absence of the 175-bp fragment and appearance of the 376-bp fragment. Diagnosis of the sickle cell trait is based on the presence of the 175- and 376-bp fragments.

Fetuses heterozygous for Hb S and other hemoglobin disorders, such as Hb C or nondeletion type β-thalassemia, would give blot hybridization patterns identical to sickle cell trait individuals. Naturally, these could be excluded by family studies. Moreover, these disorders are usually less severe than sickle cell anemia.

We have presented a feasible approach for detection of sickle cell anemia. To date, this analysis has been conducted on DNA isolated from peripheral blood lymphocytes. We have been able to perform our analysis on as little as 20 μg of genomic DNA. Because we normally obtain a minimum of 20–30 μg of DNA from fetal cells obtained by amniocentesis at 14–18 weeks of gestation, this procedure should be directly applicable to prenatal diagnosis of sickle cell anemia.

We have observed in blot hybridization experiments with human genomic hemoglobin genes that the use of recombinant DNA molecules, as opposed to isolated restriction fragments, increases the sensitivity of the procedures. This is probably the result of enhanced specific activity and the formation of more extensive hybridization networks. It should be possible to insert the 5’-specific probe into recombinant DNA molecules. This would be expected to increase the sensitivity of this method. Also, this would result in a purification of the 5’-specific probe, eliminating the occurrence of additional high molecular weight bands. Those recombinant DNA molecules containing the Alu I (737-bp) probe should soon be available to other laboratories on request.

In conclusion, additional studies are necessary to determine the degree to which polymorphism in the natural population will interfere with the proposed method. The occurrence of polymorphisms that create or eliminate Dde I recognition sites could change the expected blot hybridization pattern. However, because we are using a probe of only 737 bp to assay fragments of 175 and 201 bp, we expect the occurrence of specific polymorphisms to be minimal.

**Note Added in Proof.** Recombinant DNA molecules containing the 737-bp 5’-specific probe are available from this laboratory. Blot hybridizations using this plasmid (pSS737) as a probe eliminate all high molecular weight bands above 376 bp. These studies have now been extended to 25 individuals (including DNA isolated from amniotic fluid), all of which show the expected banding pattern.

We thank Mike Summer and Candy Rossignol for their excellent technical assistance. We thank Dr. T. Maniatis and his laboratory for the use of the genomic β-globin clones. This investigation was supported in part by National Science Foundation Grant PCM 7909054 and National Institutes of Health Grant HL-23294.