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Highly sensitive and rapid gene mapping using miniaturized blot hybridization: application to prenatal diagnosis

(Rapid amniocyte DNA extraction; mini-gel electrophoresis; mapping sickle cell genes; fetal genotype; restriction analysis)

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SUMMARY

We have developed a protocol for the preparation and analysis of amniocyte DNA which permits more sensitive and more rapid antenatal detection of sickle-cell anemia (SCA) than previously has been possible. After rapid extraction of DNA from amniotic cells, only 50 ng of *Mst*II-digested DNA need be analyzed by mini-gel electrophoresis and hybridization detection to determine reliably the fetal genotype. Under these conditions, the entire gene-mapping procedure can be performed within 5 days. When larger amounts of DNA ($>$ 500 ng) are analyzed, the minimal diagnosis time is reduced to 2 days. The resolution of restriction fragments on mini-gels is comparable to that obtained with larger gels. The 1.15-kb β^A and 1.35-kb β^S *Mst*II fragments are well separated. The technique is useful whenever rapid and sensitive analysis of genomic DNA is desired.

INTRODUCTION

Techniques for the prenatal diagnosis of SCA by restriction endonuclease analysis of amniocyte DNA have improved recently. In earlier gene-mapping studies segregation analysis of polymorphic restriction sites linked to the β^S mutation allowed prenatal diagnosis in up to 86% of the pregnancies at risk for SCA (Kan and Dozy, 1978a,b; Phillips et al., 1980; Boehm et al., 1983). Later, direct analysis of the β^S allele became possible with the discovery of the restriction enzyme *Dde*I (Makula and Meagher, 1980), whose recognition sequence (CTNAG) is present in codons 5 and 6 of normal DNA, but is lost in β^S DNA (Geever et al., 1981). However, the relatively small *Dde*I β^A and β^S fragments required the digestion of 10–20 μg of genomic DNA (Chang and Kan, 1981; Geever et al., 1981; Wilson et al., 1982).

Prenatal diagnosis of SCA can be performed with greater sensitivity by employing the restriction endonuclease *Mst*II, which also differentiates β^A and β^S alleles (Chang and Kan, 1982; Chang et al., 1982; Orkin et al., 1982; Wilson et al., 1982). Since the *Mst*II recognition sequence occurs less frequently than does the *Dde*I sequence, using conventional

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Abbreviations: *AA*, *AS*, β-globin genotypes (see MATERIALS AND METHODS, section a); β^A, normal β-globin allele; β^S, sickle β-globin allele; E buffer, 36 mM Tris base, 30 mM NaH_2PO_4, 1 mM EDTA, pH 7.8; EtBr, ethidium bromide; kb, kilobase pairs; SCA, sickle-cell anemia; SSC, 150 mM NaCl, 15 mM Na_3·citrate, pH 7.6; SDS, sodium dodecyl sulfate; SSPE, 180 mM NaCl, 10 mM NaH_2PO_4, 1 mM EDTA, pH 7.4.

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gene mapping procedures, as little as 1 μg of genomic DNA can be analyzed within about 2 weeks (Chang and Kan, 1982).

We have improved both the sensitivity and efficiency of the *Mst* II prenatal assay for SCA. A rapid DNA extraction procedure, combined with mini-gel electrophoretic analysis of *Mst* II fragments permits unambiguous diagnosis with as little as 50 ng of genomic DNA. Depending upon the amount of DNA analyzed, the entire procedure can be completed within 2–5 days. The procedures we describe are generally useful for rapid and sensitive analysis of genomic DNA.

MATERIALS AND METHODS

(a) Rapid DNA extraction

The cells contained in 5–20 ml of amniotic fluid obtained at 16–18 weeks gestational age are pelleted at 12000 × *g* (10000 rev./min, Sorvall SS-34 rotor) at 4°C for 5 min, washed twice with 5 ml of 0.9% NaCl, and resuspended in 400 μl of lysis buffer (20 mM Tris · HCl, 0.3 M sodium acetate, 1 mM EDTA, pH 7.5) in a 1.5-ml eppendorf tube. Proteinase K is added to 150 μg/ml, and SDS is added to 0.2%. Complete lysis occurs within 1–6 h incubation at 37°C.

The lysates are extracted three times (6 min each) with phenol–chloroform (1:1), and three times (6 min each) with an equal volume of anhydrous ethyl ether. The aqueous and organic phases are separated by centrifugation for 3 min in a Beckman microfuge. Nucleic acids are precipitated by the addition of 2.5 vols. of cold (−20°C) ethanol, and by incubation for 20 min at −80°C. To estimate DNA concentration, a sample is electrophoresed on a minigel with known amounts of undigested λ DNA. The rapid extraction procedure can be used with minor modifications for extraction of DNA from tissues and from peripheral blood buffy coats. Control human genomic DNAs with known β-globin genotypes (*AA*, *AS*, *SS*) were extracted as described previously (Bruzdzinski et al., 1983).

(b) Isolation and labeling of the human 5′-β-globin probe

The probe used for this study is the 1.15-kb *Mst* II fragment which extends from the *Mst* II site at codons 5–7 of the normal human β-globin gene (β^A) to the closest *Mst* II site in the 5′-flanking region (Fig. 1). We purified this fragment from plasmid

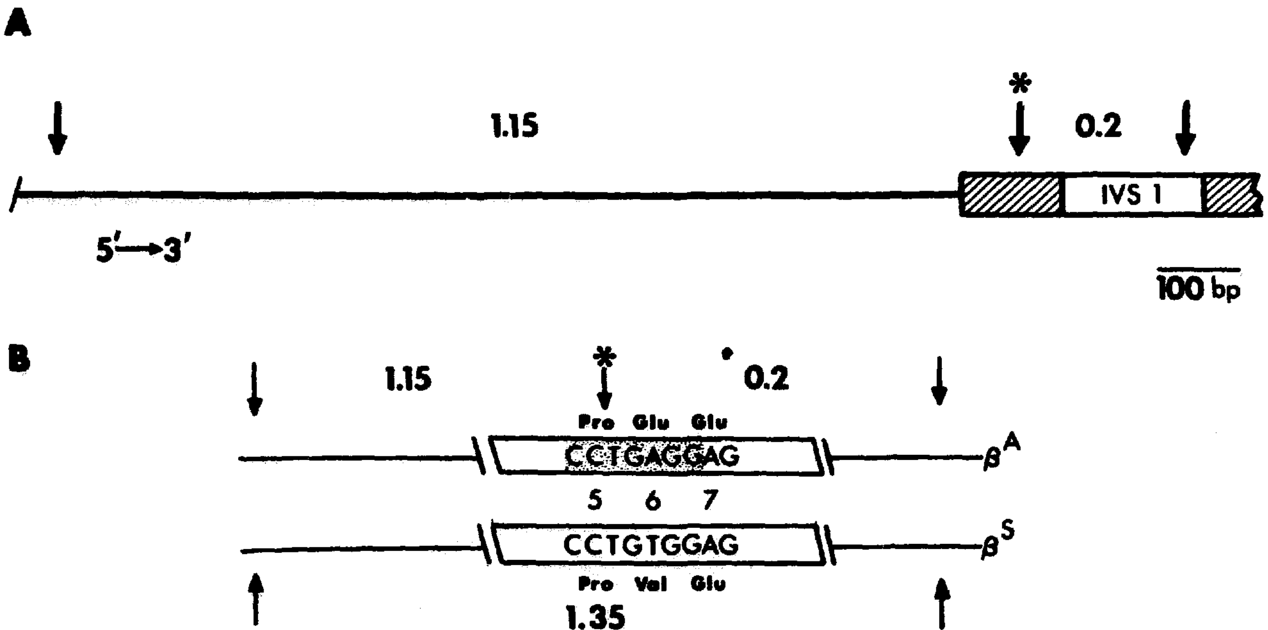

Fig. 1. *Mst* II restriction map of human β-globin genes. (A) *Mst* II sites (↓) in the 5′-flanking (heavy line) and coding (hatched box) regions of the normal (β^A) gene. Fragment sizes (kb) and scale are indicated; IVS, intervening sequence. (B) Enlarged diagram (not to scale) of the sequences at codons 5–7 in the normal and sickle (β^S) alleles. Stipled area, *Mst* II recognition sequence; asterisk indicates *Mst* II site present in normal but absent in sickle gene.

Hβ1S (Lawn et al., 1978), filled its termini with Klenow fragment of DNA polymerase I (BRL), and subcloned it into the *Sma*I site of plasmid pUC-8 (Messing and Vieira, 1982). Either the entire subclone (pHβ 1.15 M) or the gel-purified 1.15-kb insert is labeled with [α-^{32}P]dCTP (Amersham, 400 Ci/mmol) by nick translation (Rigby et al., 1979) using a kit supplied by BRL. Specific activities $\geq 5 \times 10^7$ cpm/μg are suitable for detection.

(c) Sample preparation and mini-gel electrophoresis

DNA is digested with *Mst*II (New England Biolabs) under conditions recommended by the supplier for $\geq$3 h. Digested DNA samples are electrophoresed in horizontal, 6 × 10 × 0.5 cm, 1.4% agarose gels in E buffer, at 60 V for 2–3 h with buffer recirculation and cooling.

(d) DNA transfer, hybridization, and detection procedures

DNA fragments are denatured for 10 min in two changes of 0.5 N NaOH, 1.5 M NaCl, and neutralized by soaking 2 × 5 min in 1 M ammonium acetate, pH 7.0. The fragments are blotted (Southern, 1975) onto nitrocellulose (0.45 μm, Schleicher and Schuell, Inc.) in 20 × SSC for $\geq$2 h at room temperature and are fixed to the filters for 2 h at 80°C in vacuo. Filters are prehybridized for 2–4 h at 42°C in plastic bags containing 5 × SSPE, 35% formamide (MCB), 5 × Denhardt's solution (5 × = 0.1% bovine serum albumin, 0.1% Ficoll, 0.1% polyvinyl polypyrrolidone), and 250 μg/ml of sheared, denatured salmon sperm DNA, using 0.5 ml of solution per cm^2 filter area. Hybridization is performed for $\geq$ 10 h at 42°C in 5 × SSPE, 35% formamide, 1 × Denhardt's solution, 10% dextran sulfate, 100 μg/ml denatured salmon sperm DNA, and 100 ng of the nick-translated 1.15-kb *Mst*II fragment (see above and Fig. 1), using 0.2 ml of solution per cm^2 filter area.

Filters are washed for 2 × 10 min at room temperature in 2 × SSC, 0.1% SDS, and for 2 × 20 min at 65°C in 2 × SSC, 1 × Denhardt's solution, 0.1% SDS. Autoradiography is performed with XAR-5 film (Kodak) and Cromex intensifying screens (Dupont) at −80°C for appropriate intervals (Table I).

TABLE I

Improved efficiency and sensitivity in gene-mapping procedures

Procedure	Minimum time required (h)
(1) Pellet, wash and lyse amniocytes	3
(2) Extract DNA, estimate yield	2
(3) *Mst*II digestion	3
(4) Mini-gel electrophoresis	2
(5) Blot transfer	2
(6) Fix, pre-hybridize and hybridize	16
(7) Wash filter	1
(8) Autoradiography: >500 ng	8
200–500 ng	24
50–200 ng	48

RESULTS

(a) Rapid extraction of DNA from amniotic cells

With a goal to improve the rapidity and sensitivity of the prenatal *Mst*II assay for SCA, we developed a more rapid amniocyte DNA extraction procedure. Previously, when washed amniocytes were resuspended in a lysis buffer containing SDS, clumps of cells formed and these required prolonged incubation to be dispersed and lysed. In the present procedure, cells are uniformly suspended before lysis is initiated by the addition of proteinase K and SDS, resulting in complete lysis within 2 h. The small lysate volume (0.4 ml) permits rapid DNA extraction in eppendorf tubes so that nucleic acids can be extracted and the DNA yield can be estimated within 2 h. Although significant RNA contamination is evident on a stained gel, this material in no way interferes with restriction enzyme digestion, or with blotting and detection procedures. Control DNAs, extracted from peripheral blood samples by a conventional procedure (Bruzdzinski et al., 1983), and amniocyte DNA, extracted by the rapid procedure, are indistinguishable in their suitability for gene mapping (see Fig. 2). DNA yields also are unaffected by the rapid lysis and extraction procedures.

(b) Resolution of *Mst* II β-globin gene fragments on mini-gels

In an attempt to further improve the efficiency and sensitivity of SCA prenatal diagnosis, we tested the usefulness of mini-gel electrophoresis. Our first concern was that the mini-gels provide sufficient resolution to clearly distinguish a 1.15-kb β^A fragment from a 1.35-kb β^S fragment. When 300-500-ng samples of *Mst* II-digested DNA are electrophoresed for 2 h in a 1.4% agarose gel, hybridization analysis shows that the 1.15- and 1.35-kb bands are clearly separated (Fig. 2).

Fig. 2 also shows that use of the 1.15-kb *Mst* II β^A fragment as a hybridization probe reduces the number of cross-hybridizing fragments and results in more uniform hybridization intensity with the β^A and β^S fragments than when larger β-globin probes are used (Chang and Kan, 1982; Orkin et al., 1982).

(c) Increased sensitivity of hybridization detection with the mini-gel format

When using standard-sized gels, the limit of detection for a single-copy gene fragment in human genomic DNA is on the order of 1–2 µg. As seen in Fig. 3, the use of mini-gels in the *Mst* II assay for SCA, permits the detection of the β^A and β^S fragments within 200 ng of *AS* genomic DNA in an overnight exposure. With increased exposure time (≥2 days) sensitivity is increased further to 50 ng.

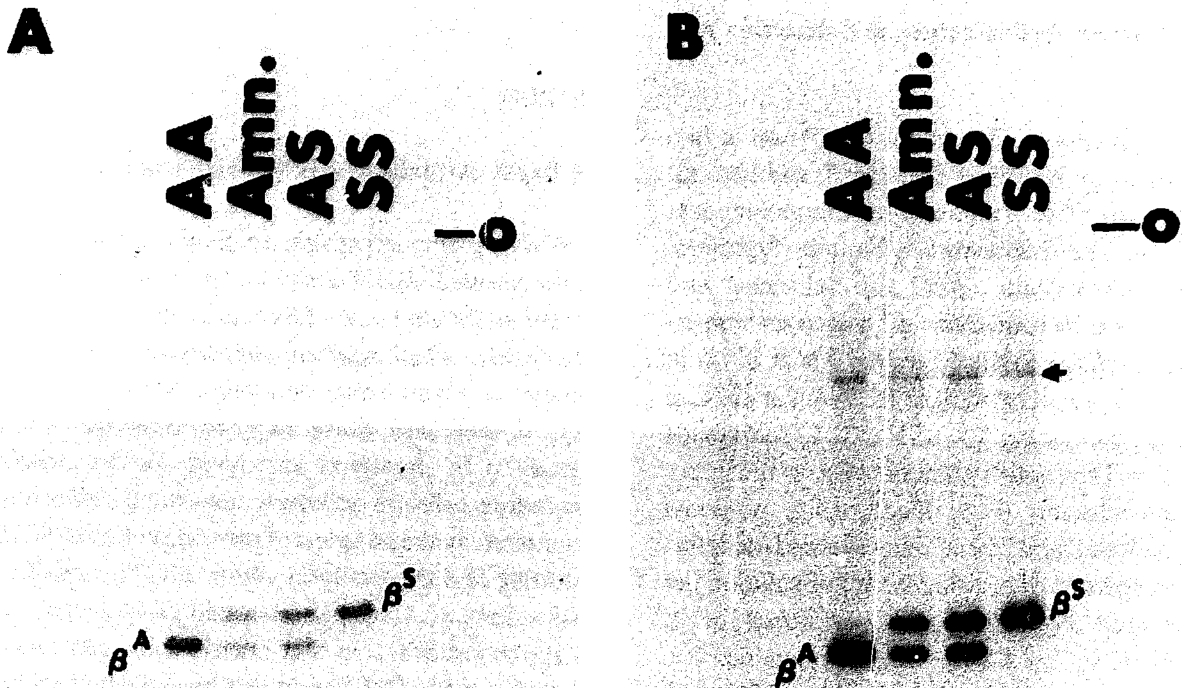

Fig. 2. Resolution of *Mst* II β^A (1.15 kb) and β^S (1.35 kb) fragments by mini-gel electrophoresis and hybridization detection. 500 ng samples of control DNAs (*AA*, *AS*, *SS*) and a 300 ng sample of amniocyte DNA (Amn.) were digested with *Mst* II, electrophoresed in a 1.4% agarose mini-gel, and transferred and hybridized with 100 ng of the nick-translated 1.15-kb, β^A, *Mst* II fragment (5×10^7 cpm/µg) as described in MATERIALS AND METHODS, sections b–d. (A) 12-h autoradiogram; (B) 60-h autoradiogram. A fainter, 6-kb, cross hybridizing δ-globin gene fragment also is detected (arrow). O, origin.

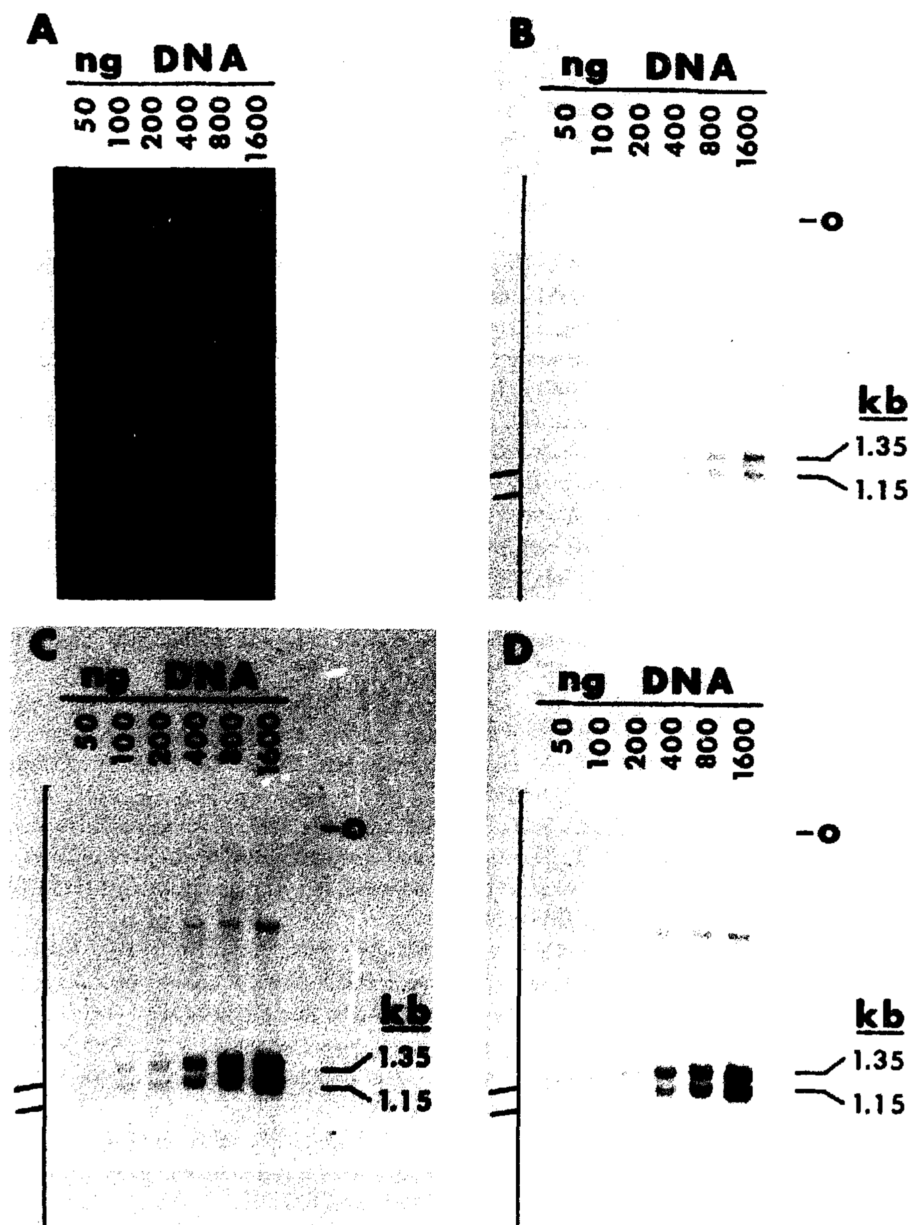

Fig. 3. Sensitivity of mapping single-copy gene fragments in DNA from a heterozygote. (A) Photograph of an EtBr-stained mini-gel containing *Mst*II-digested genomic *AS* DNA in the amounts indicated. (B–D) 16-h, 2.5-day, and 5-day autoradiograms, respectively, of the transferred and hybridized β^A (1.15-kb) and β^S (1.35-kb) fragments.

DISCUSSION

Previously, antenatal detection of SCA by *Mst*II mapping of the β^A and β^S alleles has required the analysis of at least 1 μg of fetal DNA over a period of about 2 weeks (Chang and Kan, 1982; Chang et al., 1982; Orkin et al., 1982; Wilson et al., 1982). With the DNA-extraction and mini-gel procedures we have developed, as little as 50 ng of genomic DNA can be analyzed to provide a reliable diagnosis within 5 days. When larger amounts of DNA are analyzed ($\geq$200 ng) diagnosis time can be reduced to 2–3 days (Table I). In addition to shortening diagnosis time, the increased sensitivity of the mini-gel assay permits diagnosis with less amniotic fluid. Thus far, we have isolated ample DNA from the cells contained in as little as 5 ml of amniotic fluid obtained at about 16 weeks gestational age. The volume

and the gestational age limits for the efficient recovery of DNA from amniotic fluid are still being investigated.

The increased sensitivity provided by mini-gel analysis does not involve significant loss of resolution. Under the conditions described, the resolution of DNA remains comparable to that obtained with larger gels. Together, these improvements offer several advantages: (1) antenatal detection at earlier gestational ages and/or with less amniotic fluid; (2) ability to perform several assays on the same DNA sample should the need arise; (3) no need to culture amniotic cells; and (4) conservation of enzymes and reagents.

These DNA extraction and mini-gel analysis procedures are generally useful for mapping gene fragments in genomic DNA, particularly when speed and sensitivity are desired. In addition to amniotic cells, we have used the rapid extraction procedure with minor modifications to prepare DNA from human peripheral blood buffy coats, from human fetal liver, and from whole mice. Apart from the *Mst*II assay for SCA, we have applied these techniques to: (1) mapping β^A and β^S alleles with *Dde*I; (2) mapping the $\beta^{D\ Punjab}$ allele with *Eco*RI; (3) mapping the β^E allele with *Hph*I; and (4) screening large numbers of DNA samples for restriction fragment length polymorphisms.

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