

Direct Detection of the Common Mediterranean β -Thalassemia Gene with Synthetic DNA Probes

AN ALTERNATIVE APPROACH FOR PRENATAL DIAGNOSIS

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ABSTRACT The most common form of β -thalassemia among Mediterraneans results from a single nucleotide substitution within the first intervening sequence (IVS-1) of the β -globin gene. This particular mutation is not detectable in uncloned DNA by restriction enzyme analysis. Using synthetic DNA of 19-nucleotides in length corresponding to the normal and mutant IVS-1 sequences as probes, we have developed a direct assay for this gene defect. Under carefully controlled experimental conditions these synthetic probes detect only their homologous sequences in restriction digests of both cloned and uncloned DNA samples. The method is sufficiently sensitive to establish the genotype of individuals with respect to this defect using $\sim 20 \mu\text{g}$ total DNA. This assay provides an alternative to fetal blood and DNA linkage analysis for the prenatal diagnosis of this variety of β -thalassemia, particularly among Greek families where it is especially common.

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INTRODUCTION

Recombinant DNA methods have permitted characterization of specific mutations in disorders of β -globin synthesis, the β -thalassemias (1). The most common form of β -thalassemia among Mediterranean patients is due to a single nucleotide change ($G \rightarrow A$) in the first intervening sequence (IVS-1)¹ of the β -globin gene (2, 3). This substitution generates a new splicing signal and leads to the formation of abnormally processed RNA that is unstable in vivo (4, 5). Since some normal messenger (m)RNA is produced as well, albeit in small amounts, this gene has a β^+ -thalassemia phenotype (4, 5). On the basis of gene cloning, DNA sequencing, and examination of linked DNA polymorphisms we estimated that about two-thirds of Greek and one-third of Italian β -thalassemia genes are of this type (1). Independent studies by Fukamaki et al. (5) and Ley et al. (6) are consistent with these findings.

As the DNA substitution in this common form of β -thalassemia does not alter a restriction enzyme site useful for gene mapping, we have explored the direct

¹ Abbreviations used in this paper: IVS, intervening sequence; kb, kilobase.

detection of this gene defect in DNA by small, synthetic DNA fragments (oligonucleotides) as hybridization probes. In pioneering studies Wallace et al. (7) have shown that a single nucleotide mismatch between a cloned gene sequence and an oligonucleotide is sufficient to destabilize the DNA-DNA hybrid relative to a perfect hybrid under carefully controlled conditions. Point mutations in DNA can then be detected by hybridization with appropriate oligonucleotides. In studies presented in abstract form Conner et al. (8) have described the detection of the β^s -gene mutation in uncloned DNA by this strategy.

Using synthetic oligonucleotides for the normal and mutant genes, we now report detection of the common Mediterranean β^+ -thalassemia gene in cellular DNA samples. Our findings form the basis of an alternate method for the prenatal diagnosis of this and other varieties of β -thalassemia.

METHODS

DNA samples. Suitable conditions for distinguishing normal and mutant gene sequences by hybridization were established using plasmid subclones that contained either a normal β -globin gene (9) or the common β -thalassemia gene found among Mediterraneans. This particular mutant β -globin gene, originally identified by Spritz et al. (2) and Westaway and Williamson (3), was independently isolated in our previous studies of β -thalassemia (1).

Genomic DNA were prepared by proteinase K digestion and phenol extractions (10) from peripheral blood leukocytes of individuals with known genotypes with respect to this particular β -thalassemia mutation. In prior work we have shown that this thalassemia gene is found on a chromosome with a specific pattern of polymorphic restriction sites that was designated haplotype I (1, 11). About 90% of haplotype I chromosomes of β -thalassemics of Mediterranean origin have this mutant gene (reference 1 and unpublished observations). Therefore, we selected a β -thalassemic Greek individual homozygous for haplotype I as a homozygote for the common β -thalassemia mutation. Her parents, both carriers for β -thalassemia, were chosen as obligate heterozygotes. The DNA of an unrelated Greek individual with β -thalassemia who was heterozygous for haplotype I and from whom we cloned the specific β -thalassemia gene provided an additional heterozygote sample in initial experiments.

Oligonucleotide synthesis and labeling. Oligonucleotides were prepared by a solid phase phosphotriester method (12) and labeled at the 5'-end with ^{32}P - γ -ATP (sp act 7,500 Ci/mmol, New England Nuclear, Boston, MA). 50–80 ng of oligonucleotide was incubated in standard kinase buffer (13) plus 100–150 μCi ^{32}P - γ -ATP and 5 U polynucleotide kinase (Boehringer Mannheim Biochemicals, Indianapolis, IN) in 10 μl total volume for 1 h at 37°C. Labeled oligonucleotide was separated from unincorporated ATP by passage through Bio-Gel P-4 (Bio-Rad Laboratories, Richmond, CA) in 10 mM Tris-HCl pH 7.5 and 0.1 mM EDTA. The specific activity of the probes used was 7–10 $\times 10^8$ dpm/ μg .

Blot hybridization. DNA samples were digested with the restriction enzyme BamHI (Bethesda Research Laboratories) and electrophoresed in 1% agarose at 1.5 V/cm for 16 h. DNA was blotted onto nitrocellulose filter sheets (Schleicher & Schuell, Inc., Keene, NH) as described by Southern (14)

in 6 \times SSC (0.9 M NaCl, 0.09 M Na citrate). Following baking at 80°C under vacuum for 2 h, filters were prehybridized in 6 \times SSC, five times concentrated Denhardt's solution (0.1% Ficoll, serum albumin, and polyvinylpyrrolidone [15]), 0.5% sodium dodecyl sulfate, 0.05% sodium pyrophosphate, and 100 $\mu\text{g}/\text{ml}$ sonicated and denatured salmon sperm DNA at 65°C for at least 3 h. Filters were then hybridized with labeled oligonucleotide at a concentration of 5 ng/ml in 6 \times SSC, two times concentrated Denhardt's solution (0.04% Ficoll, serum albumin, and polyvinylpyrrolidone), (100 $\mu\text{g}/\text{ml}$ yeast transfer (t)RNA, 100 $\mu\text{g}/\text{ml}$ salmon sperm DNA, 0.05% sodium pyrophosphate for 36–48 h at 41°C. Filters were rinsed in 500 ml 6 \times SSC plus 0.05% sodium pyrophosphate at room temperature, 37°, and 41°C for at least 30 min each. Final washing consisted of a 3-min incubation at 47° and 49°C for filters hybridized with the mutant and normal probes, respectively. Filters were exposed to x-ray film at –70°C with a lightening plus intensifying screen for 2–7 d.

RESULTS

As probes for the normal β -globin gene and the common β -thalassemia gene we prepared two 19 nucleotide long fragments, shown in Fig. 1. For simplicity, the normal gene probe is referred to as the "G" probe and the β -thalassemia probe as the "A" probe. Conditions were first established in which the normal and the common β -thalassemia genes could be distinguished by hybridization with these probes. Plasmid DNA containing these genes were digested with BamHI, electrophoresed in agarose, and blotted onto nitrocellulose filters. Replicate filters were hybridized separately with the oligonucleotide probes. Results are shown in Fig. 2. Upon digestion of normal or β -thalassemia gene DNA with BamHI the IVS-1 sequences that include the oligonucleotide sequence are found in a 1.8-kilobase (kb) fragment (9). The G probe, corresponding to the normal gene sequence, hybridized stably to this fragment in digests of the cloned normal β -gene, but hardly at all to the fragment from the

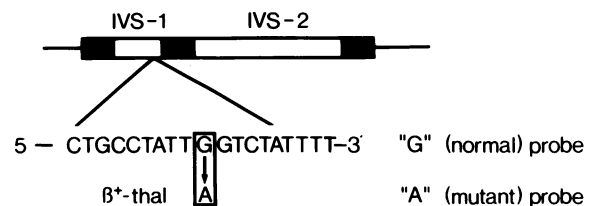


FIGURE 1 Synthetic DNA probes prepared for molecular hybridizations. The β -globin gene is depicted at the top with its three coding blocks (blackened) and two intervening sequences, IVS-1 and IVS-2. Below, the normal DNA sequence is depicted (9) and the single base substitution in the common β -thalassemia gene (2, 3) among Mediterraneans is enclosed in a box. The "G" probe corresponds to the normal 19-nucleotide sequence and the "A" probe to the mutant sequence for the same region.

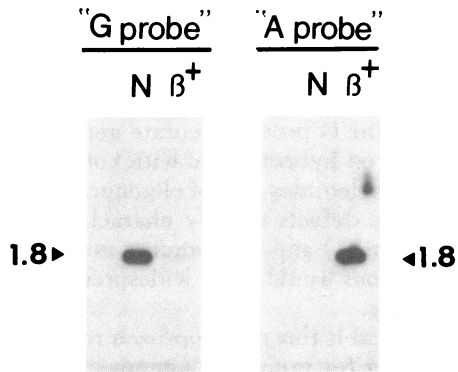


FIGURE 2 Hybridization of cloned normal and β -thalassemia genes to the synthetic oligonucleotides. 0.1 μ g plasmid DNA containing either the normal (N) or β -thalassemia genes was digested with BamHI, electrophoresed, and hybridized with the G and A probes (Methods). The location of the 1.8-kb DNA fragment carrying the portion of the β -gene with IVS-1 is shown by the arrows.

β -thalassemia gene. Conversely, the A or mutant gene probe hybridized stably with the corresponding fragment of the β -thalassemia gene and not with the normal fragment. These experiments establish that hybridization and washing conditions were chosen that satisfactorily discriminate between normal and β -thalassemia genes.

The usefulness of these oligonucleotide probes for direct detection of the common β -thalassemia mutation in uncloned total cellular DNA was analyzed by studying their hybridization to DNA samples of known genotypes. Replicate filters were prepared containing 10–15 μ g of BamHI digested DNA per lane, and then hybridized separately with the G and A probes in parallel. In these experiments, all genotypes with respect to the β -thalassemia mutation were included on a single filter to permit meaningful comparisons of hybridization patterns. Fig. 3 shows the results of this analysis. Attention should be directed to the region of

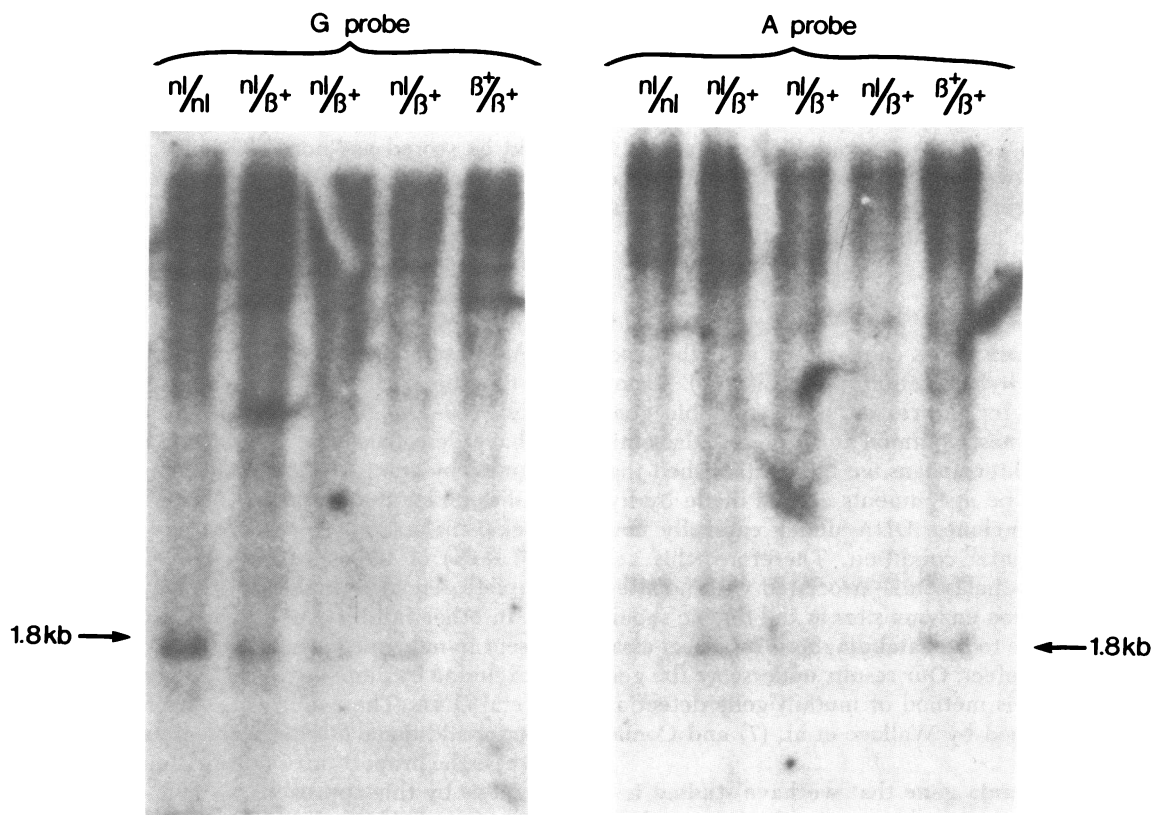


FIGURE 3 Hybridization of total cellular DNA with the synthetic oligonucleotides. Human DNA samples of known genotypes with respect to the common β -thalassemia mutation were assayed with the G and A probes. DNA samples were digested with BamHI, divided in half, and then subjected to electrophoresis and hybridization. 10–15 μ g DNA was applied per lane in the same relative order on each portion of the gel. Genotypes are normal (nl/nl), heterozygous (nl/β^+), and homozygous β^+ -thalassemia (β^+/β^+). Autoradiography was 6 d.

the autoradiogram corresponding to DNA fragments of 1.8 kb in length. Normal DNA (*nl/nl*) hybridized only with the G, or normal, probe. DNA of heterozygous individuals (*nl/β⁺*) hybridized more weakly with both G and A probes. DNA from a homozygote (*β⁺/β⁺*) hybridized only with the A, or mutant, probe. The hybridization results establish the genotypes of these DNA independent of other data. Additional hybridizing fragments of different lengths and identical in all samples seen with each probe represent other DNA fragments in the human genome with sequences very nearly identical to those of the oligonucleotides. The absence of the 1.8-kb band in homozygous mutant DNA hybridized with the normal probe and the absence of this band in normal DNA hybridized with the mutant probe demonstrates directly the validity of the assay. The additional, nonglobin fragments that are seen do not interfere with the interpretation but can serve as internal controls for proper enzyme digestion, electrophoresis, and hybridization in each lane. Seven other DNA samples were analyzed blindly with the oligonucleotide assay and the deduced genotypes compared with those assigned independently by other criteria. In no instances did the findings conflict. Our results indicate that genotypes can be defined confidently with autoradiographic exposures of 7 d or less when at least 20 μg DNA is available for study (10 μg per lane and two probes). Less DNA might suffice using much longer autoradiographic exposures, but this would not be practical for diagnostic purposes.

DISCUSSION

Synthetic oligonucleotides can be used as highly specific molecular hybridization probes (7, 8). Using probes prepared for the region of the β -globin gene mutated in the most common form of β -thalassemia seen among Mediterraneans we have established that definitive genotype assignments can be made by hybridization of uncloned DNA under carefully controlled experimental condition. Therefore, this and other forms of β -thalassemia associated with no alterations in restriction enzyme sites in the β -gene should now be amenable to prenatal diagnosis by direct assay of the primary defect. Our results underscore the general nature of this method of mutant gene detection, originally proposed by Wallace et al. (7) and Conner et al. (8).

The β -thalassemia gene that we have studied is of particular importance due to its high prevalence among Mediterranean patients (1, 5, 6). Among many families of Greek or Italian ancestry the method described here should provide a reasonable alternative to fetal blood assay (16) and DNA linkage analysis (17, 18) for prenatal diagnosis. In practice, examination of

parental DNA will be required to establish the type of thalassemia mutation carried by each. This is essential because β -thalassemia mutations other than the common variety studied here will appear normal when assessed with the G probe. Accurate genotype assignments depend on hybridization with both normal and mutant oligonucleotides. Sets of oligonucleotide probes for other gene defects already characterized among Mediterraneans (1) and for common mutant genes in other populations would allow widespread application of this strategy.

How practical is this new approach relative to fetal blood sampling for prenatal diagnosis of hemoglobinopathies? Several strengths of this method should be noted. First, it identifies the primary gene mutation, rather than a phenotypic consequence. Second, the method is quite sensitive; ~20 μg of DNA is required to permit hybridization with both probes. Third, the absence of DNA sequence polymorphism nearby any of the known β -thalassemia mutations (9) indicates that normal sequence variation will not interfere with this approach. On the other hand, there are two practical drawbacks. First, study of parental DNA is required to permit complete genotyping of the fetus due to genetic heterogeneity. This is necessary because all β -thalassemia mutations other than that studied here would be scored as "normal" genes using the G and A oligonucleotides, as their defects reside in other regions of the β -globin gene. Second, the requirement for 20 μg DNA necessitates culture of amniotic fluid cells to provide sufficient material for diagnosis. A substantial increase in the specific activity of the probes would circumvent this problem and permit assay of DNA extracted directly from amniotic fluid cells, as can now be performed in the sickle-cell anemia diagnosis (19–21). Nevertheless, at present, the method we have described here should be useful in prenatal diagnosis by amniocentesis, particularly among Greek families. Since the frequency of this mutation among Greek β -thalassemia genes is roughly 67%, nearly half (0.67×67) of Greek families would be suitable for complete genotype analysis of this mutation in the fetus. In other families in which this mutant gene was present in only one parent, severe β -thalassemia could be excluded by demonstrating the absence of this gene in fetal DNA. The availability of sets of oligonucleotides for additional β -thalassemia mutations will greatly increase the proportion of families suitable for prenatal diagnosis by this approach.

The use of direct detection of β -thalassemia mutations by hybridization with synthetic probes is likely to become substantially more attractive as an alternative to midtrimester fetal blood sampling if the safety of first trimester chorionic villi biopsies (22) is established. Early experience with this procedure has

been promising. Specimens yielding $\sim 7 \mu\text{g}$ DNA/villus, or several times that obtainable directly from uncultured amniotic fluid cells, have been obtained at 6–10 wk gestation (22). If the safety of this procedure is demonstrated in more extensive studies, early and reliable diagnosis of β -thalassemia could be then achieved in all cases by direct DNA assays of the type described here.

Note added in proof. A full account of detection of the β^+ -gene with oligonucleotides has recently appeared: Conner, B. J., A. A. Reyes, C. Morin, K. Itakura, R. L. Teplitz, and R. B. Wallace. 1983. Detection of sickle cell β^+ -globin allele by hybridization with synthetic oligonucleotides. *Proc. Natl. Acad. Sci. USA.* 80: 278–282.

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