note on methodology

Detection of the apoB-3500 mutation (glutamine for arginine) by gene amplification and cleavage with MspI

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Summary A single primer-template mismatch 2 bp from the apoB-3500 (G to A) mutation permits introduction of a cleavage site for MspI (C/CGG) in normal alleles but not in mutant alleles (CCAG). After amplification, cleavage, and polyacrylamide gel electrophoresis, normal and mutant alleles could be unambiguously distinguished. We constructed a positive (homozygous mutant) standard by site-directed mutagenesis. A negative standard was DNA from a homozygous normal subject. The method enables us to screen for the mutation with 12 μl of spotted whole blood as the source of DNA. —**Hansen, P.S., N. Rüdiger, A. Tybjaerg-Hansen, O. Faergeman, and N. Gregersen. Detection of the apoB-3500 mutation (glutamine for arginine) by gene amplification and cleavage with MspI.** *J. Lipid Res.* 1991. **32:** 1229–1233.

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Apolipoprotein B-100 (apoB-100) is a protein constituent of low density lipoproteins (LDL) and very low density lipoproteins (VLDL); binding of apoB-100 to the LDL receptor is necessary for the normal removal of LDL from plasma (1). A single nucleotide mutation in the apoB gene at codon 3500 (CGG to CAG) causes a glutamine for arginine substitution in apoB. This mutation has recently been reported to be associated with or to cause "familial defective apoB-100 (FDB)." The disorder is inherited by autosomal co-dominant transmission and is associated with hypercholesterolemia (2–4). The apoB-3500 mutation has been found in the USA, Canada, the UK, Denmark, Italy, Germany, and Austria (5). Although estimates of frequency are based on studies in different populations, the mutation may be at least as frequent as heterozygous familial hypercholesterolemia, which, by mainly clinical criteria, occurs in 1:500 in most populations (1). The apoB-3500 mutation seems to increase LDL-cholesterol (5–8), but because of differences in the design of published studies, the impact of the FDB mutation on atherosclerosis cannot be established at present (for review, see reference 8).

The mutation has been demonstrated by probing DNA, amplified by the polymerase chain reaction (PCR), with radiolabeled allele-specific oligonucleotides with a slot-blot technique (2, 6). Because this technique is laborious and requires ^{32}P-labeled probes, it is not suitable for the large scale studies necessary to determine the frequency of the mutation, the relationship of the mutation to type and degree of hyperlipidemia, and the contribution of the mutation to atherosclerotic disease.

In the following report we describe a technique for rapid and reliable detection of the mutation in heterozygous or homozygous form, which may be suitable for such studies.

SUBJECTS AND METHODS

DNA was prepared from two groups of subjects: a Danish patient, who previously was shown to have the FDB mutation by use of allele-specific oligonucleotides (6), and two normolipidemic volunteers.

Genomic DNA was initially prepared from whole blood by phenol–chloroform extraction and ethanol precipitation (9). After optimizing the conditions of the PCR, we used DNA prepared from blood spots as described by Jinks et al. (10). Approximately 50 μl of whole blood was spotted on Whatman 3 MM filter paper. After drying, the spot was fixed with methanol. Approximately a quarter of the blood spot was mixed with 100 μl of water and placed in a boiling water bath for 15 min to lyse the cells, and 50 μl of this mixture was used as DNA source for amplification. This DNA preparation technique allowed us to screen for the mutation in DNA from only 12 μl of whole blood.

Oligonucleotides were synthesized with an Applied Biosystem DNA-synthesizer (**Fig. 1**). All cDNA positions are defined by Yang, Chan, and Gotto (11). Oligonucleotide "3", ending at the base 5' to the mutation, was constructed to generate an MspI endonuclease cleavage site when the first base incorporated by extension was a guanine (G) (wild type). In contrast, incorporation of an adenine (A) (mutant) did not create a recognition site for MspI (**Fig. 2**). Oligonucleotide "5" was synthesized so that an adenine (A) was introduced at the position of the apoB-3500 mutation (cDNA position 10.658 (11)), thereby creating an "artificial mutant" by amplification (**Fig. 3**).

Abbreviations: apoB-100, apolipoprotein B-100; FDB, familial defective binding apoB-100; RFLP, restriction fragment length polymorphism; bp, base pairs; LDL, low density lipoprotein; VLDL, very low density lipoprotein; PCR, polymerase chain reaction; A, adenine; C, cytosine; G, guanine; T, thymine; DNA, deoxyribonucleic acid; FH, familial hypercholesterolemia.

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Oligonucleotides	cDNA Position
1. CCTACTGTCTCTTTCCTCATGG	10.431-10.452
2. CTAAGGATCCTGCAATGTCAAGGT	11.073-11.050
3. CCAACACTTACTTGAATTCCAAGAGCACCC	10.628-10.657
4. CTGTGCTCCCAGAGGGAATATATGCGTTGG	10.776-10.747
5. CAACACTTACTTGAATTCCAAGAGCACGCA	10.629-10.658

Fig. 1. Sequences and cDNA position of oligonucleotides used; cDNA position according to reference 11.

Oligonucleotides "3" and "4" were used for typing the apoB-3500 mutation in one PCR followed by cleavage with MspI (**Fig. 4**).

PCR was conducted in an automated Perkin-Elmer/Cetus thermal cycler with denaturation at 94°C for 1 min, annealing at 58°C for 1 min, and primer extension at 74°C for 4 min for a total of 40 cycles. *Thermus aquaticus* DNA polymerase was obtained from Cetus and used at a concentration of 2 U/100 μl. The reaction was performed in a total volume of 100 μl containing 20,000 pmol dCTP, dGTP, dATP, and TTP, 10 μl amplification buffer (10 × ampl. buffer: 500 mM KCl, 100 mM Tris-HCl, 15 mM $MgCl_2$, 0.1% (w/v) gelatin, pH 8.3), 0.2 μg (22 pmol) of each primer, and 0.2–0.4 μg genomic DNA. The mixture was overlaid with 100 μl mineral oil.

The performance of the amplification was checked by electrophoresis on a 3% agarose gel of 10 μl of the final reaction mixture. To exclude contamination by previously amplified sequences, blanks composed of the total mixture, except target DNA, were included in every PCR.

MspI digestion

In early experiments, the amplified DNA was extracted with phenol and chloroform and precipitated with ethanol following standard protocols (9). Later we used the amplified DNA directly for digestion with MspI (Fig. 3). Ten units of MspI were added to a volume of 10 μl consisting of amplified DNA and a low salt buffer (10 × low salt buffer: 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM9 dithiothretiol, pH 7.5). The reaction was performed overnight at 37°C.

Polyacrylamide gel electrophoresis

The amplified and cleaved product was analyzed by electrophoresis on a 12% polyacrylamide gel followed by staining in ethidium bromide. DNA fragments were visualized on a standard UV-transilluminator.

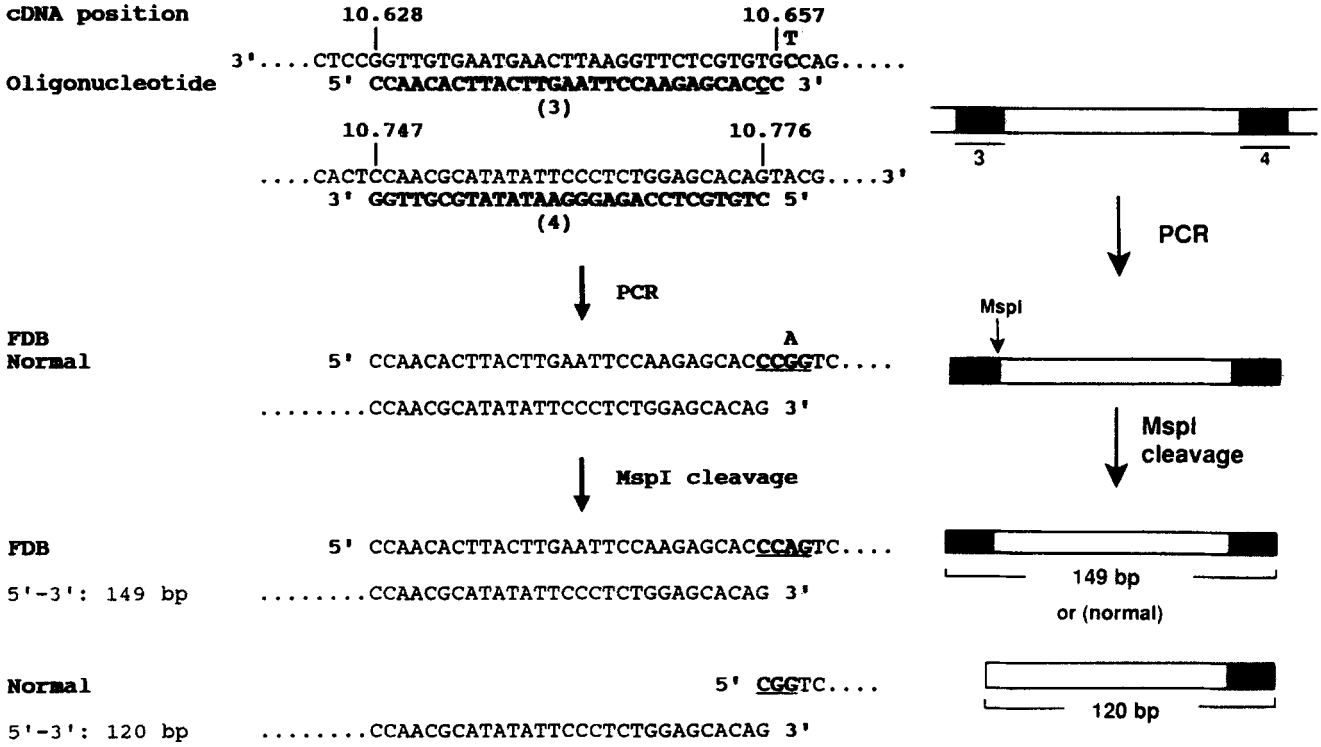

Fig. 2. Schematic presentation of the introduction of an MspI cleavage site. Amplification of a normal allele with oligonucleotide "3" creates an MspI cleavage site that is not present when the mutant allele is amplified. After cleavage with MspI, either a 120-bp fragment (wild type) or a 149-bp fragment (mutant type) is produced.

Construction of mutant DNA

Routine use of the method requires availability of negative (homozygous wild-type) and positive (homozygous mutant) standards. In the absence of naturally occurring homozygous mutant DNA, we constructed, by site-directed mutagenesis, pure artificial mutant DNA. DNA representing either the normal or the mutant allele was prepared using oligonucleotides "1", "5", and "2" in a PCR followed by GeneClean (Bio 101) extraction from a 3% agarose gel. In the assay, the extracted DNA was further amplified with oligonucleotides "3" and "4" under the conditions described above before phenol–chloroform extraction and ethanol precipitation of the final product. MspI was added to a 10-μl aliquot for total digestion at 37°C overnight. The digested material was analyzed by polyacrylamide gel electrophoresis (Fig. 3).

RESULTS

Fig. 3 shows gel-separated products of apoB gene amplification and MspI digestion of DNA prepared from whole blood by conventional phenol–chloroform extraction and ethanol precipitation from a homozygous normal subject and a patient heterozygous for the FDB mutation (lanes 2,3 and 4,5 −/+ MspI digestion). Lanes 6–9 show the results of the analysis of control DNA either homozygous for a G (6,7) or an A (8,9), −/+ MspI digestion. Fig. 3 thus shows a typical gel pattern for screening procedures, including DNA from normal subjects and an affected individual as well as controls known to be homozygous for either the normal G or the mutant A.

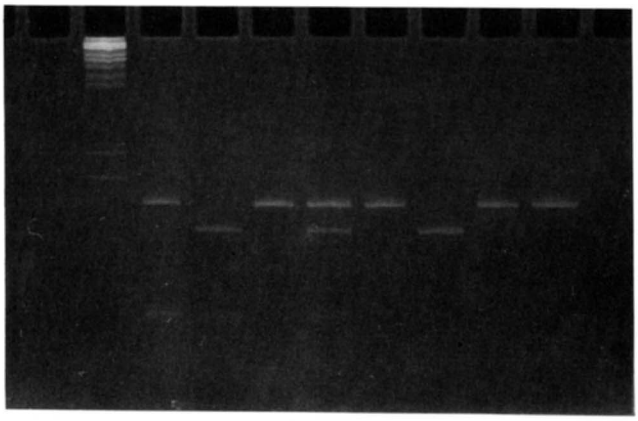

Fig. 3. Site-directed mutagenesis creating PCR products with or without the actual point mutation. Lane 1: lambda-dra marker; lanes 2-5: normal DNA (lanes 2 and 3) and heterozygous mutant DNA (lanes 4 and 5) −/+ MspI digestion; lanes 6–9: PCR produced "normals" and "mutants" −/+ MspI digestion.

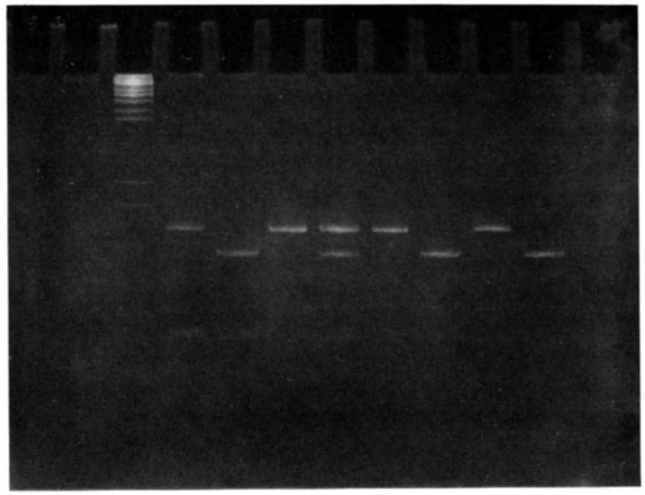

Fig. 4. Polyacrylamide gel electrophoresis of amplified normal and FDB DNA cleaved with MspI as well as cleaved products from different preparations of filter-paper DNA. Lane 1: Lambda CI 857, digested with DraI, used as a marker (lambda-dra). Lanes 2 and 3: Normal DNA isolated by phenol–chloroform extraction and ethanol precipitation −/+ MspI digestion. Lanes 4 and 5: DNA from the FDB proband −/+ MspI digestion. Two bands are seen, respectively, 149 bp and 120 bp long (heterozygous for the mutation). Lanes 6–9: Normal DNA isolated from filter paper by boiling (lanes 6 and 7) with phenol–chloroform extraction and ethanol precipitation of the PCR product before MspI digestion; lanes 8 and 9, −/+ MspI digestion without extraction and precipitation of the PCR product.

In an attempt to simplify the mutation detection assay, we tried different methods of DNA preparation as well as different methods of preparing the PCR product before MspI digestion. Fig. 4 shows gel-separated products of apoB gene amplification and MspI digestion of DNA prepared from whole blood by conventional phenol–chloroform extraction and ethanol precipitation (lanes 2–5), of blood spot DNA amplified and prepared with phenol–chloroform–ethanol before MspI digestion (lanes 6–7) as well as from amplified and digested blood spot DNA without further procedures (lanes 8–9).

Wild-type DNA gives a band of 120 bp (both alleles cleaved). DNA from the FDB patient, who is heterozygous for the mutation, gives bands of 149 bp (mutant allele) and 120 bp (wild type allele). The wild-type pattern is easily recognized whether we use conventionally isolated DNA from whole blood leukocytes, blood spot DNA which is phenol–ethanol-treated after amplification, or untreated amplified blood spot DNA. In some of the lanes, a weak low molecular weight band is seen representing "primer-dimer" formation. However, this band did not lead to problems in typing.

DISCUSSION

The aim of the present study was to establish a rapid and reliable method for detecting the apoB-3500 muta-

tion in a manner suitable for studies of the prevalence of the mutation in the population as a whole or in subgroups with hyperlipidemia and/or clinical atherosclerosis. Slot-blot analysis of radiolabeled allele-specific oligonucleotides gives reliable results, but the technique is time-consuming and costly, and it involved the use of ^{32}P. It is therefore not well suited for large-scale epidemiologic studies.

An alternative approach is based on selective amplification of the normal or the mutant allele. Several investigators (12–14) have demonstrated that a single primer-template mismatch at the 3' position of an oligonucleotide, under optimal conditions, markedly reduces the yield from the PCR (Amplification Refractory Mutation System - ARMS). We tested the ability of the ARMS assay to detect the FDB mutation with an oligonucleotide containing a G as the 3' nucleotide and another containing an A at the 3' position. Even after introduction of another destabilizing mismatch two bases from the mutation, we could not demonstrate sufficient selectivity of the amplification to discriminate normal from mutant alleles. Instead we exploited this non-selectivity to introduce the G to A mutation at cDNA position 10.658, thereby constructing artificial DNA strands containing the mutant sequence as a positive control in the assay.

In some instances the point mutation of interest generates or eliminates sites of cleavage for restriction endonucleases. This is the case for the apoE polymorphism detectable with the restriction endonuclease HhaI (15). In other instances, as in the case of the apoB-3500 mutation, the point mutation of interest does not condition cleavage by currently available endonucleases. However, by using appropriate oligonucleotides in the PCR, a cleavage site can be introduced into the PCR product by changing a single base. Cleavage with restriction endonucleases will then be a function of presence or absence of the point mutation of interest (16).

For the FDB sequence a single alteration of an A to a C produces a C-T mismatch one base from the 3' nucleotide in oligonucleotide "3". Extension of the first copy of genomic DNA from the oligonucleotide "3" therefore produces a base composition of CCGG in wild type alleles and CCAG in mutant alleles. Normal but not mutant alleles can then be cleaved by the endonuclease MspI, as demonstrated with artificially constructed DNA as well as with DNA from normolipidemic subjects and a patient previously shown to be heterozygous for the FDB mutation by use of allele-specific oligonucleotides (Fig. 3).

As demonstrated (Fig. 3), the method can be used upon DNA from only 12 μl of whole blood, prepared by methanol fixation and boiling before amplification and MspI digestion. The method may therefore be suitable for large-scale epidemiologic studies of the FDB mutation.

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