Identification of apolipoprotein E polymorphism by using synthetic oligonucleotides

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Summary A procedure based on selective hybridization with allele-specific oligonucleotides was developed for typing apolipoprotein E variants from human genomic DNAs. Two sets of oligonucleotides were synthesized and used to discriminate either between $\epsilon 3$ and $\epsilon 4$ alleles or between $\epsilon 3$ and $\epsilon 2$ alleles. Combination of the allele-specific oligonucleotide hybridization with the method for in vitro DNA amplification (Polymerase Chain Reaction) (Saiki, R. K. et al. 1985. *Science.* **230:** 1350–1354) (1) dramatically improved the sensitivity and the reliability of the procedure. Adaptation of a simple strategy involving direct cloning and DNA sequencing of in vitro amplified DNA enables rapid identification of any mutation within the apoE gene area encoding the receptor binding domain. — **Smeets, H. J. M., J. Poddighe, P. M. J. Stuyt, A. F. H. Stalenhoef, H. H. Ropers, and B. Wieringa.** Identification of apolipoprotein E polymorphism by using synthetic oligonucleotides. *J. Lipid Res.* 1988. **29:** 1231–1237.

Supplementary key words DNA typing with oligonucleotides • DNA in vitro amplification • rapid cloning of gene variants

Apolipoprotein E (apoE) is a constituent of plasma lipoproteins such as chylomicrons, very low density lipoproteins (VLDL), and high density lipoproteins (HDL). The major physiological function of apoE is its mediation of uptake of lipoproteins through specific cell surface receptors and its regulation of cholesterol metabolism (2). In addition, apoE may play a role as regulator of cellular activity in the immune system (3) and in development and regeneration of the nervous system (4). In humans, three major apoE isoproteins, apoE-2, E-3, and E-4 are known (5). The synthesis of these isoproteins is genetically controlled by three co-dominant alleles ($\epsilon 2$, $\epsilon 3$, and $\epsilon 4$) on one polymorphic locus assigned to chromosome 19q. From epidemiological studies, the alleles $\epsilon 2$ and $\epsilon 4$ are found associated with hypertriglyceridemic and hypercholesterolemic states, respectively (6). Type III hyperlipoproteinemia, characterized by an accumulation of remnants of triglyceride-rich proteins, is associated with the apoE phenotype E2/2 (7). ApoE-2 has a reduced ability to bind apoB,E receptors (8). In addition, there is a questionable relationship between type V hyperlipoproteinemia and apoE4/4 (9, 10).

Besides apoE-2 (cys_{112}, cys_{158}), E-3 (cys_{112}, arg_{158}) and E-4 (arg_{112}, arg_{158}) (11), several other rare apoE isoproteins have recently been described. Those variants which bear a conformational distortion of the receptor binding domain (amino acid residues 140–160 (12)) predominantly are found associated with type III hyperlipoproteinemia (12–17). Others are associated with different types of hyperlipoproteinemia or with no disease at all (18–20).

Most of our present knowledge of apoE protein variants has been gathered through amino acid analysis and protein sequencing (reviewed in ref. 21). ApoE phenotyping is routinely done by isoelectric focusing of VLDL apolipoproteins. As this method is based on charge differences between the apoE isoproteins, it is not possible to identify neutral amino acid substitutions or neutralizing double mutants, nor to locate the site of the mutation. Moreover, this apoE protein typing cannot be applied in those cases where only limited amounts of serum are available. Since many apoE variants have recently been characterized at the DNA level (20, 21), it is now possible to solve these problems by application of DNA typing methods. In this report we present a new method for routine detection of apoE alleles based on hybridization with allele-specific oligonucleotides and the polymerase chain reaction (PCR) technique of Saiki et al. (1). The method can be applied to detect any pretyped mutation within the gene area encoding the receptor binding region of apoE or, after modification, for any other relevant portion of the gene. The potential value of the method is demonstrated by genotyping individuals homo- or heterozygous for the common E3 (cys_{112}, arg_{158}), E2 (cys_{112}, cys_{158}), and E4 (arg_{112}, arg_{158}) proteins. In addition, we present a rapid technique for the identification of new mutations in the portion of the apoE gene encoding the receptor binding domain. For either application only minute quantities of human DNA are required. The potential of these techniques in the discovery and typing of apoE variants in patients at risk for hyperlipoproteinemia and atherosclerosis will be discussed.

MATERIALS AND METHODS

Sera and DNAs

Blood samples were obtained from patients who visited the lipid clinic in our hospital. ApoE typing in sera of patients and normal individuals was as described earlier (22, 23). Plasmid pEB4 containing a 930-bp insert of human apoE3 cDNA (24) was a gift from Dr. S. Humphries, London. High molecular weight DNA from peripheral blood was isolated according to Aldridge et al. (25) with minor modifications.

Abbreviations: apo, apolipoprotein; ϵ, apolipoprotein E allele; VLDL, very low density lipoproteins; HDL, high density lipoproteins; PCR, polymerase chain reaction.

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Oligonucleotides and in vitro amplification of genomic DNA

Oligonucleotides used as starting primers for the polymerase chain reaction and for detection of ε3, ε4, and ε2 alleles were synthesized by the phosphoramidite method using a Cyclone DNA synthesizer from Biosearch Inc., New Brunswick Scientific Co. Oligonucleotides were eluted from the column-support and deprotected by treatment in 25% (w/v) ammonia, purified by acrylamide gel electrophoresis where necessary, recovered by precipitation in four volumes of ethanol, and finally dissolved in water at a concentration of 1 μg/μl and stored until use at −20°C.

The PCR technique used to amplify local areas of the apoE gene in genomic DNA was a slightly modified version of the original method of Saiki et al. (1). The reaction was started with 1 μg human DNA in a buffer containing 7 mM Tris-HCl, pH 7.5, 7 mM $MgCl_2$, 20 mM NaCl, 0.1 mM EDTA, 1.5 mM DTT, 10% v/v dimethylsulphoxide (DMSO), 1.5 mM dATP, 1.5 mM dGTP, 1.5 mM dCTP, 1.5 mM dTTP, and 1 μM each of the amplification primers (oligonucleotides AE-1 and AE-2, see text) in a total volume of 100 μl. For the first cycle the reaction mixture was heated at 95°C for 5 min to denature the DNA prior to annealing the primers at 41°C for 2 min. Next, 1 unit of Klenow DNA polymerase I (BRL) (in buffer as specified above) was added and primer elongation was allowed to proceed for 4 min at 41°C. For all subsequent cycles the denaturation step was shortened to 2.5 min. The total number of consecutive cycles used is further specified in the text. The amplified DNA was recovered by ethanol precipitation and one third was electrophoresed on a 4% (w/v) agarose minigel in 40 mM Tris-acetate, pH 7.8, 1 mM EDTA, until the bromophenol blue marker dye reached 4 cm.

Southern blotting and oligonucleotide analysis

Restriction endonuclease-digested DNA or DNA resulting from PCR amplification was immobilized on either Biotrace-RPM (Gelman) or Gene Screen Plus (New England Nuclear) membranes by conventional Southern blotting, using conditions as given by the manufacturers. For dot-blotting, DNA mixtures were denatured in 0.25 N NaOH, diluted to 0.125 N NaOH, 0.125 × SSC (10 × SSC = 0.15 M Na citrate, 1.5 M NaCl, pH 7) and immobilized on Gene Screen Plus membrane using a Schleicher and Schuell dot-blot manifold.

Oligonucleotides were 5'-end-labeled with T4-polynucleotide kinase (11.5 U, Pharmacia–P&L Biochemicals) in a 10-μl reaction mixture containing 2 pmol of oligonucleotide, 4 pmol of γ-^{32}P ATP (Amersham, > 5000 Ci/mmol) in 50 mM Tris-HCl, pH 9.5, 10 mM $MgCl_2$, 5 mM DTT for 30 min at 37°C. Unincorporated γ-^{32}P ATP was removed by passage through a Sephadex G50 column. Specific activity was between 10^8 and 10^9 cpm per μg.

Filter-immobilized DNA was prehybridized in 5 × SSPE (20 × SSPE = 3.0 M NaCl, 200 mM NaH_2PO_4, 20 mM EDTA, pH 7.4), 0.3% (w/v) SDS, and 10 μg/ml single-stranded herring sperm DNA (Gene Screen Plus) or 0.5 mg/ml (Biotrace). Hybridization was performed overnight (16 hr) under similar conditions at probe concentrations of 0.3–1 × 10^6 cpm/ml and temperatures as specified below or in the text. Stringency conditions of hybridization and washing were dependent on the set of oligonucleotides used. ApoE-3 and apoE-4 specific oligonucleotides AE-t112 and AE-c112 were hybridized at 65°C and washing was done in two cycles of 5 × SSPE, 0.3% (w/v) SDS for 5 min each, followed by one wash at 65°C for 10 min in 1 × SSPE, 0.3% (w/v) SDS. ApoE-2- and apoE-3-specific oligonucleotides AE-t158 and AE-c158 were hybridized at 60°C and washed three times for 5 min at 63°C in 5 × SSPE, 0.3% (w/v) SDS. Autoradiographic exposure to Kodak X-Omat S film was at −70°C with two intensifying screens (Cronex Lightning Plus, Dupont) for the periods given.

Cloning and sequencing of in vitro amplified apoE DNA

One tenth of the amplified DNA (equivalent of 15 μl reaction mixture, either diluted directly or following ethanol precipitation) was digested with 5 units of endonuclease PstI (BRL) in a total volume of 50 μl for 1 hr at 37°C. Next, the DNA was phenol-extracted, recovered by ethanol precipitation, and dissolved in 10 μl sterile water. An amount of DNA equivalent to 1% of the amplified DNA was ligated into 20 ng pGEM3 plasmid vector (Promega Biotec). Vector DNA was PstI-cleaved, treated with calf intestine alkaline phosphatase, and purified on 1% (w/v) low gel temperature agarose (Bio-Rad). Ligation was for 16 hr in 66 mM Tris-HCl, pH 7.2, 7 mM $MgCl_2$, 10 mM DTT, 0.2 mM ATP, and 400 U T4-ligase (New England Biolabs) in a volume of 10 μl at 16°C in the presence of the agarose (26). The ligation mixture was heated to 65°C for 5 min, diluted, and transfected into *E. coli* HB101. Resulting Amp^r recombinant colonies were replicated onto nitrocellulose filters and screened with a 5'-^{32}P-labeled oligonucleotide (5'-gcgcacccgcagctcctcg, codons 131–137) at 55°C in 5 × SSPE, 0.3% w/v SDS, 10 μg/ml herring sperm DNA. Washing was done in the same buffer without herring sperm DNA at 55°C. Positively hybridizing colonies identified by autoradiography were purified and grown for DNA preparation. Plasmid DNA was purified and sequenced by the dsDNA method essentially as described by Hattori and Sakaki (27), using T7 and/or SP6 sequencing primers (Promega Biotec).

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RESULTS

Identification of apoE DNA polymorphisms

From gene sequencing data it is known that the apoE-2 and apoE-3 genes differ only by having either a C or T nucleotide at the first nucleotide of codon 158. In the apoE-3 and apoE-4 genes only the first nucleotide of codon 112 (C or T, respectively) is at variance. In analogy to methodology already published (28, 29), we have used allele-specific oligonucleotides for the identification of these three apoE gene variants. The two sets of synthetic oligonucleotides used to distinguish the apoE-3 and E-4 specific codons (oligomers AE-t112 and AE-c112) and the apoE-2 and E-3 specific codons (oligomers AE-t158 and AE-c158) are given in **Fig. 1.** The most fruitful conditions for discriminating between mismatched and perfectly matched duplexes (see Materials and Methods) were empirically established for each set of oligonucleotides on dot-blots of plasmid pEB4 DNA (results not shown). Subsequently, we tested hybridization and washing conditions on genomic DNAs from individuals with known apoE phenotypes. For this, restriction endonuclease EcoRI-cleaved DNA was resolved by agarose gel electrophoresis and subjected to the discriminative hybridization and washing cycles in a conventional Southern analysis procedure with the AE-c112 and AE-t112 oligonucleotides. As shown in **Fig. 2,** results were in agreement with the apoE phenotype inferred from protein typing, though the hybridization signals at the 2-kbp band were rather weak, especially with the E3-specific AE-t112 oligonucleotide.

We therefore decided to employ the PCR method for DNA amplification in vitro (1, 30, 31). The amplification-priming oligonucleotides selected (oligonucleotides AE-1 and AE-2) are displayed in Fig. 1. They are 188 bp apart and bracket the complete receptor binding domain encoding region of apoE. The reaction conditions for the PCR procedure were established by tests of the AE-1 and AE-2 primers on plasmid pEB4 DNA (24). The conditions

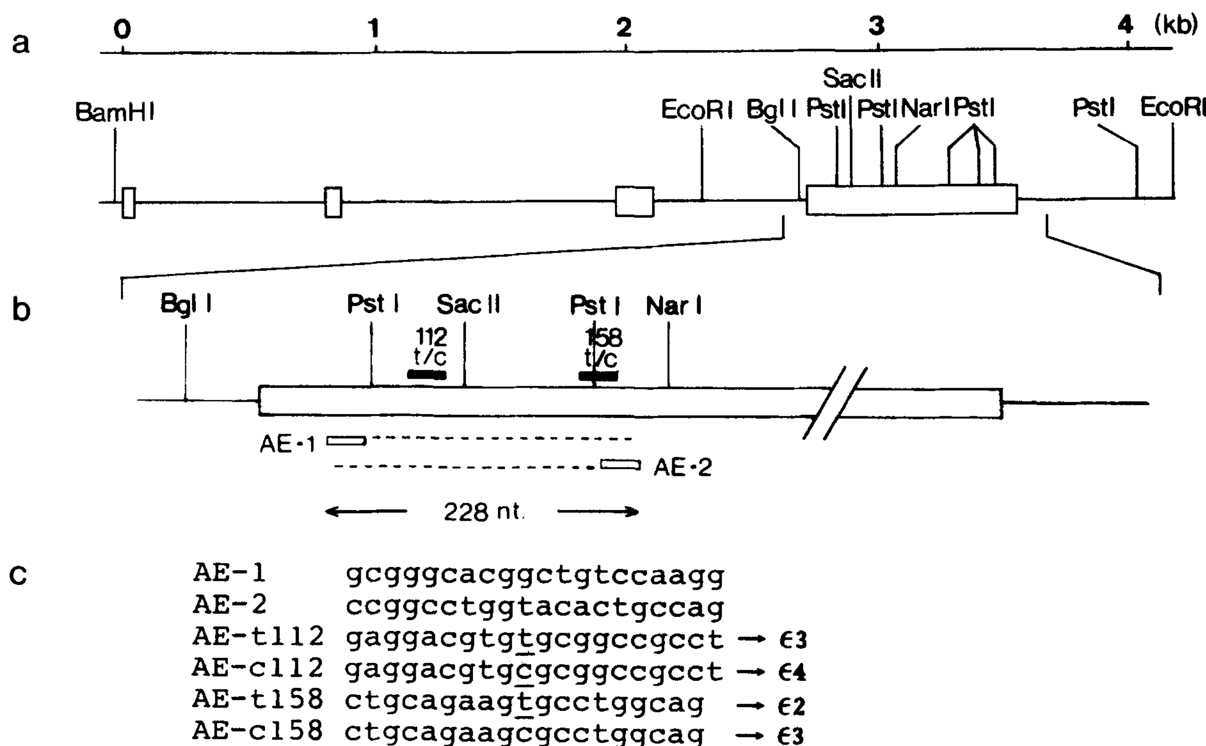

Fig. 1. Schematic representation of various demarcation points in the apoE gene. 1a) Gene organization and restriction enzyme map of the apoE gene. 1b) Structure of the relevant portion of exon IV and positioning of the amplification primers AE-1 and AE-2 (given by open boxes). Closed boxes indicate the positions of the oligonucleotides used to discriminate between ϵ3 and ϵ4 alleles and between ϵ3 and ϵ2 alleles. 1c) Sequences of the amplification primers and allele specific oligonucleotides.

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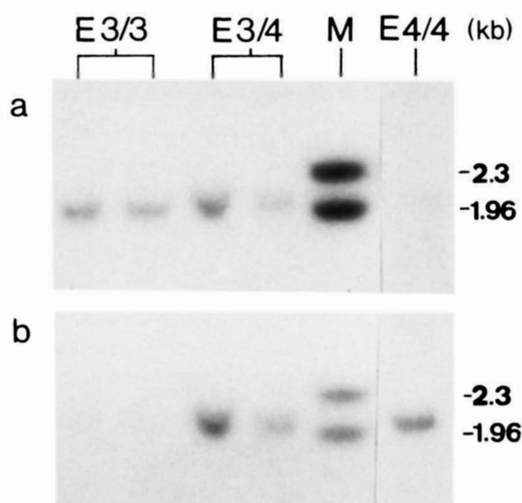

Fig. 2. Direct blot analysis of genomic DNAs with the ε3-ε4 allele-specific oligonucleotides. Chromosomal DNAs of individuals typed to be heterozygous (E3/4) or homozygous (E3/3; E4/4) for apoE isoproteins were EcoRI-digested and subjected to Southern blot analysis using the allele-specific AE-t112 (panel a) and AE-c112 (panel b) oligonucleotide probes as described in Materials and Methods. ApoE genotypes are indicated above each lane. M, ^{32}P 3'-end labeled DNA marker fragments (HindIIIxλDNA). Autoradiography was for 7 days with two intensifying screens.

finally chosen are only slightly different from the conditions originally established by Saiki et al. (1), the main differences being the extended reaction times for denaturation and DNA polymerase-mediated elongation and the somewhat higher annealing and elongation temperature. After 10 amplification cycles the plasmid DNA (100 ng) showed a distinct band at 228 bp which could be visualized by ethidium bromide staining (**Fig. 3a**). Amplification of chromosomal DNA resulted in a broad smear of DNA in which the apoE-specific band shows up only after blotting and hybridization with an oligonucleotide complementary to sequences comprised in the 228 bp segment (Figs. 3b and 3c). We observed that a series of 15–16 consecutive amplification cycles gave optimal signal intensity. For unknown reasons, prolonged incubation (20–25 cycles) did not result in further increase of hybridization intensity at the 228-bp band (results not shown). We estimate that the sensitivity of the analysis was improved at least 10,000-fold by the PCR technique. **Fig. 4a** shows the result of amplification of 300 ng of genomic DNA from E3/3 and E4/4 homozygotes and one E3/4 heterozygote and screening with the ^{32}P-5' end-labeled AE-c112 and AE-t112 oligonucleotides. Both alleles can be clearly inferred from the autoradiogram after a 3.5-hr exposure. Fig. 4b shows the oligonucleotide typing of two E2/2 homozygotes, one E3/3 homozygote, and one E2/3 heterozygote using the AE-c158 and AE-t158 oligonucleotides for which a somewhat longer autoradiographic exposure was consistently required.

Cloning and sequencing of amplified apoE DNA

To show that the amplification of the exon IV DNA domain could be utilized for rapid identification of apoE

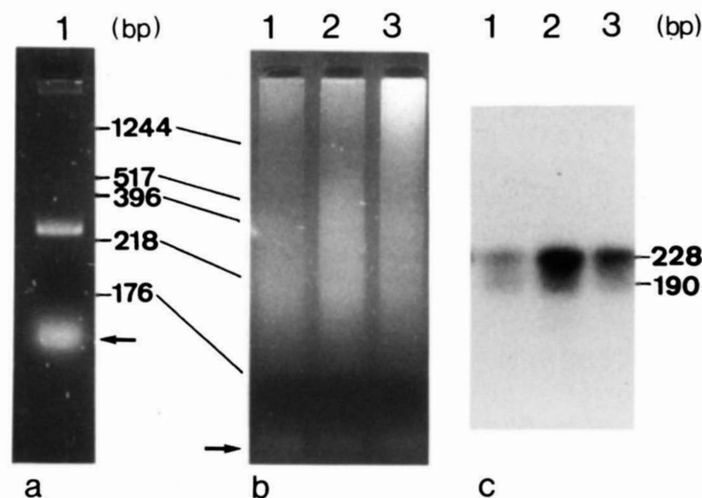

Fig. 3. Gel analysis and hybridization detection of the in vitro amplified apoE gene segment. a,b) Ethidium bromide-stained reaction product(s) of PCR-amplified plasmid pEB4 DNA (panel a) and amplified total genomic DNA of three unrelated individuals (panel b). Oligonucleotides AE-1 and AE-2 (see Fig. 1) were tested by amplification (10 cycles) of the pertinent 228 bp segment from denatured double-stranded pEB4 plasmid DNA. Stained bands of the reaction product and of the oligomer primers (arrow) are visible. Sizes of various marker fragments are indicated. Upon PCR-amplification of chromosomal DNAs (16 cycles, panel b) no distinct reaction products can be visualized. c) Hybridization screening of the reaction products shown in panel b by use of oligonucleotide AE-t112 as probe. The intensely hybridizing band at 228 bp is the expected amplification product of the apoE gene segment; the additional weakly hybridizing band at about 190 bp is of unknown origin. Autoradiography was for 4 hr.

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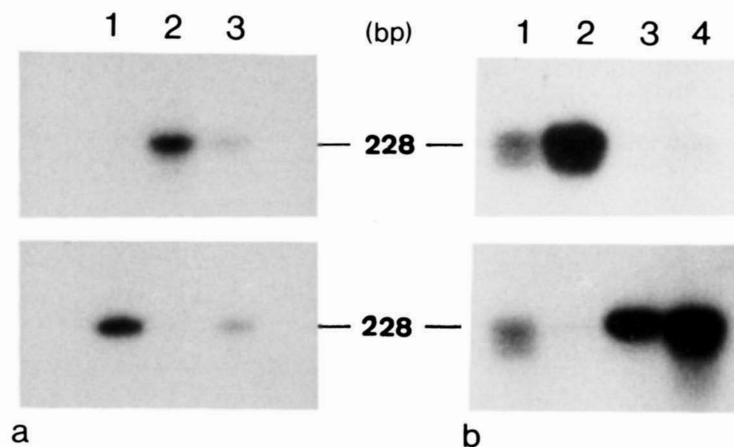

Fig. 4. Oligonucleotide-mediated apoE-allele screening of PCR-amplified genomic DNAs. Genomic DNAs obtained from individuals with known apoE types were amplified (16 cycles), resolved on agarose gels, blotted and screened with apoE allele-specific oligonucleotides. Panel a: lane 1, E3/3 DNA; lane 2, E4/4 DNA; lane 3, E3/4 DNA screened with oligonucleotide AE-c112 (upper part) or AE-t112 (lower part). Panel b: lane 1, E2/3 DNA; lane 2, E3/3 DNA; lanes 3 and 4, E2/2 DNAs screened with oligonucleotide AE-c158 (upper part) or AE-t158 (lower part). Autoradiography was for 3.5 hr (panel a) and overnight (panel b), respectively.

mutations, we performed a model cloning experiment. Therefore, the DNA of an E4/3 heterozygote individual was amplified through 16 cycles of PCR reaction and 1% of the material was cloned into a pGEM3 vector plasmid in *E. coli* HB101. Of the approximately 500 colonies obtained, 3 positively hybridizing colonies were identified with an oligonucleotide spanning codons 131–137. From direct cloning of PstI-cleaved DNA, approximately 1 in 10^6–10^7 clones would be expected to originate from apoE DNA. The observed incidence of apoE-specific clones (3 in 500) therefore indicates a specific enrichment in the order of 10,000- to 100,000-fold by the amplification step. This corroborates the estimate from direct hybridization and is in the same order of magnitude as reported from similar studies involving the human globin and HLA gene loci (30, 31). In order to prove that the insert of the three clones indeed originated from the pertinent apoE gene-domain, we sequenced the DNAs. The sequences read were completely concordant with the apoE-3 and apoE-4 sequences across the 188-bp segment already published by others (32, 33). Two ε4 alleles and one ε3 allele were identified (results not shown, DNA clones available upon request).

DISCUSSION

Our results clearly show that typing of the three major apoE variants from minute quantities of DNA can be reliably done using the PCR methodology in conjunction with allele-specific oligonucleotide hybridization. The method presented in this paper is inherently more sensitive than currently existing methods based on protein characterization and can be applied on each tissue specimen from which DNA can be obtained.

Moreover, by employing appropriate sets of oligonucleotides, the technique will allow discrimination between similar but distinct mutations in apoE variants now commonly referred to as being of either E-2, E-3, or E-4 type. For example, no less than four allelic subtypes each are known for the ε2 (13, 14, 16, 21) and for the ε3 (17, 21) variants. These alleles all have similar amino acid substitutions at distinct positions and the commonly used phenotyping based on charge alterations in the protein usually cannot discern between different sites of mutation in such subtypes. Moreover, the latter method may lead to misclassification in a small number of cases.

In considering the evident advantages of our apoE DNA typing method, however, one has to keep in mind that for each new set of allele-specific oligonucleotides the method becomes more laborious and conditions for discriminative hybridization have to be reassessed. In our experiments, discrimination of ε3-ε4 and ε2-ε3 alleles is based on the difference in thermal stability between fully matched 19- or 20-mers and duplexes with a mildly disruptive G-T mismatch or duplexes with a more destabilizing C-A mismatch. To fully exploit the discriminative power of the allele-specific oligonucleotides, optimized conditions for stringency of hybridization and washing would be required for every individual oligonucleotide. Therefore, we decided to use the PCR method for generation of signals that were much stronger and less affected by nonhomologous hybridization. We chose to combine this method with Southern analysis rather than dot-blot assays (33) to avoid further complication. Though it is in-

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herently simpler than Southern blot analysis, dot-blotting does not discriminate between genuine and background signals and would probably lead to ambiguities in the interpretation. Presumably, namely due to their rather high G + C content (a feature found throughout the entire apoE gene) and the noncritical annealing temperature, the AE-1 and AE-2 amplification primers displayed a tendency to hybridize at multiple loci on the genomic DNA. Furthermore, a pseudo-apoE gene was recently reported on chromosome 16 (34) and we cannot exclude that several other loci with homology to apoE-specific oligonucleotides exist throughout the genome. This might explain why in some experiments, in addition to the apoE-specific 228-bp signal, another band at about 190 bp and a weak background smear of hybridization were observed (Fig. 3c).

Appropriate sets of oligonucleotides can be designed for any mutation outside the crucial 188-bp domain, too. From comparison, however, of overall amplification efficiencies, we observed that regions with high C + G content consistently seem more refractory to Klenow DNA-polymerase-mediated amplification than regions with a more average base composition (not shown). Various apoE gene regions, therefore, may be particularly difficult to amplify. As reported recently, most of these difficulties can be overcome, and a more locus-specific DNA-amplification can be obtained by employing the heat-stable *Thermus aquaticus* DNA polymerase and higher temperatures for primer annealing and elongation (35).

The PCR-amplification, combined with rapid cloning and sequencing methods, will greatly facilitate the identification of new apoE mutations in individuals where alterations in apoE receptor binding affinity are evident or even in apparently healthy individuals. This abolishes the need to establish complete gene libraries from every individual with anomalous apoE sequences and will deepen the overall insight into the association between apoE haplotypes and the incidence of hyperlipidemia and atherosclerosis in the human population. ■

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