Molecular basis of five apolipoprotein B gene polymorphisms in noncoding regions

Li-Shin Huang, Michael E. Ripps, and Jan L. Breslow

Laboratory of Biochemical Genetics and Metabolism, The Rockefeller University, New York, NY 10021

Abstract Restriction fragment length polymorphisms (RFLPs) are useful in linkage and clinical association studies of human diseases. In this report, we characterize the molecular basis and frequencies of two new RFLPs, AvaII and BalI, two previously reported RFLPs, HincII and PvuII, and one new sequence polymorphism in the human apolipoprotein B gene. For the AvaII RFLP, the two alleles yield either a 1 kb fragment or 0.7 and 0.3 kb fragments, and have frequencies of 20% and 80%, respectively. The polymorphic site is about 4 kb upstream of exon 1. For the BalI RFLP, the two alleles yield either a 4.9 or 6.2 kb fragment, and have about equal frequencies. The polymorphic site is within an Alu sequence in intron 20, 146 bp 5' to exon 21. The BalI recognition sequence TGGCCA is replaced by TAGCCA. For the HincII RFLP, the two alleles yield either a 1.7 or 1.3 kb fragment and have frequencies of 80% and 20%, respectively. The polymorpic site is in intron 4, 171 bp 3' to exon 4. The HincII recognition sequence GTTAAC, present in the minor allele, is replaced by GTTACC. HincII fragments of 7.4 and 7.0 kb, previously reported for this polymorphism, are the result of partial digestion at the invariant HincII site in intron 3, 334 bp 3' to exon 3. For the PvuII RFLP, the two alleles yield either a 7.5 or 5.5 kb fragment and have frequencies of 96% and 4%, respectively. The polymorphic site is within an Alu sequence in intron 4, 523 bp 5' to exon 5. The PvuII recognition sequence CAGCTG, present in the minor allele, is replaced by CGGCTG. For the sequence polymorphism in intron 3, the alleles have either a G or T at the position 92 bp 3' to exon 3 and have frequencies of 53% and 47%, respectively. —**Huang, L-S., M. E. Ripps, and J. L. Breslow.** Molecular basis of five apolipoprotein B gene polymorphisms in noncoding regions. *J. Lipid Res.* 1990. **31:** 71–77.

Supplementary key words restriction fragment length polymorphism • haplotype analysis • apolipoprotein B gene

Apolipoprotein B (apoB) is the major protein constituent of low density lipoproteins (LDL) and is a ligand for the LDL receptor (1). Epidemiological studies have shown that both LDL-cholesterol and plasma apoB levels are positively correlated with coronary heart disease incidence (2). The human apoB gene is 43 kb is length, contains 28 introns, and resides on chromosome 2p24 (3–5). Recent studies have identified mutations in the apoB gene that alter plasma LDL-cholesterol levels (6–9). One of these studies showed that a single base substitution at amino acid 3500 causes reduced LDL receptor binding affinity and increased plasma LDL-cholesterol levels (6). Other studies showed that deletion or nonsense mutations in various regions of the apoB gene result in decreased plasma LDL-cholesterol levels in patients with hypobetalipoproteinemia (7–9). In addition to these mutations, common genetic variation in the apoB gene detected as restriction fragment length polymorphisms (RFLPs) has been reported (4, 10–13). RFLPs can result from base substitutions, deletions, or insertions that either create or destroy restriction enzyme recognition sites. They can also result from insertion or deletion of DNA sequences between two flanking enzyme sites. Several apoB gene RFLPs in exons result in codon changes and some of these have been correlated with structural variations in the apoB protein detectable with antibodies (13–18). RFLPs that do not alter the structure of the protein have also been identified in the 3' flanking region or as silent substitution in exons (12, 18). ApoB gene RFLPs have been used in clinical association studies. In various studies, apoB gene RFLPs have been associated with myocardial infarction incidence and altered total serum cholesterol and LDL cholesterol levels (10, 19–23). ApoB gene RFLPs have also been used in linkage studies to follow the segregation of apoB alleles within families (9, 24). Both association and linkage studies can be made more informative by utilizing multiple RFLPs to construct apoB allele haplotypes.

In this report five polymorphisms spanning the apoB gene were characterized. We identified two new RFLPs, AvaII and BalI, and a new sequence polymorphism in intron 3. In addition, we elucidated the molecular basis of two previously reported RFLPs (11), HincII and PvuII.

MATERIALS AND METHODS

Probe preparation

ApoB genomic clones were isolated from a cosmid library and a lambda library as described previously (12,

25). Restriction fragments derived from these clones were subcloned into pUC vectors. Inserts from these subclones were released from plasmid vectors by restriction enzyme digestion and then isolated by electrophoresis through low-melting agarose gels. These inserts were nick-translated (26) and used as probes for Southern blotting analysis.

Southern blotting analysis

Genomic DNA was isolated from white blood cells or lymphoblast cell lines as described (4). Lymphoblast cell lines were obtained from the National Institute of General Medical Sciences Human Genetic Mutant Cell Repository (Camden, NJ). These cells were derived from individuals residing in Utah. Genomic DNA samples were digested with restriction enzymes and then subjected to agarose gel electrophoresis. The DNA samples were then transferred to nylon membranes (Gelman, Biotrace). The membranes were hybridized with apoB probes which were prepared as described (9). After overnight hybridization, the membranes were washed with 0.1 × SSC/0.1% SDS to remove excess probe and then exposed to X-ray film.

Molecular cloning of the polymorphic apoB alleles

Genomic DNA samples from individuals homozygous for various RFLPs were amplified by the polymerase chain reaction (PCR) (27). The regions of the apoB gene that were amplified are shown in **Fig. 1.** Sequences flanking the polymorphic site for each RFLP were synthesized as oligonucleotide primers by phosphoramidite chemistry on a DNA synthesizer (Applied Biosystems, Model 381A). The primers used, with linkers underlined, were as follows:

region 1, 5'(CCAGAATTCGCTGTCCTTGGGAG) and
3'(TAATCTAGATCATGATTCCTATT);
region 2, 5'(TTGGAGCTCTGATTAGAGATTAA) and
3'(TCGGAGCTCTCATAATTCCCACG);
region 3, 5'(CTCGAATTCACTGGTAGTCAAGA) and
3'(CATGAATTCGAGTTTCAAGGGCC);
region 4, 5'(GGTAAGCTTCATGAAAGCAAGGA) and
3'(TTGGAATTCACTGGACAAGGTCA).

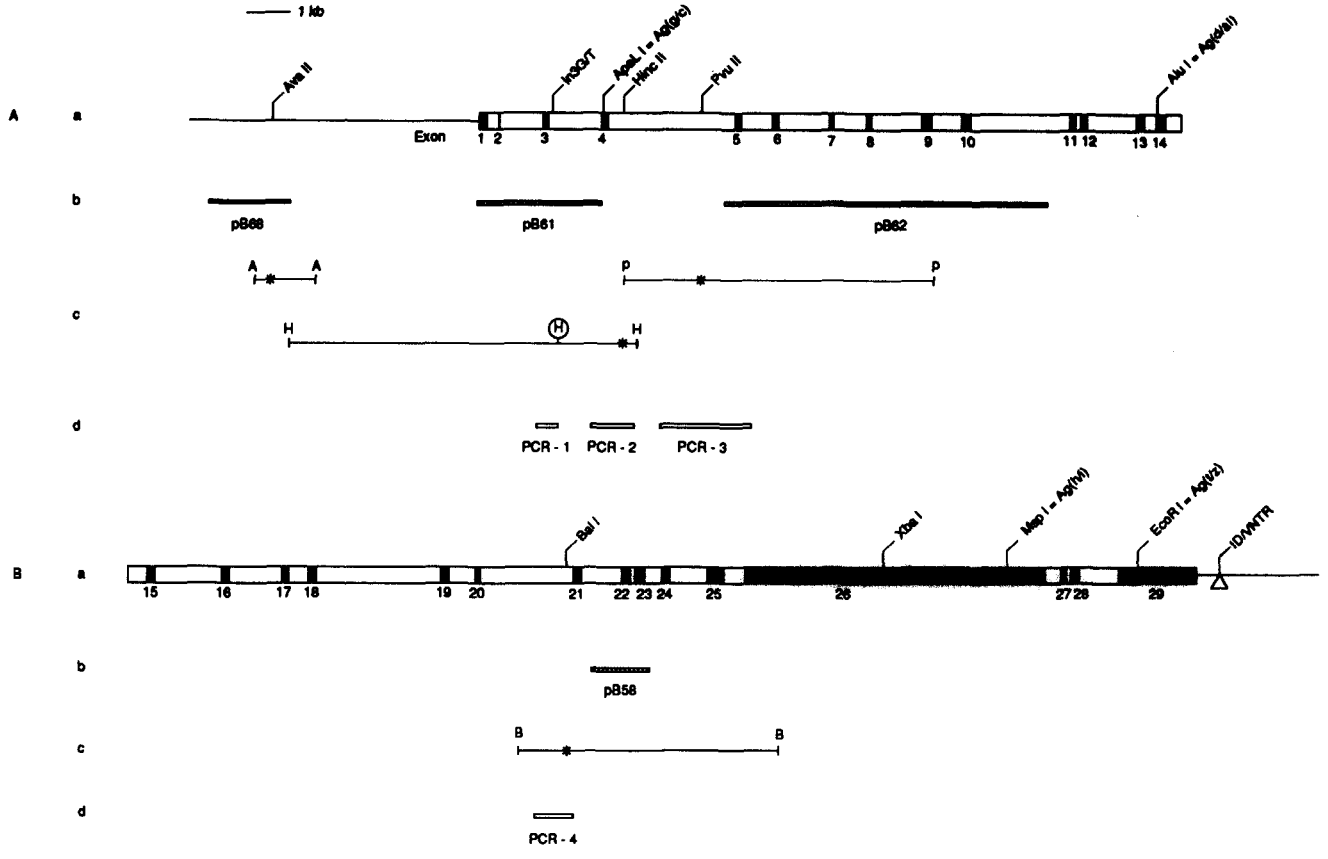

Fig. 1. Restriction map of apoB gene RFLPs. The map of the apoB gene is shown in A and B. The polymorphic sites of previously reported and new RFLPs in the apoB gene are shown in (a). The scale is indicated in kb above the map. Exons are shown in solid bars and introns are shown in open bars. The 5' and 3' flanking regions are shown as horizontal lines. The ID/VNTR RFLP is the length polymorphism containing AT-rich tandem repeats (12, 32) which is indicated as a triangle. The Ag allotypes that correspond to DNA RFLPs are also indicated. Probes that were used to identify RFLPs reported here are shown in (b). The restriction fragments responsible for each RFLP are shown in (c). The stars indicate the positions of the polymorphic enzyme sites. Regions of the gene cloned by the PCR are shown in (d).

Sequences of 3' primers are complementary to the sense strand of the apoB gene. The PCR reaction and cloning of the amplified DNA were performed as described previously (9).

DNA sequencing analysis

Multiple clones of the apoB alleles derived from the PCR-amplified DNA samples were sequenced. Double-stranded plasmid DNA isolated from these clones was denatured with an equal volume of 0.4 N NaOH for 5 min, neutralized with 100 mM ammonium acetate and 200 mM sodium acetate (pH 7.0), and precipitated by addition of ethanol. The resuspended sample was sequenced with modified T7 DNA polymerase (Sequenase, United States Biochemical Co.). (28) according to the dideoxy-termination method of Sanger, Nicklen, and Coulson (29).

Allele-specific oligonucleotide hybridization

Oligonucleotides #317 and #323 were synthesized as allele-specific oligonucleotide (ASO) probes for the sequence polymorphism in intron 3. ASO probe #317 contains the sequence CCCGGAGG<u>T</u>TGTGTACT, which is specific to sequence T (underlined). ASO probe #323 contains the sequence CCCGGAGG<u>G</u>TGTGTACT, which is specific to sequence G (underlined). DNA sequences spanning the polymorphic nucleotide were amplified by PCR using primers for region 1. The PCR amplified DNA samples were denatured with 0.5 M NaOH/1.5 M NaCl, neutralized with 1 M Tris-Cl, pH 7.5/1.5 M NaCl, and blotted onto nitrocellulose membranes using a slot-blot apparatus. The membranes were hybridized with ASO probes at 50°C overnight. The membranes were washed with 6 × SSC/0.05% sodium pyrophosphate at 56°C for ASO probe #317 and 58°C for ASO probe #323.

RESULTS

AvaII RFLP

A new RFLP, AvaII, was identified by Southern blotting analysis when probe pB68 (Fig. 1) was used. The segregation of the two alleles is shown in **Fig. 2a.** The probe hybridized to two invariant fragments of 1.2 and 0.8 kb and three polymorphic fragments of 1.0, 0.7, and 0.3 kb. The allele that yields a fragment of 1 kb is designated allele 1 and the allele that yields fragments of 0.7 and 0.3 kb is designated allele 2. In a Utah Caucasian population, allele 1 has a frequency of 20% and allele 2 has a frequency of 80% (**Table 1**). Restriction mapping analysis showed that the polymorphic AvaII site is about 4 kb upstream of exon 1 of the apoB gene (Fig. 1).

BalI RFLP

Another new RFLP, BalI, was identified by Southern blotting analysis when probe pB58 (Fig. 1) was used. The probe hybridized to polymorphic fragments of 6.2 and 4.9 kb (Fig. 2b). The allele that yields a 6.2 kb fragment is designated allele 1, and the allele that yields a 4.9 kb fragment is designated allele 2. The alleles have about equal frequency (50%) in a Utah Caucasian population (Table 1). Restriction mapping showed that the polymorphic site is in intron 20. Sequencing of clones derived from individuals homozygous for either BalI allele showed that the BalI recognition site, TGGCCA, is present in allele 2 and is replaced by TAGCCA is allele 1 (**Fig. 3a**). The base substitution is in intron 20, 146 bp 5' to exon 21 (Table 1, Fig. 1).

HincII RFLP

The HincII RFLP has been reported previously (11) using a 1 kb 5' apoB cDNA clone (30) that hybridizes to sequences that extend over 25–30 kb of the apoB gene (3). We have identified the same polymorphism using the genomic probe pB61 (Fig. 1). This probe hybridized to polymorphic fragments of 7.4 and 7.0 kg (Fig. 2c) as reported previously (11). The allele that yields a 7.4 kb fragment is designated allele 1, and the allele that yields a 7.0 kb fragment is designated allele 2. Allele 2 has a frequency of 20% in a Utah Caucasian population (Table 1). As shown in Fig. 2c, the probe also hybridized to an invariant 5.7 kb fragment. In addition, the probe hybridized to a second set of polymorphic fragments of 1.7 and 1.3 kb that cosegregated with the 7.4 and 7.0 kb fragments, respectively. Restriction mapping and DNA sequencing analysis of the PCR-2 region showed that the polymorphic HincII site is in intron 4, 171 bp 3' to exon 4 (Fig. 1a). The HincII site (GTTAAC) in allele 2 is replaced by GTTACC in allele 1 as shown in Fig. 3b. Sequencing of clones derived from the PCR-1 region showed that all individuals, regardless of their HincII genotypes, had an additional HincII site (GTTAAC) 334 bp 3' to exon 3. Therefore, the true polymorphic fragments for the HincII RFLP are 1.7 kb for allele 1 and 1.3 kb for allele 2. Restriction fragments of 7.4 kb and 7.0 kb are the result of an incomplete digestion at the HincII site in intron 3.

PvuII RFLP

The PvuII RFLP was reported previously using the same cDNA probe as for the HincII RFLP described above (11, 30). We have identified the same RFLP using the genomic probe pB62 (Fig. 1). The probe hybridized to polymorphic fragments of 7.5 and 5.5 kb. The allele that yields a 7.5 kb fragment is designated allele 1, and the allele that yields a 5.5 kb fragment is designated allele 2. Allele 2 is present at a low frequency (4%) in the Cauca-

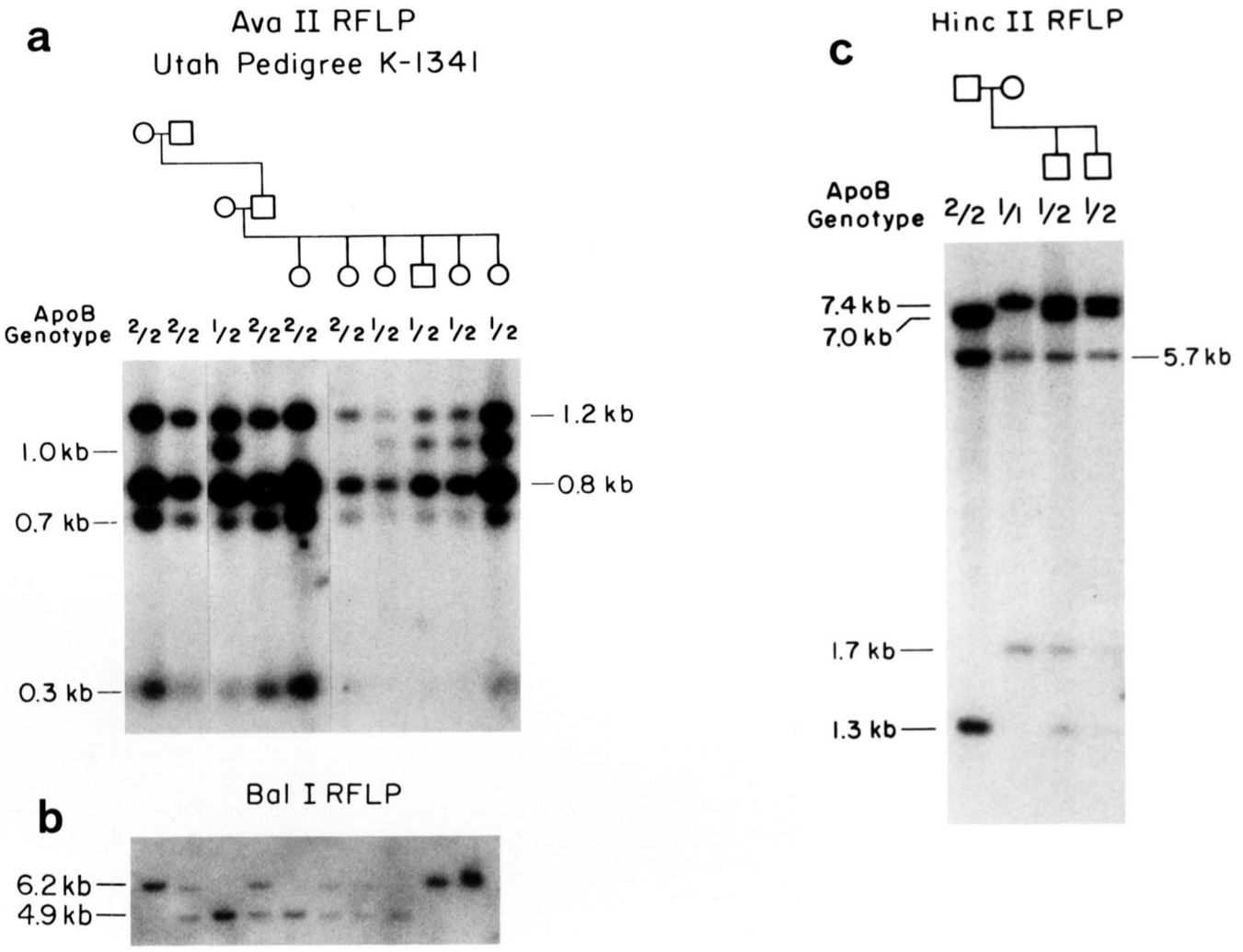

Fig. 2. Southern blots of the apoB gene RFLPs. (a) AVaII RFLP: Genomic DNA samples from a Utah kindred (pedigree K-1341) were digested with AvaII and subjected to Southern blotting analysis using probe pB68. The polymorphic fragments, 1.0, 0.7, and 0.3 kb, are shown on the left, and the invariant fragments, 1.2 and 0.8 kb, are shown on the right. Segregation of the AvaII alleles is shown in the pedigree above the autoradigram. The allele that yields a 1.0 kb fragment is designated allele 1 and the allele that yields 0.7 and 0.3 kb fragments is designated allele 2. The apoB genotype of each individual is indicated directly above each lane. (b) BalI RFLP: Genomic DNA from lymphoblast cell lines was isolated, digested with BalI, and subjected to Southern blotting analysis using probe pB58. The two alleles yield BalI fragments of either 6.2 kb or 4.9 kb. (c) HincII RFLP: Genomic DNA samples from a four-member family were digested with HincII and subjected to Southern blotting analysis using probe pB61. The polymorphic fragments, 7.4 and 7.0 kb or 1.7 and 1.3 kb (see Text for explanation), are shown on the left and the invariant fragment, 5.7 kb, is shown on the right. The allele that yields the 7.4 kb or 1.7 kb fragment is designated allele 1, and the allele that yields the 7.0 or 1.3 kb fragment is designated allele 2. The segregation of these alleles is shown here.

sian population tested (Table 1). DNA sequencing of clones derived from individuals homozygous for each allele showed that the PvuII recognition site, CAGCTG, is present in allele 2 and is replaced by CGGCTG in allele 1 (Fig. 3c). The base substitution is within an Alu sequence in intron 4, 523 bp 5' to exon 5 (Table 1, Fig. 1).

Noninformative restriction enzymes

In addition to the enzymes described above, other enzymes were also used for Southern blotting analysis using probes pB68, 61, 62, and 58 as shown in Fig. 1. When these probes were used we found no polymorphic sites for the following enzymes: BglII, EcoRV, HaeIII, HindIII, HinfI, KpnI, PstI, StuI, StyI, TaqI, and XbaI.

Intron 3 sequence polymorphism

Sequencing of clones derived from the PCR-1 region revealed a sequence polymorphism 92 bp 3' to exon 3 (Fig. 3d). The allele containing a G is designated allele 1 and the allele containing a T is designated allele 2. To determine the allelic frequencies of this sequence polymorphism, PCR-amplified DNA samples from 20 individuals in a Utah Caucasian population were used in an

TABLE 1. Summary of the apoB gene polymorphisms

Polymorphism	Probe	Fragment Size		Number[b]	Frequency		Base Change[c]	Location
		#1[a]	#2[a]		#1[a]	#2[a]		
		kb			%			
AvaII	pB68	1	0.7 and 0.3	12	20	80	–[d]	5' upstream; ~4 kb upstream to exon 1
In3G/T	ASO[e]	G(+)[e]	T(+)[e]	20	53	47	G→T	Intron 3; 92 bp 3' to exon 3
HincII	pB61	1.7 (7.4)	1.3 (7.0)	12	80	20	C→A	Intron 4; 171 bp 3' to exon 4
PvuII	pB62	7.5	5.5	12	96	4	G→A	Intron 4; 523 bp 5' to exon 5
BalI	pB58	6.2	4.9	12	50	50	A→G	Intron 20; 146 bp 5' to exon 21

[a]Alleles in which the polymorphic sites are absent or present are designated alleles 1 or 2, respectively. For the sequence polymorphism, In3G/T, alleles that have the nucleotide G are designated 1, and alleles that have T are designated 2.
[b]Number of individuals tested for determination of allelic frequencies.
[c]The base change from allele 1 to allele 2.
[d]Not determined.
[e]Sequence polymorphism was determined by ASO hybridization. Allele 1 is positive for G and allele 2 is positive for T.

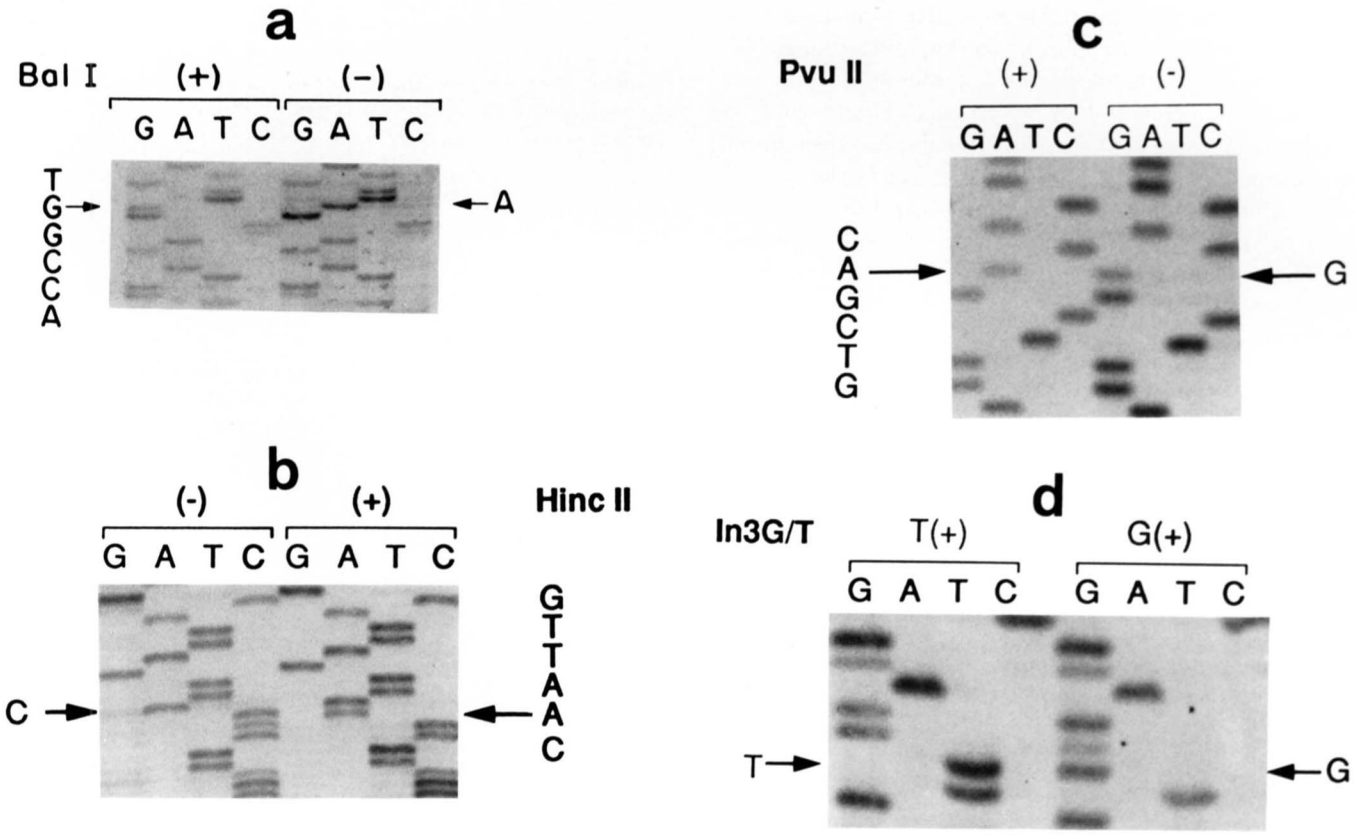

Fig. 3. DNA sequences of various polymorphic apoB alleles. Different apoB alleles were obtained as described in Methods. Sequencing analysis of these alleles revealed the molecular basis of each polymorphism. The sequence from top to bottom reads in the 5' to 3' orientation. The base difference in each polymorphism is indicated by an arrowhead. (a) BalI RFLP: A clone with the polymorphic BalI site (TGGCCA) is shown on the left, and a clone without the site and which had the sequence TAGCCA is shown on the right. (b) HincII RFLP: A clone with the polymorphic HincII site (GTTAAC) is shown on the right and a clone without the site and which had the sequence GTTACC is shown on the left. (c) PvuII RFLP: A clone with the polymorphic PvuII site (CAGCTG) is shown on the left, and a clone without the site and which had the sequence CGGCTG is shown on the right. (d) Intron 3 sequence polymorphism: A clone with the polymorphic T sequence is shown on the left and a clone with the polymorphic G sequence is shown on the right.

ASO hybridization. Allele 1 has a frequency of 53% and allele 2 has a frequency of 47% (Table 1).

DISCUSSION

In this report, we have characterized five polymorphisms spanning the human apoB gene. These include two new RFLPs, AvaII and BalI, two previously reported RFLPs, HincII and PvuII, and a new sequence polymorphism. The allelic frequencies for each polymorphism were determined in a relatively small number of individuals from a Utah Caucasian population. The allele frequencies for these polymorphisms may vary when examined in a larger sample or in different ethnic groups. The polymorphisms reported here are unlikely to be functionally significant, since they are either in the 5' upstream region not required for hepatic expression of the apoB gene (25) or in introns and do not alter the amino acid sequence of the protein. These polymorphisms probably reflect common genetic variation.

Two of the RFLPs reported here, PvuII and BalI, reside in Alu sequences in introns 4 and 20, respectively. Alu sequences are moderately repetitive sequences present at about 400,000 copies in the haploid genome (31). Due to sequence homology between members of the Alu family, these elements may be prone to homologous recombination. A total of six Alu sequences has been found in the apoB gene (3). Three of these are in the 5' to 3' orientation and located in introns 4, 14, and 15. The other three are in the reverse orientation and located in introns 16, 20, and 21. Since these Alu sequences are potential sites for recombination, they suggest regions that may be mutated in defective apoB genes. In fact, we have shown that an unequal crossing over between the Alu sequences in introns 20 and 21 resulted in the deletion of exon 21 from the apoB gene of a patient with homozygous hypobetalipoproteinemia (9). The PvuII and BalI RFLPs might be useful in detecting mutations resulting from Alu-Alu recombination events.

As summarized schematically in Fig. 1, six other apoB gene RFLPs have been characterized previously (4, 10, 12–17, 32). One of these is a length polymorphism, which consists of multiple alleles, and is due to variable numbers of AT-rich tandem repeats (VNTR) at the 3' end of the gene (12, 32). The other RFLPs are the result of base substitutions in exons of the gene. The XbaI RFLP changes the third base of codon 2488 and does not result in an amino acid change (18). The four remaining RFLPs result in amino acid changes in the protein and correspond to four of the five Ag allotypes defined by antisera (14–17). The ApaLI RFLP in exon 4 corresponds to Ag(g/c) (16), the AluI RFLP in exon 14 corresponds to Ag(d/al) (15), the MspI RFLP in exon 26 corresponds to Ag(h/i) (17), and the EcoRI RFLP in exon 29 corresponds to Ag(t/z) (14). The molecular basis of the fifth Ag allotype, Ag(x/y), is unknown but it is in linkage disequilibrium with the XbaI RFLP (18). Although the XbaI RFLP does not result in a structural variation, this polymorphism has been associated with increased risk of myocardial infarction and peripheral arterial disease, altered cholesterol levels and type III hyperlipidemia (10, 19–23). These associations are probably due to linkage disequilibrium between the XbaI RFLP and the causative mutations elsewhere in the apoB gene or in closely linked genes. The five polymorphisms reported here, together with the six previously described RFLPs, provide a spectrum of genetic markers spanning the apoB gene that can be useful for clinical association or family linkage studies.

Addendum: While this paper was being reviewed, two other apoB gene polymorphisms were reported. An MspI RFLP is identified in the promoter region (33) and a three-codon insertion/deletion polymorphism is found in the signal peptide region (34).

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