# A polymorphism in a region with enhancer activity in the second intron of the human apolipoprotein B gene

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**Abstract** A 443-base pair fragment ( + 622 to + 1064) from the second intron of the human apolipoprotein B gene was shown to contain a tissue-specific enhancer when placed in front of an apolipoprotein B promoter-chloramphenicol acetyltransferase construct in transfection experiments. To identify potential regulatory mutations in this region of the gene, DNA from various subjects was examined for the presence of point mutations by means of chemical cleavage of mismatched heteroduplexes. An A→G substitution within the second intron of the gene at position + 722 was identified in three unrelated subjects and confirmed by DNA sequencing. Although the base substitution was contained within a nuclear protein-binding site, as determined by DNase I footprinting, it did not appear to affect the protein/DNA interaction in its vicinity, as shown by gel retardation experiments. The single base substitution at position + 722 abolishes a *Sty*I restriction site, thus creating a *Sty*I polymorphism. Using allele-specific oligonucleotides, we screened the DNA of 172 subjects for the presence of this polymorphism: two other subjects carrying the polymorphism were found. In each of the five unrelated subjects, the polymorphism was associated with the same haplotype. — **Levy-Wilson, B., L. Soria, E. H. Ludwig, M. Argyres, A. R. Brooks, B. D. Blackhart, W. Friedl, and B. J. McCarthy.** A polymorphism in a region with enhancer activity in the second intron of the human apolipoprotein B gene. *J. Lipid Res.* 1991. **32:** 137-145.

**Supplementary key words** LDL • chloramphenicol acetyltransferase • regulation of apoB synthesis

Clearance of low density lipoproteins (LDL) by the LDL receptor pathway is a critical step in cholesterol homeostasis. Defects in this pathway can lead to high LDL levels and consequently to increased risk for coronary artery disease (1). Familial hypercholesterolemia, for example, is defined by mutations in the gene for the LDL receptor that reduce the efficiency of LDL clearance. Many mutations have been identified that affect various domains of the LDL receptor and impair its function by a variety of mechanisms (1). In constrast, only one mutation has been characterized that reduces the binding capacity of the ligand for the LDL receptor, apolipoprotein (apo) B (2). This mutation, in codon 3500 of the apoB gene, leads to a single amino acid substitution that drastically reduces the binding of LDL to its receptor and defines the disease familial defective apolipoprotein B-100 (3). Other mutations in the apoB gene have been identified; for example, a point mutation in the codon for amino acid 4019 was identified in a hypercholesterolemic subject but failed to segregate with hypercholesterolemia in that kindred (4), and several apoB gene defects resulting in truncated forms of apoB were found to be associated with hypobetalipoproteinemia (5, 6).

The present report describes a region within the human apoB gene that displays enhancer activity. This region of the second intron contains a DNaseI-hypersensitive site together with recognition sequences for DNA-binding proteins. The enhancer activity demonstrable in vitro suggests that this region plays a role in modulating the activity of the apoB gene and in determining the amount of apoB synthesized. Chemical probing of mismatched heteroduplexes by the method of Cotton, Rodrigues, and Campbell (7) enabled us to identify a nucleotide substitution in this enhancer region. The possibility that this substitution may influence cholesterol levels in the subjects who carry it is discussed.

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Abbreviations: LDL, low density lipoproteins; apo, apolipoprotein; CAT, chloramphenicol acetyltransferase; bp, base pair; PCR, polymerase chain reaction; ASO, allele-specific oligonucleotide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DH, DNase I-hypersensitive; HVR, hypervariable region.

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## MATERIALS AND METHODS

### Tissue culture

HepG2 cells and CaCo-2 cells were grown as previously described (8).

### Transfection of the apolipoprotein B promoter constructs into cultured cells

The reference plasmid contained the chloramphenicol acetyltransferase (CAT) gene under the control of the apoB promoter ( − 898 to + 121) (8). The 443-base pair (bp) fragment ( + 622 to + 1064) was isolated from a genomic clone by digestion with *Sma*I and *Pvu*II, followed by purification of the fragment on acrylamide gels. *Hin*dIII linkers were added to the ends of the purified fragment, which was then cloned directly upstream of the apoB promoter into the *Hin*dIII site of pPvu-CAT. Transfection and CAT assays were performed as described earlier (8). All CAT activity measurements were normalized for differences in transfection efficiency among cells by cotransfection with a plasmid containing the β-galactosidase gene under the control of the Rous sarcoma virus promoter (8).

### Synthesis of oligonucleotides

All oligonucleotides were synthesized on an Applied Biosystems DNA synthesizer, model 380B. The following single-stranded oligonucleotides were used as primers for the polymerase chain reaction (PCR): primer 1 - 5' AATGTCAGCCTGGTCTGTCCAAGTA 3' ( + 539 to + 563), and primer 2 - 5' TGAGTCCAGCTGCAGTGAT GACAG 3' ( + 1049 to + 1072). The allele-specific oligonucleotides were ASO-E1 - 5' ACACCCAAGGGATGC 3' (wild type), and ASO-E2 - 5' ACACCCAGGGGATGC 3' (mutant) ( + 715 to + 729). The sequences of the oligonucletides used in the gel retardation experiments were as follows: the 43-mer wild type - 5' AGCTTCTGCC CACACCCAAGGGGATGCCAACTCTCTTCTACCT 3', and the mutant 43-mer had a G instead of the underlined A.

### DNA amplification by polymerase chain reaction

The PCR was used to amplify a 534-bp fragment from a portion of intron 2, in an automated Eppendorf Thermocycler, with *Thermus aquaticus* DNA polymerase. First, 250 ng of genomic DNA from each of various subjects was amplified using 100 ng each of primers 1 and 2. One-tenth of this PCR reaction was then amplified using 50 ng of $^{32}$P-end-labeled primers 1 or 2. The radioactive products were ethanol-precipitated and electrophoresed on 6% acrylamide gels. The appropriate amplified band of 534 bp was excised from the gel, electroeluted, and precipitated with ethanol.

### Chemical cleavage

Chemical cleavage was performed as described by Cotton, Rodrigues, and Campbell (7). In brief, 2 × $10^5$ cpm of amplified DNA in 6 μl was incubated with 20 μl of a 2.5 M hydroxylamine solution for 2 h at 37°C. The reaction was terminated by adding 200 μl of a stop solution containing 0.3 M sodium acetate, 0.1 mM $Na_2$ EDTA (pH 5.2), and 25 μg of tRNA/ml. The DNA was recovered by ethanol precipitation. Fifty microliters of 1 M piperidine was added to the dry DNA pellets, and the mixture was incubated for 30 min at 90°C. Samples were obtained and dried. For electrophoresis on 6% acrylamide-7 M urea sequencing gels, samples were resuspended in a solution of formamide dye mix (v/v), 0.1% bromphenol blue, 0.1% xylene cyanol, 35 mM $Na_2$ EDTA (pH 7.4) and heated for 2 min at 98°C prior to loading on the gel.

### *Sty*I polymorphism

The presence or absence of the *Sty*I site at position + 722 was determined by amplifying intron 2 from genomic DNA of the various subjects using primers 1 and 2, followed by digestion of an aliquot of each sample with *Sty*I under manufacturer-recommended conditions. Digests were electrophoresed on 6% acrylamide gels for 2 h at 150 V. Gels were stained with ethidium bromide and photographed.

### Allele-specific oligonucleotide hybridization

Thirty-microliter aliquots of nonradioactive amplified DNA from various subjects and containing 25 μg of herring sperm DNA also were denatured in 1 N NaOH for 30 min at 65°C, followed by neutralization with 200 μl of 0.5 M Tris-HCl (pH 8.0), 1.5 M NaCl, and the addition of 1 ml of 20 × SSC (1 × SSC = 150 mM sodium chloride, 15 mM sodium citrate, pH 7.0). The denatured DNA samples were applied to a nitrocellulose filter using a slot apparatus from Schleicher & Schuell (Keene, NH). After prehybridization in a solution containing 0.9 M NaCl, 0.09 M EDTA, and 0.1% SDS with 250 μg of tRNA/ml, the filters were annealed at 42°C for 16 h in the same solution with $^{32}$P-labeled oligonucleotides ASO-E1 and E2. Washing was in 1 × SSC (0.15 M NaCl, 0.015 M sodium citrate), 0.1% SDS for 5 min at room temperature, followed by a 2-min wash at 44°C and autoradiography.

### DNA sequencing

Amplified DNA was cloned into M13 vectors and sequenced by the dideoxynucleotide chain determination method of Sanger, Nicklen, and Coulson (9).

### Subjects

The 132 normal subjects were volunteers from the Austrian army and from various Austrian sports clubs.

None had a history of heart disease, and all tested normal on electrocardiograms both during rest and physical stress. The mean age was 50.0 (±5.4) years. Eleven of the subjects exhibited apoB plasma levels below the 10th percentile and thus were grouped as low-apoB subjects. In addition, DNA was obtained from 40 healthy hypocholesterolemic subjects (total cholesterol <5th percentile).

### Determination of serum lipid levels and lipoproteins

The levels of serum cholesterol, triglycerides, and lipoprotein and of apoB were determined as described by Friedl et al. (10).

### Haplotyping

Haplotyping and genotyping were performed as described by Ludwig and McCarthy (11).

### Preparation of nuclear extracts, gel retardations, and DNase I footprinting

Nuclear extracts from HepG2 cells were prepared by the procedure of Dignam, Lebovitz, and Roeder (12). Gel retardation experiments were performed as described by Carthew, Chodosh, and Sharp (13) with minor modifications: 0.5 ng of double-stranded oligonucleotides was mixed with 5 μg of nuclear protein extract in a final volume of 20 μl containing 12 mM HEPES, pH 7.9, 12% glycerol, 60 mM KCl, 5 mM $MgCl_2$, 0.6 mM EDTA, 0.6 mM dithiothreitol, and 4–6 μg of poly(dI-dC). Competitor DNA was added to some reaction mixtures, as described in individual experiments. Reactions were incubated for 30 min at 37°C and immediately analyzed on a 5% polyacrylamide gel (80:1 acrylamide:bis ratio) at 10–12 V/cm in buffer containing 0.5 × TBE (44.5 mM Tris borate, pH 8.3, 44.5 mM boric acid, 1 mM EDTA). The dried gels were autoradiographed.

The DNase I footprinting assay was carried out as described by Paik et al. (14). Five nanograms of 3' or 5' end-labeled fragment and various amounts of protein extracts were used in a final volume of 100 μl. The amounts of DNase I and incubation times were optimized to produce an even cleavage pattern. The reactions were analyzed on an 8% polyacrylamide–8 M urea sequencing gel.

## RESULTS

### Second intron of the human apolipoprotein B gene contains a segment with enhancer activity

Alerted by the presence of a tissue-specific DNase I-hypersensitive (DH) site near the 5' end of the second intron that exhibits a high degree of DNA sequence conservation between the human and the mouse apoB gene (8), we searched for regulatory elements in this region that might affect expression of the apoB promoter in cultured cells. We chose a 443-bp *Sma*I-*Pvu*II fragment (between nucleotides +622 and +1064 on the gene map) encompassing the DH site (+727 to +751) and tested its effect on the apoB promoter fragment (−898 to +121) (8) in HepG2 and CaCo-2 cells. The results, illustrated in **Fig. 1**, demonstrated that this fragment enhanced expression of the apoB promoter in both orientations in HepG2 and CaCo-2 cells. The magnitude of the enhancer effect varied approximately between 4- and 6-fold in HepG2 cells and between 2- and 4-fold in CaCo-2 cells. The same 443-bp fragment had no effect on the apoB promoter in HeLa cells (data not shown). We are currently engaged in a detailed biochemical characterization of this 443-bp fragment, including the identification of the DNA sequence elements responsible for the enhancer effect and of the nuclear proteins that interact with these various sequences.

### Identification of a single nucleotide substitution in the 443-base pair fragment

Our goal is to relate the findings established in the study of regulatory elements that control the levels of apoB mRNA in liver and intestine to clinical syndromes leading to either low plasma apoB levels (hypoapobetalipoproteinemia) or high plasma apoB levels (hyperapobetalipoproteinemia). To this end, we used techniques capable of detecting point mutations within this 443-bp segment of the apoB gene.

Chemical cleavage of mismatched heteroduplexes was used as a means of screening a large number of individuals for mutations in this region. DNA of various subjects was amplified using PCR with oligonucleotide primers flanking the 443-bp fragment, followed by purification of the amplified DNA, i.e., a 534-bp fragment, and end-labeling. Subjects heterozygous for a nucleotide change can be detected by virtue of the fact that, after denaturation and reannealing, only those mismatched heteroduplexes formed by one strand from the normal allele and one from the mutant allele will be modified by hydroxylamine, which is specific for cytosines. Piperidine will then cleave the modified base, giving rise to fragments whose length can be determined. Any mutation that is present can be localized by means of a DNA sequencing gel. **Fig. 2** shows the region amplified by primers 1 and 2 (top panel) and the size of the radioactive fragments expected if a point mutation were present at position +722 (middle panel). The bottom panel of Fig. 2 shows an autoradiogram of a gel with the intact 534-bp sense strand and the 351-bp labeled band resulting from cleavage by hydroxylamine of a C at position +722 in the antisense strand of the two heterozygous individuals.

Having identified a single base change in the DNA in these two subjects, we characterized the nucleotide substitution by amplifying their DNA and sequencing it. An A→G substitution at position +722 in the transcribed sense strand and a T→C substitution in the complemen-

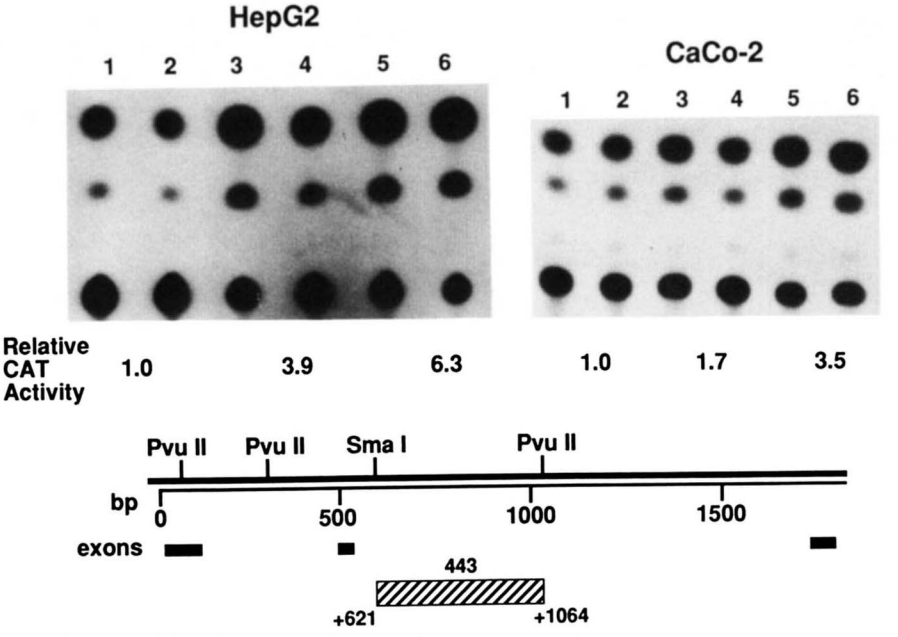


**Fig. 1.** Enhancer activity of the 443-bp fragment from intron 2 of the apoB gene. The top panel shows autoradiograms representing CAT assays performed with HepG2 cells (left) and CaCo-2 cells (right). In each case, the radioactivity at the origin (bottom spot) represents the substrate, and that at the top the product of the CAT enzymatic reaction. Lanes 1 and 2 in both left and right panels represent the CAT activity of the pPvu-CAT construct, i.e., the apoB promoter alone; lanes 3, 4, 5, and 6 depict the results with the constructs containing the 443-bp insert upstream of the apoB promoter; lanes 3 and 4 reflect the forward orientation of the enhancer fragment, and lanes 5 and 6, the reverse orientation. The CAT activities were expressed relative to that of the apoB promoter construct, which was set at 1.0, and the other numbers are the averages of the two sets of independent transfections. The bottom panel shows a restriction map of the area of interest, with a scale in base pairs below the map. Exons are shown as black boxes below the map. The location of the 443-bp enhancer fragment is shown by the hatched rectangle at the bottom of the figure; the numbers at the 5' and 3' ends represent the positions of the *Sma*I and *Pvu*II sites, respectively.

tary strand were detected. No other mutations were present within the amplified fragment. The DNA of 20 individuals exhibiting low plasma apoB levels (<10th percentile) was analyzed in this manner, and three individuals with this mutation (K111, K130, and K23 in Table 1) were identified.

### Intron 2 base substitution falls within a footprint

The base substitution at position +722 was contained within a footprint, as shown in **Fig. 3**. The sequence of the antisense strand is shown with the mutant base T encircled. Because the footprint represents the site of interaction between that DNA sequence and one or more nuclear proteins from HepG2 cells, we wished to determine whether this single base change affects binding of nuclear proteins to the footprinted sequence. Therefore, we compared the binding of nuclear proteins to two labeled oligonucleotides: one bearing the wild-type sequence, and the other, the mutant sequence.

The base substitution was placed in the middle of the mutant oligonucleotide. The results, in **Fig. 4**, lanes 2 and 7, show that both wild-type and mutant oligonucleotides bind specifically to one major protein, as evidenced by the fact that the binding is competed for by an excess of unlabeled oligonucleotides (lanes 3, 4, 8, 9) but not by pBR322 DNA (lanes 5 and 10). Binding of proteins to either the wild-type or the mutant oligonucleotide is readily abolished by a 100-fold excess of either wild-type or mutant competitor (lanes 3, 4, 8, 9). To ascertain whether small differences in the affinity of protein binding exist between the wild-type and mutant oligonucleotides, we performed a complete set of competition experiments using a wide range of concentrations of competitor wild-type or mutant oligonucleotides. Some of these experiments are shown in lanes 11–16 of Fig. 4. No differences were detectable between the two oligonucleotides in the binding to HepG2 cell proteins, suggesting that the single base change does not diminish nuclear protein binding as measured by this in vitro assay. One cannot discount the possibility that the protein responsible for binding to the 4–6 bases surrounding the mutation is present at a concentration too low to be detected in our gel retention assay, although the ease of detection of the protein binding by footprinting makes this unlikely.

### Intron 2 nucleotide substitution generates a *Sty*I polymorphism

The single base change A→G in intron 2 abolishes a *Sty*I restriction site. Thus, digestions with *Sty*I can be used to distinguish normal individuals from those possessing

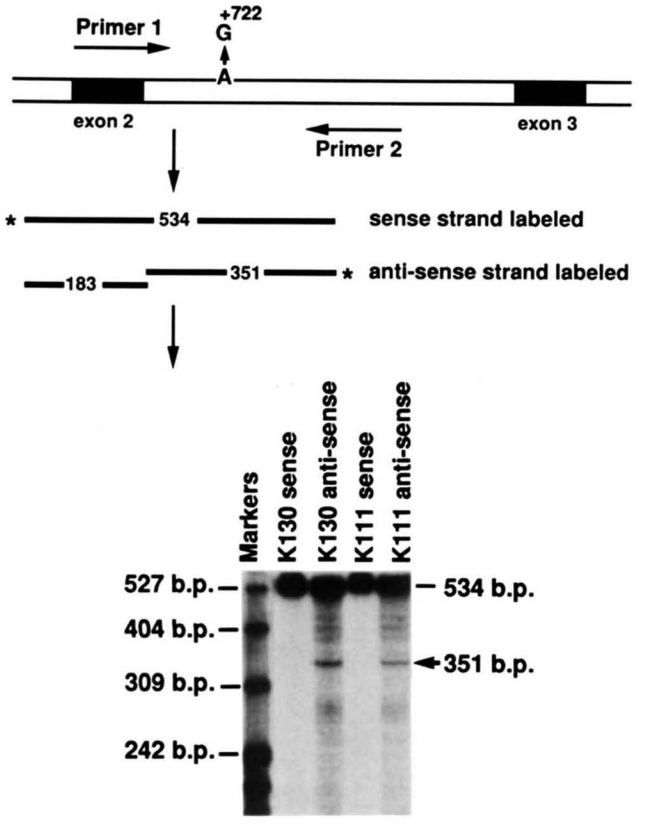


**Fig. 2.** Analysis of the cleavage of a heteroduplex by hydroxylamine with 5' end-labeled DNA. The top panel shows a schematic of the DNA region of interest, together with the locations of PCR primers 1 and 2. The A→G substitution at position + 722 is shown. The middle panel illustrates the size of the PCR fragment (534 bp) and the expected sizes of the fragments derived by cleavage at + 722 after hydroxylamine modification of the cytosine in the antisense strand. The bottom panel shows an autoradiogram of the DNA after the hydroxylamine reaction with the DNA of two heterozygous subjects, K130 and K111. The labeled 351-bp fragment is apparent in the lanes representing the labeled anti-sense strand.

the intron 2 mutation. We tested the 534-bp amplified DNA fragment from various individuals in this manner (data not shown). In normal individuals the DNA is digested to completion, yielding a 353-bp band and a 181-bp band. In individuals bearing the single nucleotide substitution, one of the alleles lacks the *Sty*I site, thus leaving a portion of the 534-bp DNA fragment uncut. Because of limitations inherent in the use of restriction enzymes for screening amplified DNA samples, we used allele-specific oligonucleotide hybridization to study the genetic transmission of this polymorphism in a family study, as illustrated in **Fig. 5**. Six individuals with the polymorphism were identified in this family by virtue of the hybridization of their DNA with the oligonucleotide specific to the mutant allele. This technique was then used to search for this polymorphism in a group of 172 subjects.

### Frequency of the intron 2 polymorphism, and clinical data on the probands

DNA from 172 subjects was subjected to amplification and hybridization with allele-specific oligonucleotides as described above. No mutants were identified in 121 normal subjects (data not shown). A group of 51 subjects classified as "hypobetalipoproteinemics" was also studied. Of these, 40 subjects were from the San Francisco Bay Area and had low levels of total plasma cholesterol and

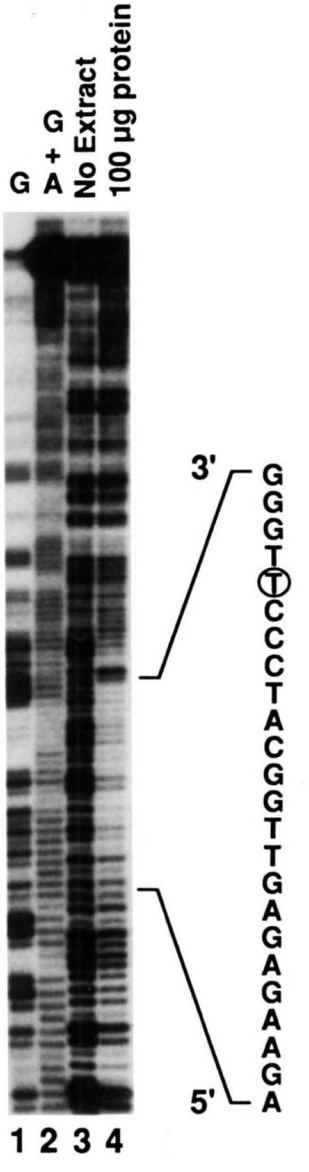


**Fig. 3.** The A→G substitution is contained within a footprint. Lanes 1 and 2 show the Maxam and Gilbert sequencing reactions. Lane 3 contains no protein extracts, and lane 4 shows the footprint obtained in the presence of 100 µg of HepG2 nuclear extract. The sequence, in brackets on the right side, corresponds to the footprint of the anti-sense strand, and the highlighted T is the position at which the mutation occurs.

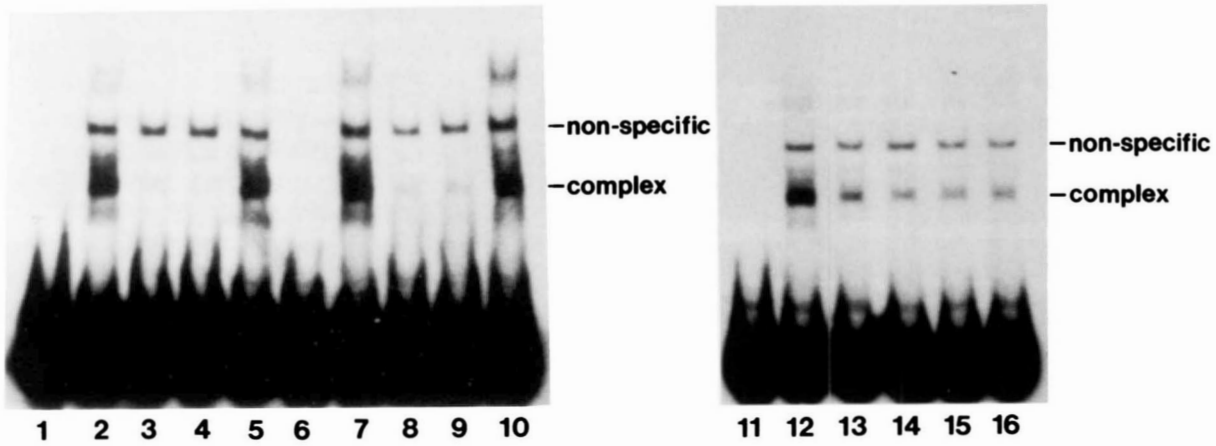


**Fig. 4.** Binding of nuclear proteins from HepG2 cells to wild-type and mutant oligonucleotides, as revealed by the gel retardation assay. Aliquots of protein extract were incubated with 0.5 ng of labeled oligonucleotide and 4.5 μg of poly(dI-dC). "Complex" indicates specific protein-DNA interactions. Lane 1, 0.5 ng of $^{32}$P-labeled wild-type oligonucleotide with no extract; lane 2, 0.5 ng of $^{32}$P-labeled wild-type oligonucleotide with 5 μg of HepG2 extract; lane 3, same as lane 2 plus 100 ng of unlabeled wild- type oligonucleotide; lane 4, same as lane 2 plus 100 ng of unlabeled mutant oligonucleotide; lane 5, same as lane 2 plus 100 ng of pBR322 DNA; lane 6, 0.5 ng of labeled mutant oligonucleotide with no extract; lane 7, 0.5 ng of $^{32}$P-labeled mutant oligonucleotide with 5 μg of HepG2 extract; lane 8, same as lane 7 plus 100 ng of unlabeled mutant oligonucleotide; lane 9, same as lane 7 plus 100 ng of unlabeled wild-type oligonucleotide; lane 10, same as lane 7 plus 100 ng of pBR322 DNA; lane 11, 0.5 ng of $^{32}$P-labeled mutant oligonucleotide; lane 12, 0.5 ng of mutant oligonucleotide plus 5 μg of HepG2 extract; lane 13, same as lane 12 plus 1 ng of mutant oligonucleotide competitor; lane 14, same as lane 12 plus 1 ng of wild-type oligonucleotide competitor; lane 15, same as lane 12 plus 2 ng of mutant oligonucleotide competitor; lane 16, same as lane 12 plus 2 ng of wild-type oligonucleotide competitor.

apoB protein (<5th percentile). None of these subjects possessed truncated forms of apoB (6). In this group, we found one individual who carried the intron 2 polymorphism. The remaining 11 subjects tested were from a well-studied Austrian population (15), and all had either plasma apoB levels or total cholesterol levels below the 10th percentile for the population. Three additional subjects with *Sty*I polymorphism were identified within this group and correspond to the same three individuals that were identified by the chemical method as carriers of the polymorphism.

The plasma apoB and cholesterol levels of the four probands described above are shown in **Table 1**. In three of the four, the apoB levels were at or below the 10th percentile (71 mg/dl). Although K130 had a total apoB level above the 10th percentile, this is probably attributable to an elevation of his very low density lipoprotein level (Table 1). His total cholesterol level and that of a second proband were below the 10th percentile (167 mg/dl). All four individuals had LDL cholesterol levels below the 10th percentile (126 mg/dl).

In an attempt to determine whether a correlation exists between the presence of the *Sty*I polymorphism and low plasma apoB levels, we studied a family whose pedigree is depicted in Fig. 5. However, all first-degree relatives within this family exhibited a rather low level of plasma apoB whether or not they carried the mutant allele, making it difficult to establish a correlation between apoB

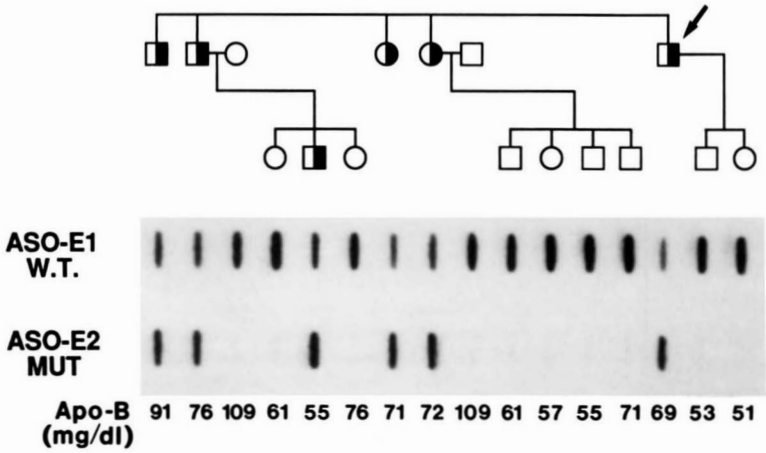


**Fig. 5.** Inheritance of the intron 2 polymorphism. The top panel shows the pedigree of the proband K111. Half-solid circles and squares represent individuals who are heterozygous for this mutation. The proband is indicated by an arrow. The slot blots in the bottom panel represent hybridization of amplified DNA from each individual with one or both of the two allele-specific oligonucleotides (ASOs); ASO-E1 represents the wild-type allele, and ASO-E2, the mutant allele. The level of apoB in the plasma of each individual is listed at the bottom of the figure.

TABLE 1. Clinical data for probands carrying the intron 2 mutation

| Subject No. | ApoB | | TC | LDL-C |
|---|---|---|---|---|
| | Total | VLDL | | |
| | *mg/dl* | | | |
| K111 | 69 | 10 | 187 | 101 |
| K130 | 83 | 34 | 145 | 85 |
| K23 | 71 | 1 | 178 | 76 |
| 115 | 50 | NA | 139 | 66 |

VLDL, very low density lipoproteins; TC, total cholesterol; LDL-C, LDL cholesterol; NA, not available.

levels and the presence of the *Sty*I polymorphism. No family members of the other probands were available.

### Haplotype analysis

To determine whether the *Sty*I polymorphism was associated with one or more apoB haplotypes, we used 10 markers to study the segregation of apoB alleles within the kindreds of subjects K111 and 102 (**Table 2**). Of these markers, eight were diallelic restriction fragment length or insertion/deletion polymorphisms within the coding region, and the two flanking markers were hypervariable minisatellite markers. This system of haplotype analysis has been described in detail elsewhere (11) and is capable of distinguishing a large number of alleles. The nomenclature uses numbers from 0 to 255 to describe the 256 possible combinations of the eight diallelic markers, followed by two other numbers that indicate the number of repeats of the 5' and 3' hypervariable regions (HVRs). The deduced haplotype of the allele with which the *Sty*I polymorphism was associated was 251/15-30. Although pedigree analysis was not performed, the genotypes of two other probands carrying the mutation (K130 and K23) were consistent with this assigned haplotype for the mutant allele, while that of a third subject (115) showed only a minor difference in the size of the 3' HVR.

To obtain an estimate of the frequency of the intron 2 polymorphism within individuals carrying haplotype 251/15-30, we screened a group of nine unrelated individuals possessing this haplotype; one additional subject with the intron 2 polymorphism was identified and is listed as 102 in Table 2.

## DISCUSSION

The high degree of polymorphism of the human apoB gene has long been evident. Multiply transfused patients produce antibodies against different LDL allotypes; five antithetical pairs of antibodies have been defined that correspond to polymorphic sites in the protein (16). A comprehensive study of several populations around the world has established the existence of 14 of the 32 possible haplotypes that could theoretically be distinguished by these five pairs of antibodies (17).

As a result of efforts in several laboratories, many base sequence variations have been described within the apoB gene. A recent review lists more than 75 positions in the coding region at which sequence differences have been reported (18). Although some of these may represent sequencing errors and others are third-position substitutions, these data emphasize the polymorphism of the apoB gene. When eight of these nucleotide polymorphisms were used as haplotype markers, 20 haplotypes were resolved in a sample of only 83 individuals within eight kindreds (19). The multiplicity of resolvable haplotypes is further increased by the use of two flanking hypervariable markers. These polymorphisms either singly or combined have also been employed as markers in several studies designed to establish associations between apoB haplotypes and susceptibility to disease (10, 11, 15, 20).

In the case of the *Sty*I polymorphism reported here, we have not established its functional significance, although two facts make this a real possibility. First, it falls within a region of the second intron that exhibits enhancer ac-

TABLE 2. Haplotype of the intron 2 mutation and genotypes of probands with the mutation

| Subject No. | 5' $(TG)_n$ | SP | ApaLI | HincII | PvuII | AluI | XbaI | MspI | EcoRI | 3' HVR |
|---|---|---|---|---|---|---|---|---|---|---|
| K111 | **15/14** | +/+ | +/+ | +/− | +/− | +/+ | −/+ | +/+ | +/+ | **30/40** |
| 102 | **15/14** | +/+ | +/+ | +/− | +/− | +/− | −/− | +/+ | +/+ | **30/34** |
| K130 | **15/14** | +/+ | +/+ | +/− | +/− | +/+ | −/− | +/+ | +/+ | **30/38** |
| K23 | **15/14** | +/+ | +/+ | +/− | +/− | +/− | −/− | +/+ | +/+ | **30/34** |
| 115 | **15/16** | +/+ | +/+ | +/− | +/− | +/− | −/− | +/− | +/− | **32/48** |
| Haplotype 251/15-30 | **15** | + | + | + | + | + | − | + | + | **30** |

Haplotype 251/15-30 designates the haplotype based on a binary numbering system using the eight diallelic markers (SP through EcoRI) and the two hypervariable regions (5'$(TG)_n$ and 3' HVR). This haplotype is always displayed in boldface. For subjects K111 and 102, the haplotypes were unequivocally deduced from pedigree analysis, while K130, K23, and 115 genotypes are listed, with 251/15-30 as the likely haplotype carrying this mutation. The haplotype for subject 115 differed only in the 3' HVR.

tivity and within a footprint defined by a DNA-binding protein. Perhaps because of the complexity of this regulatory region, where several protein-binding sites occur (A. R. Brooks, B. D. Blackhart, K. Haubold, and B. Levy-Wilson, unpublished results), we were not able to demonstrate that the base substitution affected protein binding in this in vitro assay. We have not tested for differences in the enhancer effect of wild-type and mutant constructs directly because the limited sensitivity of the CAT assays will make it impossible to quantitate a small, but real change in activity. To the extent that transcription is a rate-limiting step in apoB synthesis and that the enhancer modulates the rate of transcription, it remains possible that this single nucleotide substitution influences the amount of apoB synthesized and secreted. Even small changes in plasma apoB levels might have pronounced effects on cholesterol homeostasis. A second set of data presented here suggests, although it does not establish, that there may be a relationship between the occurrence of this polymorphism and the level of apoB in the plasma; the frequency of this polymorphism does appear to be higher in subjects displaying lower serum levels of apoB than in the overall population, although a more comprehensive study will be required to prove this contention.

The apparently increased frequency of the *Sty*I polymorphism in subjects with lower apoB levels could be directly related to this polymorphism. Alternatively, a second mutation present in haplotype 251/15-30 may be in genetic disequilibrium with the *Sty*I polymorphism and causally related to hypobetalipoproteinemia. The fact that mutant alleles of apoB exist that result in unequal representation of the amount of apoB in the plasma has been established (21, 22). Analysis of other families in which this allele is present will enable us to determine whether the intron 2 polymorphism segregates with hypobetalipoproteinemia.

Our haplotype studies to date indicate that the polymorphism is associated with haplotype 251/15-30. In a recent study with 85 subjects, we estimated the frequency of haplotype 251 to be ~10% (11). The vast majority of 251 haplotypes are associated with the 5' and 3' flanking markers 15 and 30. Thus, the combined haplotype 251/15-30 is likely to be found in no more than 10% of the population. From our screening of nine individuals with that haplotype, we found one intron 2 mutant (subject 102), thus suggesting that perhaps one in every ten 251/15-30 subjects may carry the intron 2 polymorphism.

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