

Donor Splice Site Mutation in the Apolipoprotein (Apo) C-II Gene (Apo C-II_{Hamburg}) of a Patient with Apo C-II Deficiency

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Abstract

The DNA, RNA, and protein of apo C-II have been analyzed in a patient with apo C-II deficiency (apo C-II_{Hamburg}). Markedly reduced levels of plasma and intrahepatic C-II apolipoprotein were demonstrated by immunoblotting and immunohistochemical analysis. Northern, slot blot, and in situ hybridization studies revealed low levels of a normal-sized apo C-II mRNA. No major rearrangement of the apo C-II gene was detected by Southern blotting. Sequence analysis of apo C-II genomic clones revealed a G-to-C substitution within the donor splice site of intron II. This base substitution resulted in the formation of a new Dde I and loss of a Hph I restriction enzyme cleavage site. Amplification of the mutant sequence by the polymerase chain reaction and digestion with Dde I and Hph I restriction enzymes established that the patient was homozygous for the G-to-C mutation. This is the initial report of the DNA sequence of an abnormal apo C-II gene from a patient with deficiency of apo C-II. We propose that this donor splice site mutation is the primary genetic defect that leads to defective splicing and ultimately to an apo C-II deficiency in this kindred.

Introduction

Apo C-II plays a central role in triglyceride metabolism as a cofactor for the enzyme lipoprotein lipase. The importance of apo C-II as an activator of this enzyme has been established by the absence of lipoprotein lipase activity in patients with an apo C-II deficiency. This syndrome, which is inherited as an autosomal recessive trait, has been described in several kindreds (1–8). Patients homozygous for apo C-II deficiency have marked derangements in triglyceride metabolism, including an elevation of plasma triglycerides, chylomicrons, and VLDL, as well as eruptive xanthomas and an increased incidence of pancreatitis. The diagnosis of apo C-II deficiency is established by finding a virtual absence of apo C-II in plasma associated with reduced postheparin lipoprotein lipase activity that is corrected by the addition of normal apo C-II-containing plasma. Patients with near normal levels of a nonfunctional apo C-II variant are also designated as patients with apo C-II deficiency. Transient normalization of triglyceride and lipoprotein abnormalities present in the plasma of apo C-II-deficient patients can be achieved by infusion of normal plasma

(1, 3, 7), purified apo C-II (5), or synthetic apo C-II fragments (7).

Of the various kindreds described with an apo C-II deficiency, two have been identified with an abnormal plasma apo C-II. Apo C-II_{Toronto}, a nonfunctional C-II apolipoprotein, has a different amino acid sequence at amino acids 64–74, as well as loss of amino acids 75–79 when compared with normal apo C-II (8). Apo C-II_{St. Michael} has an altered sequence starting with a proline substituted for a glutamine at position 70 and the abnormal apo C-II sequence is extended 17 residues past the normal carboxyl-terminal amino acid (9). These abnormalities are most consistent with a base deletion for apo C-II_{Toronto} and a base insertion for apo C-II_{St. Michael}, resulting in a subsequent shift of the translation reading frame; however, the precise DNA defect in these two kindreds has not been determined. Studies of the apo C-II gene in apo C-II-deficient patients at the DNA level have been limited to Southern blot hybridization analyses that have not, thus far, revealed any major rearrangements of the apo C-II gene (10, 11).

In this study, we investigate the molecular defect at the gene level in a kindred with apo C-II deficiency, apo C-II_{Hamburg}. A donor splice site mutation in the second intron of the apo C-II_{Hamburg} gene was identified by sequence analysis of the proband's DNA. We propose that this mutation is the basis for the deficiency of apo C-II in this kindred.

Methods

Clinical data. The proband is a 30-yr-old female of Turkish descent followed in the Medizinische Kern- und Poliklinik, Universitäts Klinik Eppendorf, Hamburg, FRG. She is one of three family members with a deficiency of apo C-II. The proband's parents are first cousins. Lipid values on presentation were 181 mg/dl total cholesterol and 1,848 mg/dl triglycerides. Plasma apo C-II levels by RIA were 0.01 mg/dl (normal range of 5.18 ± 0.30 mg/dl) and lipoprotein lipase activity before and after addition of normal apo C-II containing plasma was 0.9 and 7.7 $\mu\text{mol FFA/ml per h}$. Normal values were 5–15 $\mu\text{mol FFA/ml per h}$. Hepatic triglyceride lipase activity was 10.65 $\mu\text{mol FFA/ml per h}$. Plasma apo C-II concentrations were kindly determined by Dr. Moti Kashyap (Veterans Administration Medical Center, Long Beach, CA).

Electrophoretic analysis of the plasma apo C-II protein. Two-dimensional gel electrophoresis of plasma, consisting of isoelectric focusing followed by SDS gel electrophoresis, was performed as described previously (12). The gels were stained by the silver stain method (13, 14). The proteins separated by SDS gel electrophoresis were transferred to nitrocellulose paper at 80 V for 1 h (15). Apo C-II was detected by using a monospecific rabbit apo C-II antisera as the first antibody and visualized by indirect immunoperoxidase assay on nitrocellulose paper according to the manufacturer's (Bio-Rad Laboratories, Richmond, CA) instructions.

Immunohistochemistry. Frozen sections (8 μm thick) were prepared from liver biopsies of normal and apo C-II-deficient individuals. Liver tissue was obtained from the patient during open abdominal

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surgery for cholecystectomy, and from control subjects at the time of organ donation. Tissue was stored at -70°C until used. The liver tissue was embedded in OCT compound (Miles Scientific Div., Miles Laboratories, Inc., Naperville, IL) and sectioned. The sections were air dried and stored at -70°C until use. Specimens were rehydrated in PBS buffer, pretreated with 5% heat-inactivated normal goat serum, and incubated at room temperature for 60 min with an anti-apo C-II mouse MAb. After the initial incubation, unbound antibody was removed with three washes of PBS and the sections reincubated with colloidal gold-linked secondary antibody (Auro Probe LM; Janssen Life Sciences Products, Beerse, Belgium) at room temperature for 30 min. Silver enhancement was initiated by overlaying the tissue sections with a mixture of enhancer solution and initiator solution (1:1) as described by the manufacturer (Integrated Separation Systems, Hyde Park, MA) for 7 min at room temperature. After three rinses for 5 min each with distilled water, the slides were counterstained with Hanes' hematoxylin and evaluated for staining with dark pigment indicative of a positive reaction.

DNA and RNA preparation. RNA was isolated from the frozen liver using the guanidine thiocyanate method as previously described (16). DNA was isolated from white blood cells as described (10).

cDNA probes. A 354-bp Alu I restriction fragment of an apo C-II cDNA clone was used for our studies (10). For positive controls, an 850-bp Msp I restriction fragment of an apo A-I clone (17) and a 412-bp Dra I and Rsa I double-digest fragment of a 3'-untranslated β actin cDNA clone (18) were used. The β actin cDNA clone was kindly provided by Dr. Lawrence Kedes (Stanford University School of Medicine and Veterans Administration Medical Center, Palo Alto, CA).

Northern and slot blot hybridization analyses of RNA. Gels for Northern blot analysis were prepared with 1% agarose in the presence of 6% formaldehyde, electrophoresed at 25 V for 16 h, and transferred to nitrocellulose filters (Schleicher and Schuell, Inc., Keene, NH) as described previously (19). 8 μg of total RNA was analyzed and gels were stained with ethidium bromide to confirm that equivalent quantities of RNA were electrophoresed in each lane.

For slot blot analysis, serial dilutions of total RNA, (3.0, 2.0, and 1.0 μg) were loaded in duplicate onto nylon filters (Gene Screen Plus; Dupont Co., Wilmington, DE) using a slot blot apparatus (Bethesda Research Laboratories, Gaithersburg, MD). Baking, prehybridization, and hybridization conditions were as previously described except that hybridization was performed at 42°C for 24 h (20). Filters were autoradiographed and the blots were quantitated using a laser densitometer (Ultrascan XL; LKB Instruments, Bromma, Sweden). The absorbancy values were normalized with the mean of the values for normal RNA being assigned a value of 1. For reuse, filters were stripped of radiolabeled probe by incubation in $0.1\times$ SSC/SDS at 90°C for 1 h.

RNA in situ hybridization. Frozen sections were prepared as described for immunohistochemical studies. An Alu I cDNA apo C-II fragment was ligated into the Sma I cloning site of pGEM-3 vector DNA (Promega Biotec, Madison, WI), and radiolabeled sense and antisense strands were synthesized by transcribing 1 μg of template DNA with T7 and SP6 DNA polymerases (Promega Biotec) respectively (21). In situ RNA hybridization was performed as previously described (21) except that the samples were counterstained with Mayer's hematoxylin and eosin (American Histolabs, Bethesda, MD).

Cloning and screening of genomic libraries and isolation of apo C-II clones. DNA from the apo C-II-deficient subject was partially digested with the restriction enzyme Mbo I and the DNA ligated to EMBL3 vector DNA. *Escherichia coli* P2392 was then infected with the EMBL3 recombinant phage. Screening of the library with a radiolabeled apo C-II cDNA probe and large scale preparation of phage DNA were as previously described (22).

A 5-kb Bam HI and a 3.7-kb Eco RI fragment of insert genomic DNA isolated from the phage containing the apo C-II insert each were purified by agarose gel electrophoresis and ligated into the Bam HI and Eco RI sites of pGEM-3 vector DNA (Promega Biotec). Recombinant clones were selected by testing for antibiotic resistance and hybridization to the apo C-II cDNA probe.

DNA sequencing of apo C-II genomic clones. Recombinant pGEM3 plasmid DNA was digested with Bam HI and Eco RI and the fragments were subcloned into the M13 vectors mp18 and mp19 (Bethesda Research Laboratories). Sequencing was performed by the dideoxynucleotide chain termination method of Sanger et al. (23). 20-bp synthetic oligonucleotides based on the apo C-II DNA sequence or complementary to the apo C-II DNA sequence were obtained for sequencing from OCS Laboratories, Denton, TX.

Sequence amplification with Taq DNA polymerase. 1 μg of genomic DNA from control and apo C-II-deficient subjects was amplified by the automated polymerase chain reaction (PCR)¹ technique (24) for 30 cycles using Taq DNA polymerase (Perkin-Elmer Corp., Norwalk, CT) and 20-bp primers. The amplified region included bases 2,645 through 3,102 of the apo C-II gene (22). 2-min extensions at 72°C , 30-s denaturations at 95°C , and primer annealing at 55°C were performed. One tenth of the total amplified DNA was digested with two units of either Dde I or Hph I for 2 h. The digested fragments were separated on a 4% agarose (FMC BioProducts, Rockland, ME) Tris acetate EDTA (pH 8.3) minigel at 85 V for 1 h. DNA was identified by staining with ethidium bromide.

Restriction enzymes. All restriction enzyme used were from New England Biolabs (Beverly, MA) and the digestion conditions were as recommended by the manufacturer.

Results

Fig. 1 illustrates the pattern of apolipoproteins present in the plasma of normal (Fig. 1 A) and the apo C-II-deficient (Fig. 1 B) subjects. Absence of the normal apo C-II isoform in the plasma of the proband, based on silver staining, is illustrated in Fig. 1 B. The more sensitive method of immunoblotting demonstrates low levels of an apo C-II protein that exhibits normal electrophoretic mobility on two-dimensional gel analysis of the patient's plasma (Fig. 1 D).

Immunohistochemical analysis of liver incubated with an anti-apo C-II MAb revealed low but detectable intrahepatic levels of apo C-II in the patient (Fig. 2 C) when compared with normal levels (Fig. 2 B) based on the intensity of the brown staining. Normal liver, when incubated with either the preimmune (data not shown) or irrelevant MAb (Fig. 2 A), showed no cytoplasmic staining. Specificity of the procedure is also demonstrated by the lack of staining within connective tissue, which is not expected to contain the C-II apolipoprotein (Fig. 2 B).

Northern blot analysis of 8 μg of total RNA isolated from normal and patient's liver revealed the apo C-II mRNA of the patient to be of normal size but decreased in quantity when compared with the apo C-II mRNA from normal patients (Fig. 3 D). Ethidium bromide staining of the gel confirmed that equivalent amounts of nucleic acid had been electrophoresed in all lanes (Fig. 3 A). Stripping and rehybridization of the blot with nick-translated β actin and apo A-I cDNA probes followed by autoradiography revealed that nearly equal levels of both of these mRNAs were present in hepatocytes from both the apo C-II-deficient patient and normal subjects (Fig. 3, B and C).

Further quantitation of the level of apo C-II mRNA in the liver of the apo C-II-deficient patient was performed by slot blot and in situ RNA hybridization. Low but detectable levels of apo C-II message visualized as black grains by in situ hybridization are evident in the liver of the apo C-II-deficient patient

1. Abbreviations used in this paper: PCR, polymerase chain reaction.

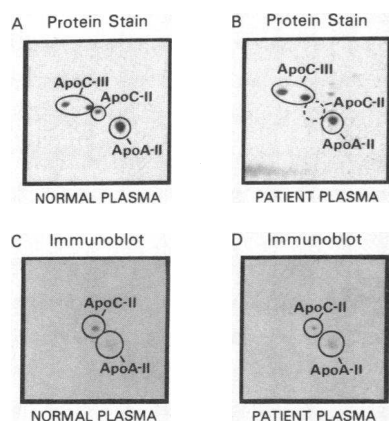


Figure 1. Two-dimensional gel electrophoretogram of plasma apolipoproteins from (A) normal and (B) apo C-II-deficient subjects analyzed with silver stain. (B) The dashed circle indicates the position of normal apo C-II not identified in the proband plasma by protein stain. Immunoblot analysis of plasma from the normal subject and the apo C-II-deficient

subject is shown in C and D. (D) A small quantity of electrophoretically normal apo C-II protein is present in the patient's plasma. Apo A-II standards are included for reference.

(Fig. 4 C) after hybridization with the apo C-II antisense RNA when compared with normal liver (Fig. 4 B). Specificity of the procedure was demonstrated by both the absence of grains within connective tissue (Fig. 4 B) the lack of hybridization of the apo C-II sense RNA with normal liver (Fig. 4 A). The apo C-II mRNA content in the patient's liver was quantitated by slot blot hybridization analysis using 1, 2, and 3 μ g of total liver RNA. The absorbancy values obtained from densitometric scanning of the autoradiographs normalized, with the mean of the values for normal RNA being assigned a value of 1 are as follows: control liver 1, 0.95 ± 0.05 ; control liver 2, 1.15 ± 0.05 ; apo C-II-deficient patient, 0.14 ± 0.03 . The level of the hepatic apo C-II mRNA from the patient thus was $\sim 14\%$ of that present in control subjects by slot blot hybridization analysis.

Southern blot analysis of the probands DNA revealed no major rearrangements of the apo C-II gene when digested with the restriction enzymes Bam HI, Bgl I, Eco RI, Hind III, Pst I, Sst I, Sph I, and Taq I (data not shown).

Sequence analysis of two apo C-II genomic clones isolated from the DNA library of the apo C-II-deficient patient revealed a G-to-C substitution at the highly conserved first nucleotide position in the 5' splice site of intron II (Fig. 5 A). Consensus donor splice site and normal apo C-II sequences are also illustrated in Fig. 5 A. Autoradiographs of sequence gels

from normal and apo C-II-deficient subjects illustrating the G-to-C substitution are shown in Fig. 5, B and C.

Computer analysis (Fristensky-Cornell DNA Sequencing Analysis Program) of the patient's apo C-II gene sequence containing the G-to-C substitution revealed a new Dde I site at position 2,715 and the loss of a normal Hph I restriction site at position 2,725 as compared with the normal apo C-II gene (22).

Amplification of the apo C-II gene from normal and apo C-II-deficient subjects was performed using the PCR and synthetic oligonucleotides illustrated in Fig. 6 A. The amplified product was 457 bp long. Digestion of DNA amplified from a normal subject with Dde I generated two fragments of 393 and 64 bp in size, but digestion of the patient's amplified DNA with the same enzyme resulted in the formation of three fragments of 69, 324, and 64 bp in size. Digestion of DNA amplified from a normal subject with Hph I generated four fragments of 80, 202, 28, and 147 bp in size, whereas digestion of the patient's amplified DNA with the same enzyme resulted in the formation of three fragments of 282, 28, and 147 bp in length. Analysis of the restriction enzyme fragments of the amplified DNA from the patient and normal subjects by agarose gel electrophoresis is illustrated in Fig. 6 B. Absence of normal-sized restriction fragments in the DNA of the apo C-II deficient patient indicates that the proband is a true homozygote with both apo C-II alleles having the same mutation. Southern blot hybridization analysis of normal and patient DNA digested with Dde I and Hph I confirmed this finding (data not shown).

Discussion

In this study we have analyzed the DNA, RNA, and protein from the proband with apo C-II deficiency from the Hamburg kindred. RIA and immunoblot analyses have documented low levels of a plasma C-II apolipoprotein that exhibits normal mobility on two-dimensional gel electrophoresis. Markedly reduced intrahepatic levels of apo C-II protein and mRNA were demonstrated by immunohistochemistry and total liver RNA hybridization studies. Apo C-II mRNA was shown to be of normal size by Northern blot analysis and Southern blot hybridization revealed no major rearrangements of the apo C-II gene. This patient thus has low quantities of apo C-II

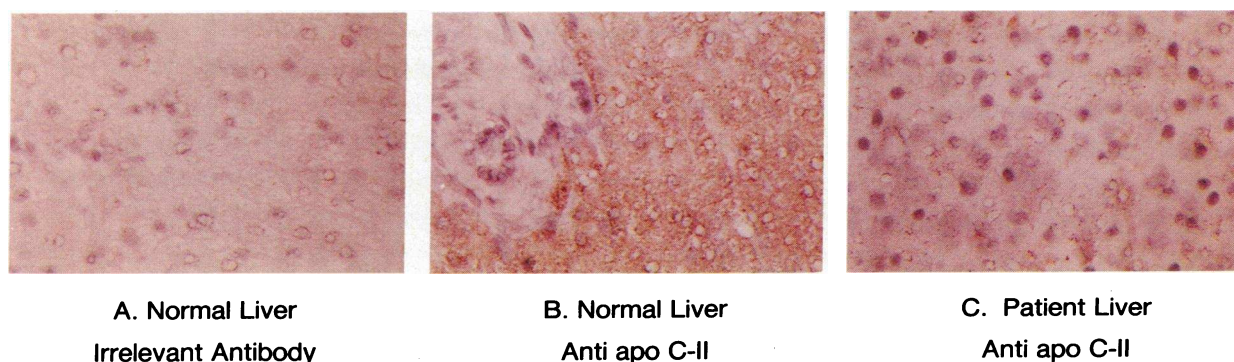


Figure 2. Immunohistochemical analysis of normal liver incubated with (A) an irrelevant antibody and (B) an anti-apo C-II MAb. (C) Markedly reduced cytoplasmic staining is present in the liver of the apo C-II-deficient patient after incubation with the anti-apo C-II MAb.

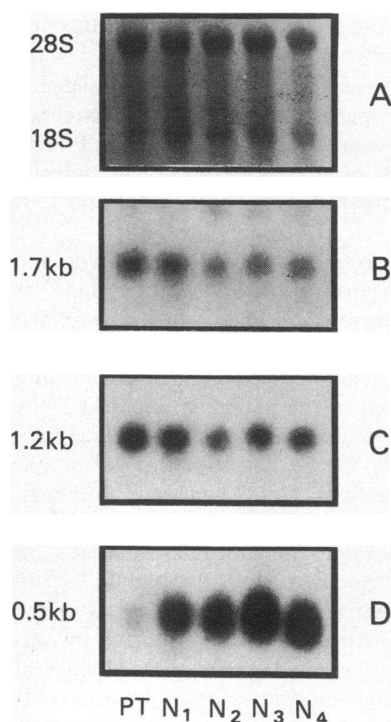


Figure 3. Northern blot analysis of 8 μ g of total liver RNA from normal (N) and apo C-II-deficient (PT) subjects hybridized with (B) β actin, (C) apo A-I, and (D) apo C-II cDNA probes. (A) Ethidium bromide staining of RNA in the agarose gel demonstrates that equivalent quantities of RNA were electrophoresed in each lane. The major bands in A are the 28S and 18S RNA bands.

mRNA and protein in the liver and low levels of the C-II apolipoprotein in plasma. These data are most consistent with a small DNA mutation that interferes with the normal transcription and processing of the apo C-II mRNA and/or destabilizes the apo C-II mRNA in the liver.

Sequence analysis of the apo C-II_{Hamburg} gene revealed a single-base substitution (G to C) at the first base of intron II. This study represents the first elucidation of a molecular defect at the DNA level in a patient with a deficiency of apo C-II. The G-to-C mutation alters the highly conserved dinucleotide invariant at the 5' splice site in structural genes and would be anticipated to result in defective processing of the apo C-II_{Hamburg} mRNA (25, 26). We thus postulate that this donor splice site mutation is the primary genetic abnormality that results in a deficiency of apo C-II in this kindred. Because this

rearrangement involves a single-point mutation, no major abnormality of the apo C-II gene is detected by Southern blot analysis.

Abnormal splicing of the β globin RNA has been described in patients with β_0 -thalassemia that have G for A or G for T mutations of the GT dinucleotide (27, 28). In these patients, splicing at the altered junction is completely abolished and alternate cryptic donor-like sequences located elsewhere in the RNA precursor are used in splicing. The resulting alternatively spliced mRNAs are frequently unstable and rapidly degraded. In a recent study, a G-to-C point mutation, identical to that present in our patient's gene, was introduced into the large β globin intron (29). In vitro expression of the altered gene revealed that a small quantity of splicing occurred one base upstream from the normal splice site, as well as in other cryptic splice sites with this mutation. In the apo C-II_{Hamburg} gene, however, it is very likely that despite the mutation in the critical GT dinucleotide, a small amount of correctly spliced message is made resulting in the low but detectable levels of normal-sized apo C-II message visualized by our Northern, slot blot, and in situ hybridization studies. Translation of this normally spliced apo C-II mRNA and secretion of the synthesized protein would result in the low plasma levels of the normal apo C-II isoform identified by our immunoblot studies.

Restriction enzyme digestion with the restriction enzymes Dde I and Hph I of the proband's genomic DNA, and DNA amplified by the PCR, confirm the presence of the G-to-C substitution within intron II identified by our sequencing studies. A new Dde I site is created and an Hph I site destroyed by the G-to-C mutation resulting in the formation of unique restriction fragments that were identified in our gel analysis studies. The absence of normal restriction fragment bands confirms that the proband is a true homozygote for the G-to-C mutation, with both apo C-II alleles having the same genetic defect. These results are consistent with the family history of consanguinity.

Thus, in this manuscript, we describe the DNA sequence of an abnormal apo C-II gene, apo C-II_{Hamburg}, from a patient with a deficiency of the C-II apolipoprotein. We conclude that the primary genetic defect in the Hamburg kindred is a G-to-C substitution within the donor splice site of the second intron of the apo C-II_{Hamburg} gene that results in abnormal splicing and eventually in a deficiency of apo C-II in plasma.

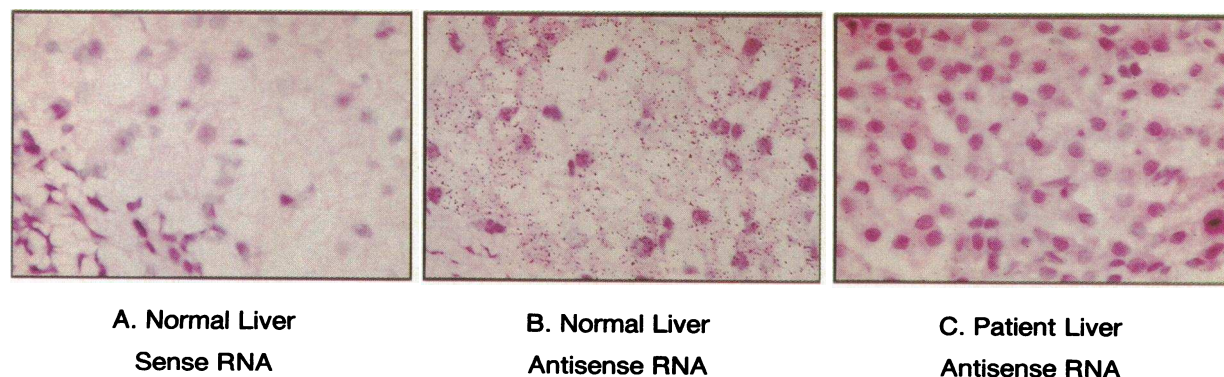


Figure 4. In situ RNA hybridization of (B) normal liver and (C) apo C-II-deficient liver hybridized with an apo C-II antisense RNA probe. Normal liver hybridized with the (A) apo C-II sense RNA probe is included as a negative control.

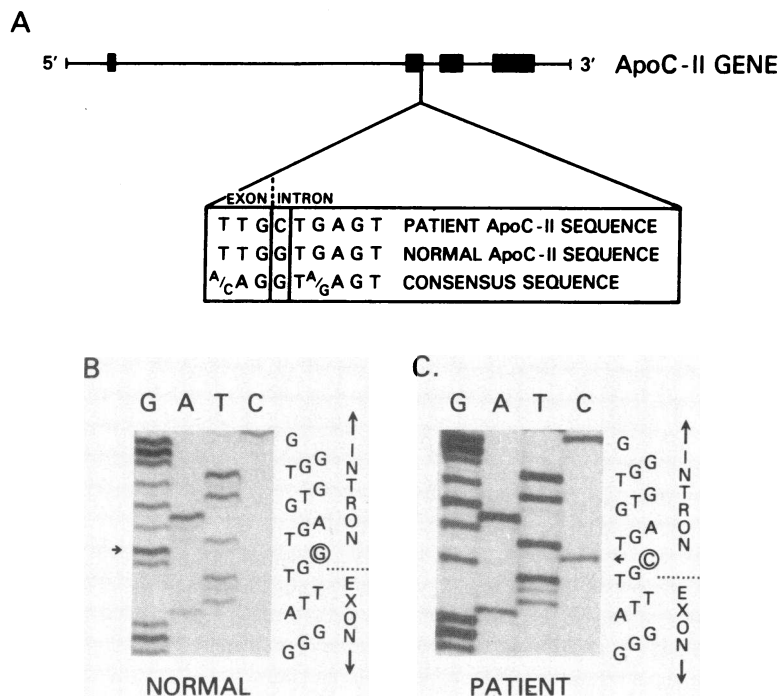


Figure 5. *A* is a schematic representation of the apo C-II gene. Exons are illustrated by the solid bars interrupted by lines that represent introns. The patient, normal, and consensus donor splice site sequences for intron II are shown. The G-to-C mutation is highlighted by a box. *B* and *C* contain the autoradiographs of sequencing gels of DNA from normal and apo C-II-deficient subjects near the region of the mutation. The G-to-C substitution is indicated by arrows.

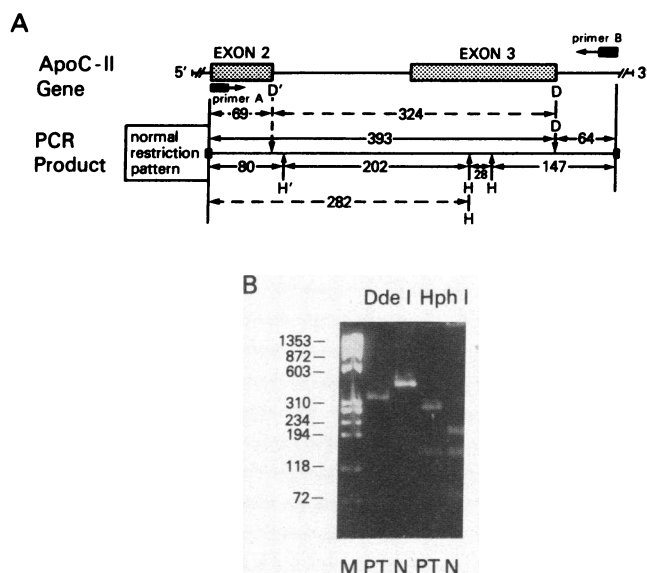


Figure 6. *A* is a schematic representation of the portion of the apo C-II gene that was amplified by the PCR. The positions of the primers (*A* and *B*) are indicated. Normal Dde I (*D*) and Hph I (*H*) sites are indicated. New Dde I (*D'*) and Hph I (*H'*) sites generated or destroyed by the mutation are indicated by the apostrophe. The horizontal solid arrows indicate the size of the restriction fragments generated by digestion of amplified normal DNA with Dde I and Hph I. The horizontal dashed arrows illustrate the size of the different restriction fragments generated when the patient's amplified DNA is digested with the same enzymes. *B* shows an electrophoretic analysis of the amplified DNA from normals (*N*) and the proband (*PT*) after restriction enzyme digestion with Dde I and Hph I. The differences in the sizes of the restriction fragments from the normal and patient DNA are illustrated. DNA molecular weight markers are shown in lane *M*.

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