ApoC-II$_{Paris2}$: a premature termination mutation in the signal peptide of apoC-II resulting in the familial chylomicronemia syndrome

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Abstract The chemical mismatch method has been utilized to screen for mutations in the apoC-II gene of a patient with familial chylomicronemia and apoC-II deficiency. Cleavage of heteroduplexes formed between normal and patient DNA strands with hydroxylamine and osmium tetroxide readily localized a mutation near base 2660 of the mutant apoC-II. Sequence analysis of PCR amplified patient DNA in the mismatched region localized by this method identified the substitution of a thymidine (T) for a cytosine (C) at base 2668 in exon 2 of the patient's gene within a CpG dinucleotide. The C to T transition in the apoC-II$_{Paris2}$ gene leads to the introduction of a premature termination codon (TGA) at a position corresponding to amino acid-19 of the signal peptide of apoC-II and the formation of a new Nla III restriction enzyme site absent in the normal apoC-II gene. Consistent with the history of consanguinity in this kindred, amplification of DNA isolated from the proband's parents by the polymerase chain reaction and digestion with Nla III established that the proband is a true homozygote for this genetic defect. Analysis of the patient's plasma by two-dimensional gel electrophoresis and immunoblotting failed to detect any plasma apoC-II. Thus, we have identified a novel mutation in the apoC-II gene of a patient with apoC-II deficiency from a Paris kindred presenting with severe hypertriglyceridemia and chylomicronemia. A single base substitution (C to T) occurring within a CpG dinucleotide present in the second exon of the apoC-II gene leads to the introduction of a premature stop codon in the signal peptide of the mutant gene, resulting in the inability to synthesize the mature apolipoprotein. The use of the chemical cleavage mismatch method to screen for mutations in the apoC-II$_{Paris2}$ gene facilitated the identification of the underlying genetic defect leading to the chylomicronemia syndrome in this kindred.—**Parrott, C. L., N. Alsayed, R. Rebourcet, and S. Santamarina-Fojo.** ApoC-II$_{Paris2}$: a premature termination mutation in the signal peptide of apoC-II resulting in the familial chylomicronemia syndrome. *J. Lipid Res.* 1992. **33:** 361–367.

Supplementary key words apoC-II deficiency • chemical cleavage • mismatch analysis • polymerase chain reaction • premature stop codon

Apolipoprotein C-II is a 79 amino acid apolipoprotein that serves as a cofactor for lipoprotein lipase (LPL), the key enzyme involved in normal triglyceride metabolism (1–3). In the presence of apoC-II, LPL hydrolyzes triglycerides present in chylomicrons and VLDL to monoglycerides and diglycerides and free fatty acids, which can be esterified or used as sources of energy. ApoC-II is synthesized primarily by the liver (4) and is normally present in human plasma at concentrations ranging from 2.2 to 5.5 mg/dl (5).

The importance of apoC-II as a physiological activator of lipoprotein lipase has been established by the identification of patients with a deficiency of apoC-II, a rare disease inherited as an autosomal recessive trait (6). Patients with apoC-II deficiency present with the familial chylomicronemia syndrome and may have eruptive xanthomas, lipemia retinales, hepatosplenomegaly, and an increased risk of pancreatitis. Chylomicrons and elevated triglycerides are present in fasting plasma. Infusion of apoC-II in these patients has resulted in transient normalization of plasma triglyceride levels and a marked improvement in clinical course (7, 8).

The underlying molecular defects that lead to a deficiency of apoC-II have been identified in several unrelated kindreds with familial chylomicronemia (7–16). To date, no major rearrangements have been identified in the apoC-II gene in patients with apoC-II deficiency. Only single point mutations have been reported and these result in either abnormal DNA splicing, the introduction of frameshift or premature termination mutations, or substitution in the initiation methionine codon. The defect in each patient studied to date has been unique; thus, there is no evidence of a founder gene effect in apoC-II deficiency.

Abbreviations: apo, apolipoprotein; PCR, polymerase chain reaction; bp, base pair; kb, kilobase; VLDL, very low density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins; LDL, lipoprotein lipase.

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In the present report we describe the underlying genetic defect in the apoC-II gene of the proband from a Paris kindred with a history of familial consanguinity. A substitution of a T for C at position 2668 of the second exon has been identified by the chemical cleavage method and sequence analysis. This novel mutation introduces a premature stop codon (TGA) that leads to the synthesis of a truncated apoC-II peptide and a deficiency of apoC-II in this kindred.

MATERIALS AND METHODS

Experimental subject

The proband is a 12-year-old female from Paris, France, who at the age of 4 months presented with a history of abnormal bloating associated with emesis. Evaluation at the Hôpital des Enfants Malades in Paris revealed fasting plasma values for triglycerides and cholesterol of 1,796 mg/dl and 153 mg/dl, respectively. The patient was then started on a low fat diet and has since remained asymptomatic. More recent lipid and plasma lipoprotein values were as follows: total cholesterol 120 mg/dl, triglycerides 1,160 mg/dl, VLDL-cholesterol 74 mg/dl, LDL-cholesterol 37 mg/dl, and HDL-cholesterol 10 mg/dl. Plasma apoC-II levels were undetectable by immunoassay and the patient's plasma when used as a source of apoC-II was unable to activate LPL in vitro. There is a history of familial consanguinity in this kindred with the patient's parents being second cousins.

Electrophoretic analysis of plasma apoC-II

Two-dimensional gel electrophoresis of plasma using isoelectric focusing followed by sodium dodecyl sulfate gel electrophoresis was performed as described previously (17). The gels were stained with Coomassie blue. For immunoblot analysis the apolipoproteins separated by sodium dodecyl sulfate gel electrophoresis was transferred to nitrocellulose paper at 80 V for 1 h. ApoC-II was detected using a monospecific rabbit apoC-II antisera as the first antibody and visualized by indirect immunoperoxidase assay on the nitrocellulose paper, according to the manufacturer's instructions (Bio-Rad, Richmond, CA).

DNA isolation

High molecular weight chromosomal DNA was isolated from white blood cells using an automated nucleic acid extractor as outlined by the manufacturer (Applied Biosystems Inc., model 340A, Foster City, CA).

DNA sequencing

Single-stranded DNA sequencing from M13 vector DNA was performed by the dideoxynucleotide chain termination method of Sanger et al. (18).

Chemical mismatch cleavage analysis

PCR amplifications of the apoC-II gene from normal and patient genomic DNA were performed as described (12, 19). PCR products obtained from normal and mutant DNA were purified from low melting agarose (BRL, Bethesda, MD) gels by phenol/chloroform extraction followed by ethanol precipitation. Radioactive templates were generated by a second amplification of the PCR products using one primer labeled at the 5′ end by T4 polynucleotide kinase and [^{32}P]ATP as reported previously (20, 21). A separate reaction was performed with either primer A (a 20-base sense primer scanning bases 2527–2546) or primer B (a 20-base antisense primer scanning bases 3102–3121) producing two stranded specific probes for each PCR region with either the sense or antisense strand labeled. Ten ng of DNA probe (sp act about 10^7 cmp/μg) and 150 ng of the unlabeled target DNA (mutant or wild type PCR products) were used to form a heteroduplex or control homoduplex (22). The

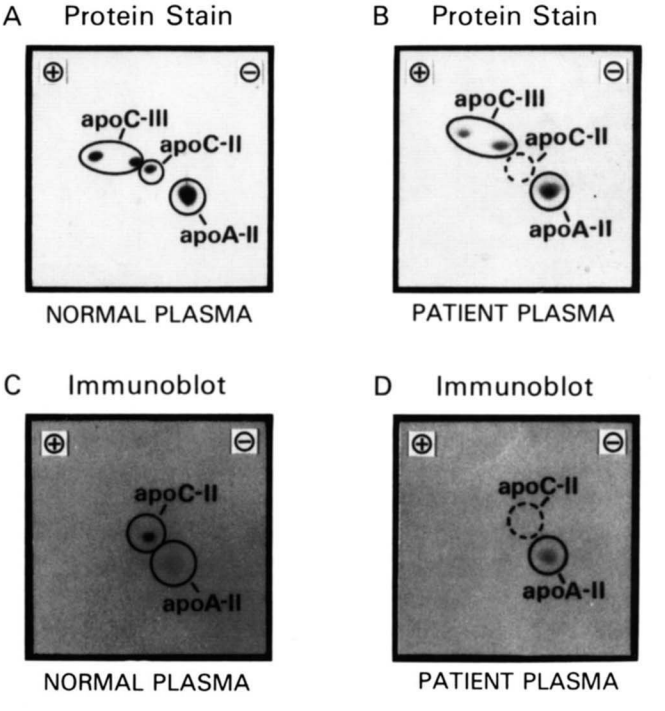

Fig. 1. Two-dimensional gel electrophoretograms of plasma apolipoproteins from (A) normal and (B) apoC-II-deficient subjects analyzed with Coomassie Blue protein staining. Immunoblot analysis of plasma from a normal subject and the apoC-II-deficient patient are shown in panels C and D. ApoA-II standards are included for reference.

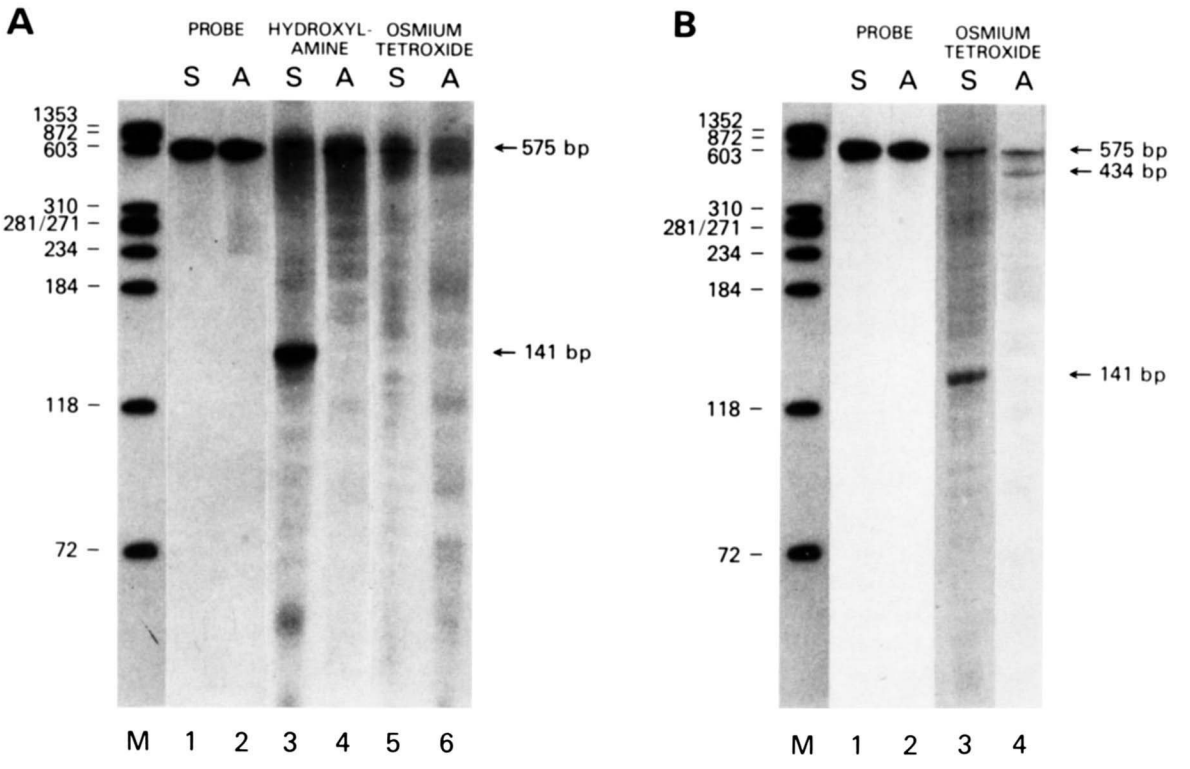

Fig. 2. Chemical cleavages of heteroduplexes of radiolabeled normal PCR template and patient PCR DNA are shown in panel A. Lanes 1 and 2 are the radiolabeled sense and antisense templates (575 bp); lanes 3 and 4 are the heteroduplexes treated with hydroxylamine; and lanes 5 and 6 are the heteroduplexes treated with osmium tetroxide. The 141 bp cleavage product is identified with an arrow. Radiolabeled molecular weight markers are shown in lane M. Panel B illustrates the chemical cleavages of heteroduplexes of radiolabeled patient PCR template and normal PCR DNA. Lanes 1 and 2 are the radiolabeled sense and antisense templates; lanes 3 and 4 are the heteroduplexes treated with osmium tetroxide showing the 141 bp cleavage product and the antisense 434 bp cleavage product. Lane M contains radiolabeled molecular weight markers.

chemical modification and cleavage reactions were performed using either 2.4% osmium tetroxide (Aldrich, Milwaukee, WI) at 37°C for 5 to 20 min or 2.5 M hydroxylamine (Sigma, St. Louis, MO) at 37°C for 15 min to 1 h for each strand. After piperidine (Dupont, Boston, MA) cleavage the fragments were analyzed by denaturing acrylamide gel electrophoresis and autoradiography. Molecular weight markers were radioactively labeled with [γ^{32}P]ATP and polynucleotide kinase.

DNA amplification with Taq DNA polymerase

One µg of genomic DNA from control, family members, and apoC-II-deficient subjects was amplified by 30 cycles using Taq I DNA polymerase (Perkin Elmer Cetus, Norwalk, CT) and two 20-base primers as reported previously (12). The amplified region included bases 2527 through 3102 of the apoC-II gene (23). Amplification was performed with 2-min extensions at 72°C, 1 min denaturation at 94°C, and primer annealing for 1 min at 55°C, followed by digestion with 2 units of Nla III (New England Biolabs, Boston, MA) for 2 h at 37°C. The digested fragments were separated on a 4% agarose TAE minigel at 85 V for 1 h. DNA was identified by staining with ethidium bromide. Synthetic oligonucleotides were synthesized by the phosphoramidite method of oligonucleotide synthesis in a DNA synthesizer (Model 380B, Applied Biosystems Inc., Foster City, CA).

RESULTS

Fig. 1 illustrates the two-dimensional gel electrophoretograms of apolipoproteins present in plasma of normal (Fig. 1A) and the apoC-II-deficient (Fig. 1B) subjects. The absence of normal apoC-II in the plasma of the patient based on Coomassie blue protein staining is illustrated in Fig. 1B. ApoC-II in the patient's plasma was not detectable by the more sensitive method of immunoblotting (Fig. 1D); whereas apoC-II was readily detected in the control plasma (Fig. 1C).

No major gene rearrangement was identified in the patient's apoC-II gene by Southern blot hybridization analysis of DNA from the apoC-II-deficient subject when compared to a normal subject (data not shown). The patient's DNA exhibited the 3.5 and 12 kb polymorphism bands for Taq I and Bgl I, respectively.

In order to rapidly screen for the presence of a small rearrangement or a single point mutation in the proband's apoC-II gene, we used the chemical cleavage mismatch method, which identifies all variants of single-base mismatches by reacting with either mismatched T or C (24). Normal and patient genomic DNA was amplified by the PCR using apoC-II-specific primers. Hydroxylamine treatment and piperidine cleavage of a heteroduplex formed between the 5′ end labeled normal probe and patient DNA identified a distinctive mismatch site involving cytosine, located 140 bp from the 5′ end of the PCR-amplified fragment which corresponded approximately to position 2660 (**Fig. 2A**). Hydroxylamine treatment and piperidine cleavage of heteroduplexes generated with the antisense normal probe and osmium tetroxide treatment followed by piperidine cleavage of heteroduplexes generated with either sense or antisense normal probe did not detect a mismatch. Analysis of heteroduplexes formed between the patient's probe radioactively labeled at the 3′ end and the normal DNA by osmium tetroxide treatment and piperidine cleavage identified a single unique mismatch. Similarly, osmium tetroxide treatment and piperidine cleavage of heteroduplexes between the 5′ end of the patient's probe and normal DNA indicated that there was a mismatch that involved the thymidine at the same site in the patient's probe (Fig. 2B). These results establish the presence of a single base substitution, C to T, in the patient's DNA sense strand at a position near base 2660.

Sequence analysis of the region of the mutation localized by the chemical cleavage mismatch method identified a single C to T substitution in exon 2 at position 2668 (**Fig. 3**). All four exons, all splice junctions, and the 5′ and 3′ untranslated regions of the apoC-II gene were subsequently sequenced, and the only mutation identified was at position 2668. This substitution leads to the introduction of a premature stop codon (TGA) at a position corresponding to amino acid -19 of the signal peptide of the apoC-II gene. Fig. 3A illustrates the genomic organization of the apoC-II gene in the region of the C to T mutation. Fig. 3B contains the autoradiogram of the sequencing gel of the normal subject and apoC-II-deficient patient. The base substitution is indicated by an arrow.

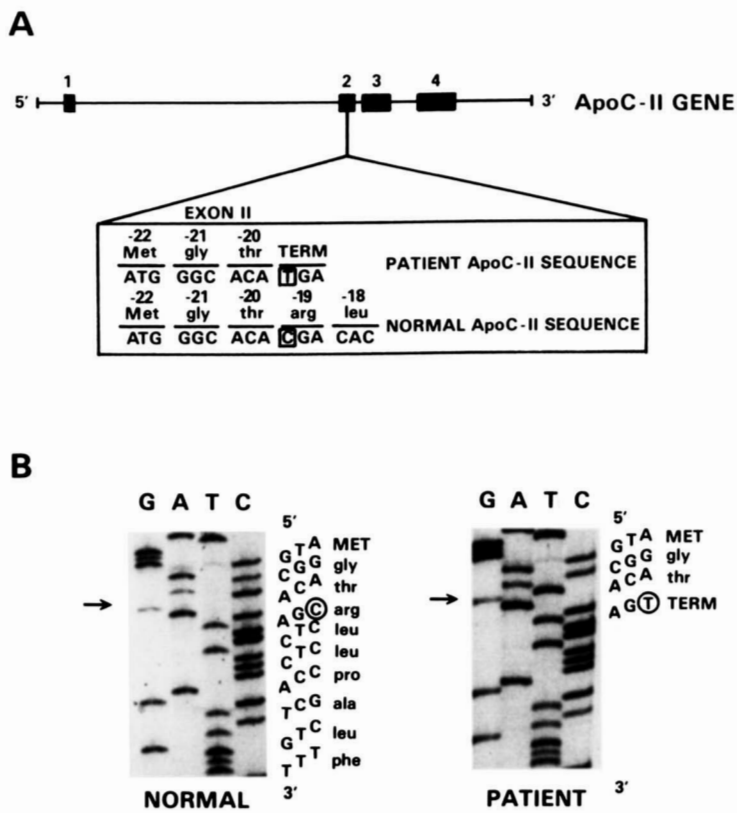

Fig. 3. Panel A contains a schematic representation of the apoC-II gene. Exons are illustrated by the solid bars interrupted by lines that represent introns. The patient and normal apoC-II sequences are shown. The C to T mutation is highlighted in a box. Panel B contains the autoradiograms of sequencing gels of DNA from a normal and an apoC-II-deficient patient in the region of the mutation. The C to T substitution is indicated by the arrow.

Sequencing was performed on multiple M13 clones derived from PCR-amplified DNA. Direct sequencing of PCR-amplified DNA revealed a T at position 2668, indicating that the substitution is present in both of the patient's apoC-II alleles (data not shown).

Fig. 4 illustrates the analysis of DNA isolated from the apoC-II-deficient patient, her mother and her father, after amplification by the PCR and digestion with Nla III. The control lane contains the normal restriction pattern consisting of fragments 481 bp and 94 bp in length. Digestion of the amplified DNA from the patient results in formation of two abnormal-sized restriction fragments, 435 bp and 46 bp in length, that result from the introduction of a new Nla III restriction enzyme site by the mutation. Digestion of amplified DNA from both parents revealed both the normal-sized restriction fragments (481 bp and 94 bp) and the abnormal-sized restriction fragments (435 bp and 46 bp). These findings establish that the parents are heterozygotes for the Nla III polymorphism and that the patient is a true homozygote for the C to T mutation at position 2668 of the apoC-II gene.

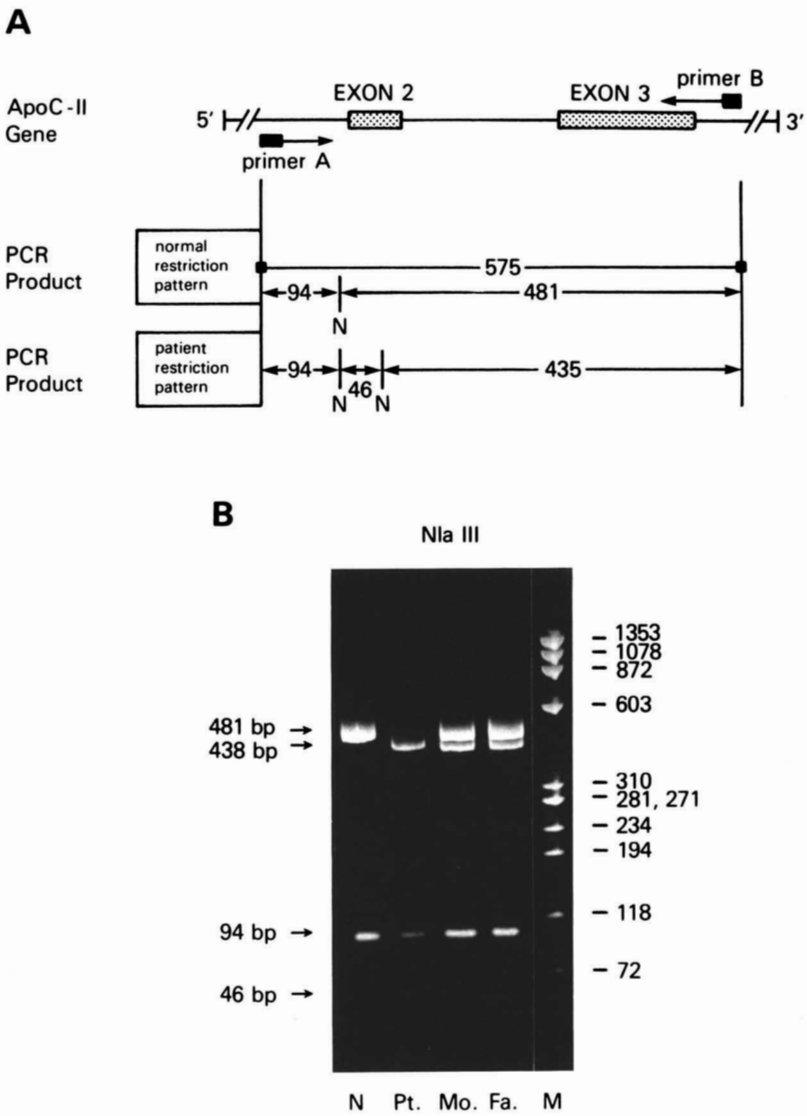

Fig. 4. A schematic representation of the region of the apoC-II gene that was amplified by the PCR using primers A and B is illustrated in panel A. The normal Nla III site is shown and the new site generated by Nla III in the patient is indicated by N′. Panel B contains the electrophoretogram of the amplified DNA of the normal (N), patient (Pt.), Mother (Mo.), and Father (Fa.) after digestion with Nla III. DNA molecular markers are shown in the lane designated M.

DISCUSSION

In the present study we have used the chemical cleavage mismatch method to screen for the genetic defect in the apoC-II gene of a patient from a Paris kindred with familial chylomicronemia and apoC-II deficiency. Treatment of radiolabeled normal or patient amplified DNA with hydroxylamine or osmium tetroxide permitted the detection of a single base mismatch involving thymidine in the second exon of the patient's apoC-II gene near position 2660. Further analysis by DNA sequencing identified a single C to T substitution at position 2668 of the mutant gene. This mutation results in the introduction of a premature termination codon at residue -19 in the signal peptide of apoC-II. Thus, no apolipoprotein is synthesized resulting in a total deficiency of apoC-II. These results are consistent with the failure to defect apoC-II in the patient's plasma by two-dimensional electrophoresis, immunoblotting, or immunoassay.

Consistent with a history of familial consanguinity, the proband from this kindred is a true homozygote for the mutation at bp 2668. Thus, direct sequencing of PCR-amplified DNA identified the C to T substitution in both of the patient's alleles and analysis of the parents DNA by the PCR, followed by restriction digestion with Nla III established that the parents were heterozygotes for the same mutation.

The mutation identified in the apoC-II gene of this French kindred is the first example of a genetic defect leading to premature termination of protein synthesis within the signal peptide of apoC-II. The fate of the short truncated signal peptide is unclear, but it is unlikely to be transported out of the endoplasmic reticulum. No evidence for the synthesis of a partial or full-length apoC-II has been detected by immunoblotting and RIA studies performed with two different polyclonal antibodies previously shown to detect the first 37 amino acid residues of apoC-II (12). Like most other defects identified in genes of different kindreds with apoC-II deficiency, the ApoC-II$_{Paris2}$ mutation consists of a unique single base substitution. Thus, unlike the mutations found in patients with deficiencies of other apolipoproteins, no major gene rearrangement has been described in probands with apoC-II deficiency (25, 26).

The mutation identified in the apoC-II$_{Paris2}$ gene occurs within a CpG dinucleotide. It has been previously reported that C to T transitions occur at high frequency within methylated gene regions, which are hot-spot areas for mutations via methylation-induced deamination of 5-methyl cytosine (27). In the case of the apoC-II$_{Paris2}$ gene mutation, DNA regulation within this CpG dinucleotide may have resulted in the C to T mutation and the substitution of the CGA (arginine) codon by a TGA (termination) codon (**Fig. 5**). This mechanism has not been previously implicated in other described mutations of the apoC-II gene (9–15).

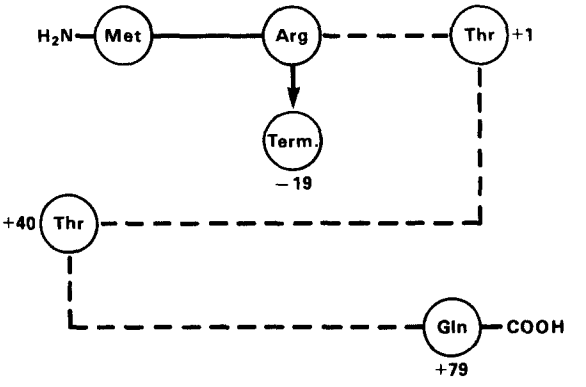

Fig. 5. Schematic representation of the C-II apolipoprotein. The formation of a premature stop codon at residue -19 of the signal peptide in the mutant apoC-II$_{Paris2}$ is illustrated.

In summary, the underlying genetic defect that leads to a deficiency of apoC-II in a patient presenting with the familial chylomicronemia from the Paris$_2$ kindred has been identified. The primary genetic defect in this kindred is a substitution of a T for a C in the second exon of the apoC-II gene which introduces a premature termination codon at position -19 of preapoC-II resulting in the synthesis of a truncated signal peptide and leading to a deficiency of plasma apoC-II and the chylomicronemia syndrome.

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