# Molecular cloning of a human apoC-III variant: Thr 74 → Ala 74 mutation prevents O-glycosylation

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**Abstract** Apolipoprotein C-III (apoC-III) is a major protein of very low density lipoprotein (VLDL). The apoC-III polypeptide contains a carbohydrate chain containing galactosamine, galactose, and sialic acid attached in O-linkage to a threonine residue at position 74. We have cloned the apoC-III gene from a subject whose serum contained unusually high amounts of apoC-III lacking the carbohydrate moiety (C-III-0). DNA sequence analysis of the cloned gene revealed a single nucleotide substitution (A → G) that encodes an alanine at position 74 instead of the normal threonine. As a result of this amino acid replacement, the mutant apoC-III polypeptide is not glycosylated. The mutation in the apoC-III gene creates a novel *Alu*I site that permits diagnosis of the change by Southern blotting of genomic DNA.—Maeda, H., R. K. Hashimoto, T. Ogura, S. Hiraga, and H. Uzawa. Molecular cloning of a human apoC-III variant: Thr 74 → Ala 74 mutation prevents O-glycosylation. *J. Lipid Res.* 1987. **28:** 1405–1409.

**Supplementary key words** apolipoprotein • apoC-III gene • human gene • gene cloning • apoC-III mutant

Apolipoprotein C-III (apoC-III) is a major protein constituent of the triglyceride-rich lipoproteins, chylomicrons, and very low density lipoproteins (VLDL), and a minor constituent of high density lipoprotein (HDL) in human serum. The precise physiological function of apoC-III in lipoprotein metabolism is not known. However, apoC-III has been shown to inhibit the in vitro activity of lipoprotein lipase (1) and to inhibit the apoE-mediated uptake of triglyceride-rich lipoproteins by liver cells (2, 3). The preprotein of apoC-III is primarily synthesized in liver cells (4–6), and consists of 99 amino acids including a 20 amino acid signal sequence. After removal of the signal sequence, the mature apoC-III is modified by the attachment of a carbohydrate chain consisting of galactosamine, galactose, and sialic acid. The molar ratio of carbohydrate to apoC-III is 1 for galactosamine, 1 for galactose, and 0, 1, or 2 for sialic acid. The variations in the molar ratio of sialic acid to apoC-III result in three apoC-III polymorphic forms designated C-III-0, C-III-1,

and C-III-2, respectively (7). We have previously described a family in which certain members contain unusually high amounts of unsialylated apoC-III (apoC-III-0) associated with their VLDL and HDL fractions (8). Family members having increased apoC-III-0 levels were asymptomatic and we demonstrated an autosomal-dominant Mendelian inheritance for this trait. Three possible mechanisms to explain the increase in unsialylated apoC-III were postulated: first, reduction of the level of sialyltransferase; second, increase of a sialidase activity; and third, reduction in the activity and/or the number of asialoglycoprotein receptors (8).

In the present study, we have cloned and sequenced the genomic DNA segments of the apoC-III gene from a member of the above family who had an increased level of serum apoC-III-0. We demonstrate that a single base substitution has occurred in the normal codon for threonine at position 74 that results in the replacement of threonine by alanine. The loss of the O-linked sugar attachment site (Thr 74) thus explains the presence of an increased amount of apoC-III-0 in this subject.

## MATERIALS AND METHODS

### Electrophoresis of apoVLDL

Venous blood was obtained after overnight fasting. Plasma VLDL particles (d < 1.006 g/ml) were isolated in a Type 50 rotor at 15°C, using a Beckman L5-50B ultracentrifuge. Polyacrylamide gel electrophoresis was carried out using 7.2% gels in 8 M urea. The gels were

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Abbreviations: VLDL, very low density lipoproteins; HDL, high density lipoproteins.
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fixed and stained overnight with 0.003% Coomassie Brilliant Blue R in 10% trichloroacetic acid.

## Preparation of DNA

Approximately 15 to 20 ml of peripheral blood was collected from the proband's elder daughter (37 years old). Following purification of white blood cells, genomic DNA was extracted according to the procedures of Kunkel et al. (9).

## Cloning of the genomic apoC-III DNA

A library of genomic DNA from the elder daughter was constructed using the EMBL 4 bacteriophage lambda cloning vector. Genomic DNA was partially digested with Sau3AI and ligated with BamHI-digested EMBL 4 phage DNA. The ligated DNA was packaged in vitro into infectious particles using a Gigapack kit (Stratagene). The library was screened by plaque hybridization (10), using $^{32}$P-labeled apoC-III cDNA. Labeling of the apoC-III cDNA (4) was carried out by nick translation using $[\alpha\text{-}^{32}\text{P}]$dCTP (400 Ci/mmol, Amersham Corp.) for 2 hr at 15°C. Phage plaques were grown on agar plates and transferred to nitrocellulose filters (BA85; Schleicher & Schuell). Following denaturation and baking, filters were hybridized with the $^{32}$P-labeled apoC-III cDNA probe (4 × 10⁵ cpm/ml) at 42°C for 16 hr in 50% formamide, 4× SSPE (0.15 NaCl, 15 mM sodium phosphate, 4 mM EDTA, pH 7.4), 0.1% SDS, 5× Denhardt's solution (0.1% bovine serum albumin, 0.1% Ficoll, 0.1% polyvinylpyrrolidone), and 50 µg/ml denatured salmon-sperm DNA. After hybridization, filters were washed twice at 60°C for 30 min in 2× SSC (1× SSC is 0.15 M NaCl, 15 mM sodium citrate, pH 7.4), and once in 0.2× SSC containing 0.1% SDS. The filters were air-dried and autoradiographed on Fuji RX film with Dupont Cronex intensifying screens at −80°C for 16 hr. Recombinant clones hybridizing with the cDNA probe were isolated by plaque purification. DNA of positive clones was digested with several restriction enzymes (EcoRI, PstI, EcoRI-PstI, EcoRI-XbaI, and EcoRI-HindIII) and analyzed by agarose gel electrophoresis. Large scale preparation of positive clones was carried out after growth in 500 ml of NZCYM medium (1% NZ-amine, 0.5% NaCl, 0.5% yeast extract, 0.1% Casamino acid, and 10 mM MgSO₄) with the host bacterial strain E. coli VCS257. The recombinant phages were precipitated with 10% (w/v) polyethyleneglycol and purified by step-wise glycerol gradient ultracentrifugation (10). Phage DNA was prepared by phenol extraction and ethanol precipitation. The DNA was digested with EcoRI and PstI, and DNA fragments were isolated after electrophoresis in low melting point agarose gels (Bethesda Research Laboratories). A 1.8-kb EcoRI-PstI fragment containing exons 1, 2, and 3 and a 2.2-kb PstI-EcoRI fragment containing exon 4 were subcloned into the EcoRI-PstI site of the pAT153 vector. For DNA sequencing, these same fragments were subcloned into the bacteriophage M13mp10 and M13mp11 for vectors (11).

## DNA sequence analysis

DNA sequence was determined according to Sanger, Nicklen, and Coulson (12). The sequencing reactions were carried out using a kit from Takara Shuzo Co. Oligonucleotide primers were synthesized using the solid-phase phosphotriester method and an Applied Biosystems DNA synthesizer and were used after desalting with a Sep-Pak column (Waters Associate, Inc., Milford, MA). The specific primers were used at 1 pmol for each DNA sequencing reaction.

## RESULTS

Plasma VLDL particles were isolated from the serum of the proband's elder daughter and analyzed for apolipoproteins by basic polyacrylamide gel electrophoresis. As shown in **Fig. 1** (lane B), in addition to the bands of apoC-II, apoC-III-1, and apoC-III-2, an intensely stained band of unsialylated apoC-III polypeptide (apoC-III-0) was observed in the VLDL sample from the daughter. The amount of apoC-III-0 was nearly the same as that of the sialylated apoC-III polypeptides, apoC-III-1 and apoC-III-2. In contrast, the band corresponding to apoC-III-0 was very faint in the serum of a control person (Fig. 1, lane A).

## Cloning of the human apoC-III gene

To analyze the DNA sequence of the apoC-III gene, DNA was extracted from peripheral blood of the daughter and used to prepare a genomic DNA library in the EMBL
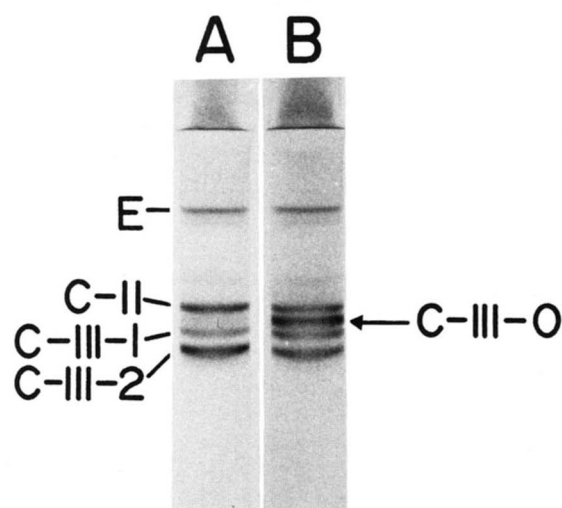


**Fig. 1.** Basic polyacrylamide gel electrophoresis (8 M urea) of apoVLDL; (A): apoVLDL from a normal individual; (B): apoVLDL from a daughter of the family having increased levels of apoC-III-0.

4 bacteriophage lambda cloning vector. We screened $8.4 \times 10^5$ recombinants from this library and isolated two clones that hybridized strongly with a $^{32}$P-labeled apoC-III cDNA probe.

Restriction maps of the human DNA segments in the two clones are shown in **Fig. 2.** The recombinant phage are designated λ apoC-IIIHK3 and λ apoC-IIIHK10. Restriction enzyme analysis and Southern blotting analysis of these clones demonstrated that λ apoC-IIIHK3 and λ apoC-IIIHK10 contained 13.3-kb and 10.1-kb inserts, respectively (Fig. 2). Restriction maps of these two clones are compatible with the results described by Karathanasis (13) and Protter et al. (14). These data indicate that both clones contain the entire coding sequences of the apoC-III gene.

ApoC-III, apoA-I, and apoA-VI genes are organized as a gene cluster within a 20-kb DNA span in humans (15, 16) and rats (17). The apoA-I gene is located about 2.6 kb downstream (3') of the apoC-III gene. The cleavage maps shown in Fig. 2 suggest that λ apoC-IIIHK3 and λ apoC-IIIHK10 contain all of and part of the apoA-I gene, respectively, in addition to apoC-III gene.

### Sequencing of the cloned apoC-III gene

The genomic DNA segments of λ apoC-IIIHK3 and λ apoC-IIIHK10 were subcloned. All four exons of the apoC-III gene and their flanking regions were analyzed

by DNA sequencing. As shown in **Fig. 3** and **Fig. 4,** only one base substitution (A→G) at the codon of position 74 was found in both clones, when compared to the DNA sequence of the normal apoC-III gene (4). This mutation was confirmed by sequencing the complementary strand of the DNA. At the protein level, this missense mutation causes the replacement of a threonine residue with an alanine at position 74. The normal threonine residue is known to be the attachment site of the carbohydrate chain (7, 18).

In addition, the mutation gives rise to a novel restriction site for *Alu*I (AGCT). To ensure that the A→G transition was present in the DNA of the daughter, we assayed for this novel *Alu*I site by Southern blotting analysis. In this DNA, a 266-bp *Alu*I segment hybridized with the *Pvu*II-*Hae*II DNA probe (582bp) derived from exon 4 of the apoC-III gene in addition to the normal 328-bp segment (data not shown). The 266-bp *Alu*I segment was not observed when DNA isolated from a normal individual was analyzed.

## DISCUSSION

Two genomic DNA clones of the apoC-III gene were isolated from a DNA library constructed from a subject with abnormally high levels of apoC-III-0. Sequence
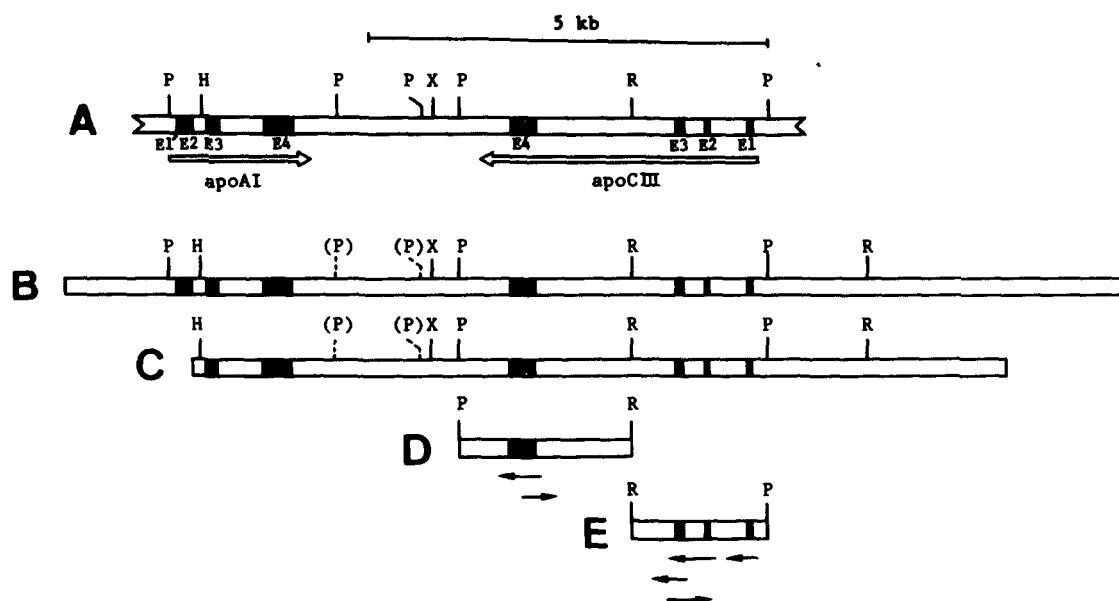


**Fig. 2.** Restriction maps of the genomic DNA segments containing the apoC-III gene. (A): The top bar represents the genomic DNA segment of the normal gene described by Protter et al. (14) and Karathanasis (13). Solid blocks indicate coding regions. Hatched regions indicate untranslated sequences. $E_1$, $E_2$, $E_3$, and $E_4$ denote exons. Open arrows indicate the direction of transcription for the apoA-I and apoC-III genes. (B) and (C): The two cloned DNA segments carried by λ apoC-IIIHK3 and λ apoC-IIIHK10, respectively. (D) and (E): DNA segments subcloned into M13 phage vectors and the strategy of DNA sequencing. Solid arrows indicate the direction and extent of nucleotide sequence determined using the dideoxy chain termination method. Relevant restriction sites are as follows: *Eco*RI (R), *Hin*dIII (H), *Pst*I (P), and *Xba*I (X). Only *Pst*I sites directly determined are illustrated by the vertical lines in (B) and (C).

|GTAA····Intron 1····GCAG|

CAGGTCAGAACCCTCCTGCCTGTCTGCTCAGTTCATCCCTAGAGGCAGCTGCTCCAGGAACAGAGGTGCC

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ATG CAG CCC CGG GTA CTC CTT GTT GTT GCC CTC CTG GCG CTC CTG GCC TCT GCC
MET Gln Pro Arg Val Leu Leu Val Val Ala Leu Leu Ala Leu Leu Ala Ser Ala
-20                                       -10
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|GTAA····Intron 2····TCAG|

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CGA GCT TCA GAG GCC GAG GAT GCC TCC CTT CTC AGC TTC ATG CAG GGC TAC ATG
Arg Ala Ser Glu Ala Glu Asp Ala Ser Leu Leu Ser Phe Met Gln Gly Tyr Met
     -1  1                              10
```

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AAG CAC GCC ACC AAG ACC GCC AAG GAT GCA CTG AGC AGC GTG CAG GAG TCC CAG
Lys His Ala Thr Lys Thr Ala Lys Asp Ala Leu Ser Ser Val Gln Glu Ser Gln
                  20                              30
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|GTAC····Intron 3····TTAG|

```
GTG GCC CAG CAG GCC AGG GGC TGG GTG ACC GAT GGC TTC AGT TCC CTG AAA GAC
Val Ala Gln Gln Ala Arg Gly Trp Val Thr Asp Gly Phe Ser Ser Leu Lys Asp
                  40                              50
```

```
TAC TGG AGC ACC GTT AAG GAC AAG TTC TCT GAG TTC TGG GAT TTG GAC CCT GAG
Tyr Trp Ser Thr Val Lys Asp Lys Phe Ser Glu Phe Trp Asp Leu Asp Pro Glu
                          60                              70
```

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GTC AGA CCA GCT TCA GCC GTG GCT GCC TGA GACCTCAATACCCCAAGTCCACCTGCCTATC
Val Arg Pro Ala Ser Ala Val Ala Ala ***
             74              79
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CATCCTGCCAGCTCCTTGGGTCCTGCAATCTCCAGGGCTTCCCCTGTAGGTTGCTTAAAAGGGACAGTATT

**Fig. 3.** Nucleotide and amino acid sequences of the mutant human apoC-III gene. A single nucleotide substitution (an A→G transition) in the codon for an amino acid 74 is present, resulting in a change of amino acid from threonine to alanine.

analysis of the isolated apoC-III gene revealed a single amino acid difference when compared to the normal gene: alanine is substituted for threonine of position 74. This replacement of alanine for threonine does not affect the isoelectric point of apoC-III (8), however, because the threonine residue at position 74 is the site of O-glycosylation in the apoC-III polypeptide (7, 18). The mutant apoC-III polypeptide is not a substrate for this posttranslational modification. The mutation (A→G) in the apoC-III gene creates a novel *Alu*I site that is readily detectable by Southern blotting. Using this technique, we were able to demonstrate directly the presence of this mutation in the proband's genomic DNA. The results also indicated that this individual was a heterozygote with both an abnormal apoC-III gene and a normal apoC-III gene in her chromosome complement. Based on previous protein analysis, other members of this family are also predicted to be heterozygous at their apoC-III loci. The availability of a rapid Southern blotting test for the mutant gene will allow us to test this hypothesis directly.

We do not yet know the functional significance of the carbohydrate moiety of the apoC-III polypeptide. Considering that the relative proportion of the unsialylated apoC-III (apoC-III-0) is essentially the same as that of the

sialylated apoC-III forms (apoC-III-1 plus apoC-III-2) in the VLDL of the heterozygotes, the mutation does not appear to affect the expression of the apoC-III gene, its secretion, or its integration into the lipoprotein particle. Future studies with the apoC-III gene described here will
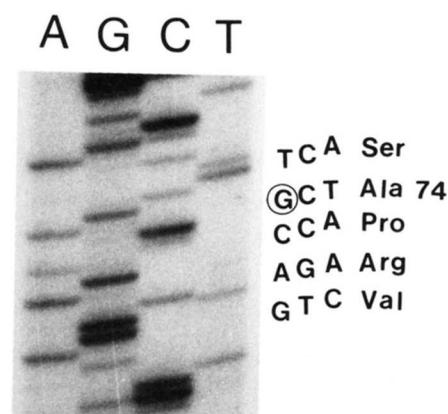
**Fig. 4.** Nucleotide sequence of the mutated apoC-III gene in the region surrounding the codon for amino acid 74 of apoC-III. DNA sequencing was performed by the dideoxy chain termination method as described in Materials and Methods.

allow us to test directly in vitro the effects of the Thr 74→Ala 74 mutation on these biological properties. In addition, insights into the mutation's effect on protein stability and ability to act as a substrate for O-linked glycosylation can be addressed. ▌▐

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