Compound Heterozygosity for an Apolipoprotein A1 Gene Promoter Mutation and a Structural Nonsense Mutation With Apolipoprotein A1 Deficiency

Akira Matsunaga, Jun Sasaki, Hua Han, Wei Huang, Mari Kugi, Takafumi Koga, Sadanori Ichiki, Tomoko Shinkawa, Kikuo Arakawa

Abstract—Apolipoprotein (apo) A1 plays a central role in the metabolism of HDL. We describe a novel genetic variant of the apoA1 gene identified in a patient with low concentrations of plasma HDL cholesterol. The proband, a 12-year-old Japanese boy, exhibited markedly low levels of both plasma apoA1 and HDL cholesterol. Genomic DNA sequencing of apoA1 genes of the patient showed a compound heterozygosity for an A to C substitution at 27 bp upstream of the transcription start site of 1 apoA1 allele, and a C to T substitution in another allele at residue 84 resulting in aberrant termination. The point mutation at nucleotide position ~27 changed ATAAATA of the putative TATA box signal sequence to ATACATA. In addition to this mutation, the patient was heterozygous for a G to A substitution at position ~75. Immunoblotting of an isoelectric focusing electrophoresis gel of the proband’s plasma showed a trace amount of normal apoA1. No measurable plasma apoA1 and HDL cholesterol in a patient with homozygosity for nonsense mutation at residue 84 has been reported previously. To determine the effects of substitution either at position ~27 or ~75, plasmids containing the 5’-flanking region of the human apoA1 promoter fused to the CAT reporter gene were constructed and transfected in HepG2 cells. A construct with the A to C substitution at position ~27 showed 41.8±4.2%, and G to A substitution at position ~75 showed 72.8±15.2% (means±SD, n=3) of CAT activities, compared with the wild-type promoter sequence. A construct with the double substitutions at positions ~27 and ~75 showed only 22.8±1.3% (mean±SD, n=3) activity relative to the wild type. Our patient is the first case with a TATA box mutation etiologically related to lipoprotein disorders. (Arterioscler Thromb Vasc Biol. 1999;19:348-355.)

Key Words: apoA1 ■ CAT assay ■ mutation ■ HDL deficiency ■ TATA box

Several epidemiological and clinical studies have demonstrated that the concentration of plasma HDL correlates inversely with the risk of coronary artery disease (CAD) and that HDL is a powerful predictor of CAD. Studies of transgenic mice and rabbits have shown that overexpression of apoA1 protects them from the development of vascular lesions. The evidence in transgenic animals indicates that apoA1 is the major factor responsible for the antiatherogenic effects of HDL. ApoA1 is known to play a central role in the regulation of the efflux and transport of cholesterol from peripheral tissues to the liver, and as a cofactor for lecithin: cholesterol acyltransferase (LCAT), which is responsible for the formation of most cholesteryl esters in plasma. Recently, scavenger receptor BI (SR-BI) was identified as a HDL receptor. The apoA1 gene is adjacent to the genes encoding apoC3 and A4 on chromosome 11q23. Mature apoA1 is a 243–amino acid single polypeptide chain synthesized by the liver and intestine. ApoA1 gene mRNA translates the 267–amino acid prepropeptide, which undergoes intracellular cleavage leading to a 249–amino acid propeptide. The propeptide is secreted and undergoes extracellular proteolysis giving rise to mature apoA1.

To date, >10 pedigrees of patients with the apoA1 mutation and extremely low concentrations of HDL cholesterol have been described. Eight different mutations of the apoA1 gene have been identified in homozygous forms which cause almost a complete absence of plasma HDL cholesterol and apoA1. One such mutation was shown to be caused by a deletion of the entire apoA1/C3/A4 gene complex, and others were caused by the synthesis of truncated apoA1, presumably because of premature termination of translation. Recently, we reported apoA1 Sasebo, which showed extremely low concentrations of HDL cholesterol caused by truncated apoA1. On the other hand, Miccoli et al reported that compound heterozygosity for an apoA1 (Leu141Arg)Pisa, as well as a frameshift mutation in exon 3 of apoA1 gene, contributed to the absence of HDL cholesterol, corneal opacities, and CAD. Heterozygous deletion mutation of amino acid 146 to 160 of apoA1/C3/A4 gene complex was shown to be associated with extremely low plasma HDL cholesterol levels, but whether this mutation causes premature translation termination of apoA1 transcript needs to be clarified.

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apoA1 Seattle with extremely low concentrations of plasma apoA1 and HDL cholesterol has also been reported. A common G to A transition at –75 bp from the transcription start site of the apoA1 gene is associated with altered levels of HDL cholesterol, although these finding have not been consistent. Apart from the –75 G/A mutation, no mutation in proximal 5′-flanking region of apoA1 gene related to plasma apoA1 or HDL cholesterol levels has been reported. In this report, we describe a compound heterozygous TATA box and nonsense mutation of apoA1 gene in a patient with a markedly low concentration of plasma HDL.

Methods

Subjects

A 12-year-old Japanese boy developed hematuria, fatty liver, and obesity 4 years before presentation. Hematuria disappeared 1 year after the point out. At presentation, he was 145 cm tall and weighed 59 kg (body mass index, 28 kg/m²). Physical examination showed no xanthoma, tussilary hypertrophy, or corneal opacity. Laboratory tests showed plasma aspartate aminotransferase and alanine aminotransferase of 133 and 76 IU/L, respectively. The concentrations of total plasma cholesterol, triglyceride, and HDL cholesterol were 3.93, 1.08, and 0.20 mmol/L, respectively. The paternal uncle had sudden death when he was 54 years old, but the other family history was consistent. Apart from the –75 G/A mutation, no mutation in 5′-flanking region of apoA1 gene related to plasma apoA1 or HDL cholesterol levels has been reported. In this report, we describe a compound heterozygous TATA box and nonsense mutation of apoA1 gene in a patient with a markedly low concentration of plasma HDL.

Lipoprotein Analysis

Plasma lipoproteins were separated by ultracentrifugation, and the concentrations of cholesterol and triglyceride were determined by an enzymatic method. The concentrations of apolipoproteins were determined by the single immunodiffusion method. Isoelectric focusing gel electrophoresis (IEF) was performed by the IEF system. ApoA1 bands were determined by the single immunodiffusion method. 20 Isoelectric precipitation in this study.

DNA Amplification and Single-Strand Conformation Polymorphism

DNA was extracted from peripheral blood leukocytes as described previously.25 Polymerase chain reaction (PCR) primers listed in Table 1 were used for single-strand conformation polymorphism (SSCP). The nucleotide numbers are based on the published sequence for apoA1 DNA.23 PCR was performed for 35 cycles of 96°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 minute. To screen for a possible apoA1 gene mutation, we used SSCP analysis as described by Orita et al24 with minor modifications. For this purpose, 0.5 µL of purified PCR products were diluted with 12.5 µL of 50% formamide solution, heated at 95°C for 5 minutes, and applied (8 µL/lane) to 7.2% polyacrylamide gel. During electrophoresis at 45 W, the temperature of the gel was kept at 24°C or 4°C with a built-in water jacket connected to an internal thermostat-regulated water circulator. After electrophoresis, the bands were detected by silver staining (Bio-Rad Laboratory).

DNA Sequencing

DNA segments carrying all 4 exons of apoA1 were amplified by PCR.23 PCR primers listed in Table 1 were used for sequencing. The PCR-amplified DNA was isolated by electrophoresis on a 0.8% agarose gel and ligated into pT7Blue-T vector (Novagen). Twelve clones from each allele were sequenced using T7 DNA polymerase (Sequenase; United States Biochemical Co) according to the dideoxynucleotide chain termination method or using an ABI 373A DNA sequencer.26

PCR-Mediated Site-Directed Mutagenesis and Restriction Fragment Length Polymorphism

Because 3 different transcriptional start sites for human apoA1 gene have been reported,27,28 the A to C substitution of 5′-flanking promoter can be shown by 3 different numbers from each transcriptional start site as –27, –28, or –30. In this study, we used number –27 from the transcriptional start site in the liver.29 To identify point mutations, a G to A substitution at position –75, an A to C substitution at –27, a G to A substitution at codon 37, and a C to T substitution at codon 84, PCR-mediated site-directed mutagenesis was performed.23 Leukocyte DNA was amplified by PCR using primer A (5′-AGGACCCATGACGACCAATATGCC-3′) and B (5′-GGCAAGGGCCTGAGCTGAC-3′) for nucleotide position –75 polymorphism.26 Primer C (5′-GTCTGTGGTGTGCGTCACT-3′) and D (5′-CCAGCTTCTGCAAGGGCGCAAT-3′) for nucleotide

| Table 1. Oligonucleotide Primers Used in PCR-SSCP and Sequence of apoA1 Gene |
|---------------------------------------------|-------------|
| Primer Sequence | Position |
| 1: | 5′-GCTTGGCTGTGGCCACACTCT-3′ | 37–56 |
| 2: | 5′-TCACCTGGCTGAATGAAGGGG-3′ | 344–366 |
| 3: | 5′-GTGGCTGGTATGCTGGTCT-3′ | 643–661 |
| 4: | 5′-ACCACCCCGCGTACCAACCA-3′ | 867–849 |
| 5: | 5′-GATATTAGTGAGGACTCGGGCA-3′ | 928–906 |
| 6: | 5′-GTGGCTGGGAGAATGCTGGGCAAC-3′ | 1308–1330 |
| 7: | 5′-AGCCCTACAACCTCTTCTGC-3′ | 1394–1412 |
| 8: | 5′-GCGCTACAGCAGGACTC-3′ | 1589–1607 |
| 9: | 5′-AAGTGGCAAGGAGAGATGAGCAC-3′ | 1614–1635 |
| 10: | 5′-GGCTCAGCTTCTTCGG-3′ | 1723–1706 |
| 11: | 5′-TGAGGGGCCCAGGGCTTCC-3′ | 1857–1841 |
| 12: | 5′-CGTTITTTCTGAGCCAGGGAAAG-3′ | 2069–2047 |

Primers 1 to 4 and 7 to 12 were used for PCR-SSCP. Primers 1, 2, 4 to 6, and 12 were used for PCR and sequencing.
ApoA1 Promoter Mutation With ApoA1 Deficiency

Construction of CAT Fusion Genes

The wild-type and mutant sequences of the apoA1 promoter were obtained by PCR amplification of the genomic DNAs from normal control, the proband or homozygous carrier of mutation at position –27, followed by the Kunkel method of site-directed mutagenesis.29 Amplification of the apoA1 promoter region was accomplished by PCR with primers 1 (5'-CTCAGG-3') and 2 (5'-ATGGAC-3') for the G to A substitution at codon 37, and primer 3 (5'-AAAGAGACAGAGGCGCTGAGC-3') and 4 (5'-GCGGCATCCTCCTGCCCCAGG-3') for the C to T substitution at codon 84. The regions of primer pairs, 1 and 2, 3 and 4, and 5 and 6, were amplified by 35 cycles of PCR at 96°C for 30 seconds, 65°C for 30 seconds, and 72°C for 1 minute and digested with MscI, TspEI, TaqI, and AfaI, respectively. After electrophoresis using 5% polyacrylamide gel, these gels were visualized with ethidium bromide.

Transient Expression of Recombinant Reporter Constructs

Plasmid DNAs (10 μg/dish) from various constructs were prepared and transfected into cultured cells by the lipofectin method (Gibco). All cultured cells were maintained in DMEM (Gibco) and supplemented with 10% FCS, penicillin, and streptomycin at 37°C in 5% CO2. Human hepatoma (HepG2) cells were seeded (1.5 × 104 cells/100-mm dish) for 40 hours before transfection. To correct for variation in DNA uptake by cells, 3 μg of the plasmid pRSV-β-gal30 were cotransfected with each test construct. After transfection, the cells were incubated for 24 hours, then gently scraped into precooled extraction buffer. Protein extracts from transfected cells were prepared by 3 cycles of freeze-thaw, and the CAT and β-galactosidase enzyme activities of the cell extracts were assayed. In each experiment, the CAT enzymatic activity was normalized relative to that of the β-galactosidase activity. The CAT assay mixture contained 0.1 μCi of [14C]chloramphenicol, 0.25 mmol/L Tris-HCl (pH=7.8), 0.8 mmol/L acetyl-CoA, and 80 μL of extract in a final volume of 160 μL. The reaction mixture was incubated at 37°C for 1 hour and extracted with 0.5 mL of ethyl acetate. Ethyl acetate was evaporated and the pellets redissolved in 20 μL of ethyl acetate and analyzed by thin-layer chromatography on aluminum sheet Silica Gel 60 (20×20 cm, E. Merck Darmstadt, Germany) in chloroform/methanol (95:5) mixture for 45 minutes. CAT activity was evaluated by visualizing the extent of conversion of [14C]chloramphenicol to its acetylated form, and quantitated by cutting out the respective spots followed by counting.

Statistical Analysis

Differences between groups in the concentrations of lipids, lipoproteins, and apolipoproteins were evaluated for statistical significance with a 2-tailed unpaired test. A P value <0.05 was considered statistically significant. Data are expressed as mean±SD.

Results

Plasma Lipids and Apolipoproteins

The concentrations of plasma lipids and apolipoproteins of the proband and other family members are presented in Table 2. The concentrations of plasma HDL cholesterol and apoA1 were extremely low in the proband, and the levels of apoA2, apoC2, and apoC3 were low. However, plasma LDL cholesterol and apo B were normal. The father (II-2) had low levels of plasma HDL cholesterol and apoA1 (45% to 55% of normal controls). The proband’s grandmother (I-1) who was 78 years old and had moderate hypertriglyceridemia, showed slightly low concentrations of plasma HDL cholesterol and apoA1. The father (II-2) had moderate hypertriglyceridemia and aunt (II-1) had moderate hypercholesterolemia. High concentrations of plasma VLDL cholesterol were observed in the father (II-2) and the grandmother (I-1) who had the E2 allele. Agarose gel electrophoresis of the proband’s plasma showed a very weak α-migrating band (data not shown). IEF and immunoblotting of the proband’s plasma also showed a trace of apoA14 isofrom, in agreement with normal apoA1 (Figure 2).

SSCP Analysis

Figure 3 shows SSCP results for exon 4 of the apoA1 gene in the proband and control. Denatured fragments of exon 4 from the proband were separated into 4 distinct bands of different mobility (Figure 3, lane 2). This mutation was detected the codon 84 mutation by DNA sequence.

DNA Analysis

To determine the molecular basis of the apoA1 variants, we amplified genomic DNA by PCR and sequenced all 4 exons and the promoter region of apoA1 gene in the proband. Sequencing of 12 separate clones of exon 4 showed a C to T position –27 mutation, primer E (5'-AGAGACTATGTTGCCAGTGGAACTCCTG-3') and F (5'-GATATTAGTGAGAGATCGGCC-3') for the G to A substitution at codon 37, and primer G (5'-AAAGAGACAGAGGCGCTGAGC-3') and H (5'-GCGGCATCCTCCTGCCCCAGG-3') for the C to T substitution at codon 84. The regions of primer pairs, A and B, C and D, E and F, and G and H, were amplified by 35 cycles of PCR at 96°C for 30 seconds, 65°C for 30 seconds, and 72°C for 1 minute and digested with MscI, TspEI, TaqI, and AfaI, respectively. After electrophoresis using 5% polyacrylamide gel, these gels were visualized with ethidium bromide.

### TABLE 2. Concentrations of Plasma Lipoprotein and Apolipoprotein in Different Members of Family

<table>
<thead>
<tr>
<th>Subject</th>
<th>Sex/Age</th>
<th>Total Cholesterol (mmol/L)</th>
<th>VLDL</th>
<th>LDL</th>
<th>HDL</th>
<th>HDL2</th>
<th>HDL3</th>
<th>Triglyceride (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>III-1</td>
<td>M/12</td>
<td>3.88</td>
<td>0.47</td>
<td>3.28</td>
<td>0.14</td>
<td>0.08</td>
<td>0.06</td>
<td>1.08</td>
</tr>
<tr>
<td>Heterozygote of TATA box mutation</td>
<td>II-2</td>
<td>M/49</td>
<td>4.58</td>
<td>1.66</td>
<td>2.33</td>
<td>0.59</td>
<td>0.34</td>
<td>0.26</td>
</tr>
<tr>
<td>Noncarriers</td>
<td>I-1</td>
<td>F/78</td>
<td>3.85</td>
<td>1.45</td>
<td>1.50</td>
<td>0.91</td>
<td>0.57</td>
<td>0.34</td>
</tr>
<tr>
<td>II-1</td>
<td>F/52</td>
<td>6.03</td>
<td>1.06</td>
<td>3.57</td>
<td>1.40</td>
<td>1.06</td>
<td>0.34</td>
<td>1.57</td>
</tr>
<tr>
<td>Normal controls (n=15)</td>
<td></td>
<td>4.68±0.49</td>
<td>0.51±0.18</td>
<td>2.48±0.41</td>
<td>1.34±0.36</td>
<td>1.03±0.36</td>
<td>0.34±0.05</td>
<td>0.85±0.28</td>
</tr>
</tbody>
</table>

*Compound heterozygote for the TATA box mutation and the codon 84 nonsense mutation. See Figure 1 for identification of each family member.
substitution at codon 84 in 5 clones that led to a Gln to stop codon (Figure 4A). This substitution was identical with the mutation detected by SSCP analysis of exon 4. The second substitution was found in exon 3, 5 of 12 separate clones showed a G to A transition at codon 37 that leads to an Ala to Thr substitution (data not shown). The third substitution was found in 27 bp upstream from the transcriptional start site of apoA1 gene; 8 of 12 separate clones showed an A to C substitution (Figure 4B). The transversion at position –27 led to a ATAAATA to ATACATA substitution in the TATA box sequence. A heterozygous common G to A substitution at position –75 of apoA1 gene was also found in the same allele that showed the substitution at position –27, and substitutions at codon 37 and codon 84 were in another allele (data not shown). Polyacrylamide gel electrophoresis was used to identify variants at position –27 and codon 84 after digestion with restriction enzymes, TspEI and AfaI. Restriction fragment length polymorphism (RFLP) was used in other members of the family to identify additional carriers of the mutation (Figure 5A and 5B). The proband and his father (Figure 5A, lanes 3 and 4, respectively) were confirmed as heterozygotes of the substitution at position –27 by the loss of the TspEI site. PCR-amplified DNA of exon 4 of the apoA1 gene from the proband (Figure 5B, lane 1) was subjected to AfaI digestion to confirm the nonsense mutation characterized by the creation of an AfaI site.

DNA sequencing and RFLP analysis showed that only the proband had a compound heterozygous mutation, whereas the father had a heterozygous A to C substitution at position –27, and other members of the family did not have these mutations (Figure 1 and Table 3). All examined family members were heterozygous or homozygous carriers of apoA1 (Ala37Thr). It was reported that the frequency of apoA1 (Ala37Thr) was about 8% in healthy Japanese persons.25

### Transcription Efficiency by Mutation of ApoA1 Promoter

To determine whether an A to C substitution at position –27 of the apoA1 gene is involved in the regulation of its expression, we constructed plasmids containing fusions between the bacterial CAT gene and DNA fragments spanning 119 to –333 of the apoA1 5′-flanking region (Figure 6). We compared the promoter regions of the apoA1 gene, including a substitution at the nucleotide position –27 mutation (−75G/−27C), substitutions at the nucleotide position −75 and −27 (−75A/−27C), substitution at position −75 (−75A/−27A), and wild type (−75G/−27A). The results of assay for CAT enzymatic activity showed that the wild-type sequence (−75G/−27A) in the −333/+119CAT construct expressed substantial levels of CAT activity in HepG2 (Figure 7). The mutant sequences, (−75G/−27C) and (−75A/−27C), in the CAT constructs expressed 41.8±4.2% and 22.8±1.3% (n=3) of CAT activity relative to the wild-type sequence, respectively. On the other hand, the substitution at nucleotide position −75 (−75A/−27A) had 72.8±15.2% (n=3) of CAT activity relative to the wild type. These results suggest that nucleotide −27 of the apoA1 gene is important and sufficient for expression in HepG2 cells.

### Table 2. Continued

<table>
<thead>
<tr>
<th>Apolipoprotein (mg/dL)</th>
<th>A1</th>
<th>A2</th>
<th>B</th>
<th>C2</th>
<th>C3</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td>13.3</td>
<td>7.4</td>
<td>106</td>
<td>1.9</td>
<td>4.0</td>
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</tr>
<tr>
<td>77</td>
<td>23</td>
<td>110</td>
<td>4.3</td>
<td>10.0</td>
<td>6.5</td>
<td></td>
</tr>
<tr>
<td>96</td>
<td>20</td>
<td>64</td>
<td>5.3</td>
<td>11.3</td>
<td>4.9</td>
<td></td>
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<tr>
<td>108</td>
<td>23</td>
<td>129</td>
<td>3.9</td>
<td>7.7</td>
<td>5.7</td>
<td></td>
</tr>
<tr>
<td>138±18</td>
<td>33±4</td>
<td>84±24</td>
<td>2.6±1.3</td>
<td>7.5±1.8</td>
<td>3.8±0.8</td>
<td></td>
</tr>
</tbody>
</table>

**Figure 2.** Isoelectric focusing gel electrophoresis and immuno-blotting. Plasma (1 µL) was incubated for 1 hour at room temperature with 25 µL of 10 mmol/L Tris-HCl (pH=8.2) containing 1% sodium deoxy sulfate, 2% ampholyte (pH=4 to 6), and 2 µL of mercaptoethanol. Used for IEF was 7.2% polyacrylamide gel containing 5 M urea and 2% ampholyte, pH 4 to 6. Lane 1 indicates normal control; lane 2, proband.

**Figure 3.** SSCP analysis for DNA fragments of exon 4 of apoA1 gene from the proband and control. Purified PCR products (0.5 µL) with primers 7 and 10 in Table 1 were diluted with 12.5 µL of 50% formamide solution, heated at 95°C for 5 minutes, and applied (8 µL/lane) to 7.2% polyacrylamide gel. Single strands of DNA fragments from the proband with a G to A substitution at residue 84 shows abnormal migration. Visualization was achieved by silver staining. Lane 1 indicates normal control; lane 2, proband; arrows, abnormal bands.

**Figure 4.** A diagram illustrating the position of substitutions in the apoA1 gene. The first substitution at codon 84 in 5 clones that led to a Gln to stop codon (Figure 4A). The second substitution was found in exon 3, 5 of 12 separate clones showed a G to A transition at codon 37 that leads to an Ala to Thr substitution (data not shown). The third substitution was found in 27 bp upstream from the transcriptional start site of apoA1 gene; 8 of 12 separate clones showed an A to C substitution (Figure 4B).
We identified apoA1 mutations in a 12-year-old Japanese boy with apoA1 and HDL deficiencies. DNA sequence analysis of the apoA1 gene in our patient showed the substitution Gln to stop codon at residue 84 in 1 allele as previously reported.\(^\text{11}\) The other allele of our patient was affected by an A to C substitution at position –27 which was involved in the TATA box and a common polymorphism of G to A substitution at position –75 in the apoA1 promoter region. The results of DNA sequence analysis were confirmed by digestions with restrictive enzymes. The proband had an additional mutation in the apoA1 gene, apoA1 (Ala37Thr), which was not related to plasma concentrations of HDL cholesterol and apoA1.\(^\text{11,25}\) The proband must have inherited the apoA1 (Ala37Thr) from his mother, not his father (II-2), because the grandmother (I-1) was a homozygous carrier of this mutation and did not have the codon 84 mutation. Therefore, apoA1 (Ala37Thr) was not related to low plasma HDL cholesterol and apoA1 in this case.

In the patient described here, determination of lipoprotein showed reduced plasma concentrations of apoA1 and HDL cholesterol. The low level of HDL cholesterol reflected reductions in both HDL2 and HDL3 fractions (Table 2), although a more marked reduction was present in the HDL2 fraction. IEF and immunoblotting of the proband’s plasma showed a trace of normal apoA1 band (Figure 2). These results suggest that the proband secreted a low amount of normal apoA1 in the plasma.

The heterozygous carrier of the substitution at position –27 of apoA1 gene showed about 45% to 55% of plasma apoA1 and HDL cholesterol, whereas in the compound heterozygous carrier the levels were about 10% of the wild type. In addition, no measurable levels of plasma apoA1 and HDL cholesterol were reported previously in a homozygosity for the same nonsense point mutation at codon 84 of apoA1.\(^\text{11}\) Heterozygosities for other nonsense and frameshift mutations in apoA1 were reported to have about 50% of apoA1 and HDL cholesterol levels.\(^\text{17,31}\) Thus, the mutation of TATA box region of apoA1 gene may exert a negative effect on HDL cholesterol levels similar to that of the nonsense mutation at codon 84, as well as affect the synthesis of apoA1.

The genes coding for apoA1 and 2 other apolipoproteins, apoC3 and apoA4, are closely linked and tandemly organized within a 15-kb DNA segment in the long arm of human chromosome 11.\(^\text{6}\) Certain regions of the chicken, rabbit, rat, and human apoA1 5'-flanking sequences are highly conserved.\(^\text{32}\) One of these regions corresponds to the typical eukaryotic gene TATA box. The TATA box is important for the correct initiation of gene transcription. In this regard, 2 different human genes with TATA box mutations were reported previously.\(^\text{33,34}\) Several different TATA box point mutations of human \(\beta\)-globin gene caused \(\beta\)-thalassemia,\(^\text{33,35}\) and 1 point mutation in the TATA box of 17\(\beta\)-hydroxysteroid

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**Figure 4.** Sequence analysis of PCR-amplified DNA of apoA1 gene from the proband. The PCR-amplified DNA was sequenced after subcloning into T7Blue-T vectors. The sequence shown is the sense strand. A, Graphic representation generated by ABI 373 analysis software (version 1.2.0) shows a C to T substitution at codon 84. B, Autoradiography of sequencing gels of DNA from the proband shows an A to C substitution at position –27. The substituted nucleotide is marked with an asterisk.

**Figure 5.** Restriction fragment length analysis of apoA1 gene. A, analysis for the A to C substitution at position –27. PCR products generated with use of primers C and D as described under Methods and digested with TspEI were electrophoresed on 5% polyacrylamide. Lane M, marker from a digest of \(\phi X174\); lane 1, grandmother (I-1); lane 2, paternal aunt (II-1); lane 3, proband (III-1); lane 4, father (II-2); lane 5, nondigested control. PCR-amplified DNAs of the promoter region from the proband and his father (lanes 3 and 4) subjected to TspEI showed 170 bp and 150 bp bands, indicating loss of a TspEI site, and DNAs from the other family members (lanes 1 and 2) showed 150 bp band resulting from complete digestion of TspEI. B, analysis for the C to T substitution at codon 84. PCR products generated with use of primers G and H and D were digested with AfaI. Lane M, marker from a digest of \(\phi X174\); lane 1, proband (III-1); lane 2, father (II-2); lane 3, normal control; lane 4, nondigested control. The proband (lane 1) DNA subjected to AfaI showed 173 bp and 156 bp bands, indicating heterozygosity for a nonsense mutation at codon 84.
dehydrogenase type 1 was reported in a patient with hereditary breast cancer.\textsuperscript{34} It has also been reported that these TATA box mutations in the \(\beta\)-globin gene showed 20\% to 55\% production of \(\beta\)-globin mRNA in transient expression compared with normal \(\beta\)-gene.\textsuperscript{36,37} The apoA1 gene was the third human gene that showed a point mutation in the TATA box.

Previous studies of the expression of human apoA1 gene in several different human cell lines by RNA-blotting analysis indicated that the gene is expressed only in certain cell lines of hepatic (HepG2, Hep3B, and PLC/PRF/5) and intestinal (Caco-2) origin.\textsuperscript{38} Detailed analysis of the nucleotide sequences involved in the apoA1 gene transcription has been performed, and some of the transcription factors have been identified.\textsuperscript{28,39 – 41} Taylor et al\textsuperscript{42} reported that a minimal segment of the rat apoA1 gene was nucleotides – 46 to –7 including the TATA box. Sastry et al\textsuperscript{27} reported that sequences within the –256 to –41 nucleotide and –2052 to –192 nucleotide, located 5’ but not 3’ to the TATA box of the human apoA1 gene, were necessary and sufficient for expression in HepG2 and Caco-2 cells, respectively. Recently, Harnish et al\textsuperscript{39} reported that apoA1 gene expression in liver cells was activated synergistically by hepatocyte nuclear factors 3 and 4 bound to 3\textsuperscript{cis}-acting elements (–214 to –192; –169 to –146; and –134 to –119). In the present study, the constructs of apoA1 gene promoter including these \textsuperscript{cis}-acting elements was used for expression of the CAT reporter gene.

We demonstrated that an A to C substitution at position –27 within the TATA box and a G to A substitution at position –75 of the human apoA1 gene were associated with reduced CAT activities.

A common polymorphism caused by a G to A transition at position –75 of apoA1 gene has been studied previously.\textsuperscript{43,44} Studies in Caucasians have shown that individuals with the A allele at position –75 have higher levels of HDL cholesterol or apoA1 than do individuals homozygous for G allele, the common allele.\textsuperscript{43,44} However, other studies including our previous report found no effect of A allele on the concentrations of HDL cholesterol.\textsuperscript{26,45,46} In vitro analysis of the effects of –75 polymorphism on transcription has yielded conflicting results. For example, Smith et al\textsuperscript{47} reported that G to A substitution at position –75 in the apoA1 promoter showed 68\% of CAT activities. Tuteja et al\textsuperscript{48} reported that the G to A substitution in a promoter construct including –330 to +1 showed about 50\% of CAT activities, but CAT activities of the same substitution in a construct spanning the region from –1469 to +397 were similar to those of the wild-type construct. On the other hand, Angotti et al\textsuperscript{49} reported that the G to A substitution increased transcription by about 5- to 7-fold in a construct spanning the region from –256 to +29. In the present study, the G to A substitution at position –75 showed 72.8\% of CAT activity, a finding similar to that of Smith et al.\textsuperscript{47} Furthermore, the construct with the mutant

![Figure 6](http://atvb.ahajournals.org/)

**Figure 6.** Structures of the apoA1 promoter–CAT fusion genes. The transcriptional start site of apoA1 gene is indicated by +1. The coordinates of the constructs are –333 to +119 from the transcriptional start site.

![Figure 7](http://atvb.ahajournals.org/)

**Figure 7.** Effect of nucleotide substitutions of the apoA1 promoter on the transcription of the CAT gene. A, CAT assays in HepG2 cells. Analysis was performed as described under Methods. The designations, –75G/–27A, –75A/–27C, and –75A/–27A, indicate the mutated promoter sequences shown in Figure 6. Negative control is the pCAT vector. B, schematic representation of CAT assays. The results shown are averages of 3 independent transfections.

**Table 3.** Haplotypes of Different Family Members

<table>
<thead>
<tr>
<th>Subject</th>
<th>Sex/Age</th>
<th>Positions in Promoter</th>
<th>Mutations of Exon</th>
<th>Apo E Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>–75</td>
<td>–27</td>
<td>Codon 37</td>
</tr>
<tr>
<td>Compound heterozygote*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>III-1</td>
<td>M/12</td>
<td>G/A</td>
<td>A/C</td>
<td>+/-</td>
</tr>
<tr>
<td>Heterozygote of TATA box mutation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>II-2</td>
<td>M/49</td>
<td>G/A</td>
<td>A/C</td>
<td>+/-</td>
</tr>
<tr>
<td>Noncarriers</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I-1</td>
<td>F/78</td>
<td>G/G</td>
<td>A/A</td>
<td>+/-+</td>
</tr>
<tr>
<td>II-1</td>
<td>F/52</td>
<td>G/A</td>
<td>A/A</td>
<td>+/-</td>
</tr>
</tbody>
</table>

*Compound heterozygote for the TATA box mutation and codon 84 nonsense mutation. See Figure 1 for identification of each family member.
ApoA1 Promoter Mutation With ApoA1 Deficiency

(−75A/−27C) showed only 22.8% activities of the wild type. These data suggest that the −75 and −27 nucleotide substitutions had the additive effect for reduced CAT activities. This is the first case of TATA box mutation in human apolipoproteins.

Despite a nearly complete absence of HDL, a highly variable susceptibility to premature CAD is present in patients with apoA1 deficiencies. In 4 different families including the nonsense mutation at codon 84, homozygous patients for different apoA1 null alleles suffered from CAD.1,3,8,11,16 In contrast, CAD was absent in 5 apoA1-deficient patients.10–12–15 Possible reasons for such variability include age of the carriers, the small sample size of each kindred, presence of other coronary risk factors, and presence of abnormal circulating apoA1 variants. Because the proband described here was still 12 years old, we could not obtain any assessment of susceptibility to CAD despite the very low concentrations of plasma HDL cholesterol. The patient was obese and had a fatty liver, but no relationship is expected between apoA1 variants and these abnormalities. The number of family members in this study was too small to allow establishment of a firm genotype/phenotype association with coronary atherosclerosis.

In conclusion, the compound heterozygous mutant of an A to C substitution at position −27 in addition to common G to A substitution at position −75 together with nonsense mutation at residue 84 of apoA1 gene may together contribute to lowering plasma apoA1 and HDL cholesterol levels.

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References


Compound Heterozygosity for an Apolipoprotein A1 Gene Promoter Mutation and a Structural Nonsense Mutation With Apolipoprotein A1 Deficiency
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