Human apolipoprotein A-I gene promoter polymorphism: association with hyperalphalipoproteinemia

F. Pagani,[1] A. Sidoli,* G. A. Giudici, L. Barenghi, C. Vergani,† and F. E. Baralle*

Laboratory of Biochemistry and Molecular Biology, Fondazione Rivetti, Milan, Italy; Department of Biotechnology,* Istituto Sieroterapico Milanese S. Belfanti, Milan, Italy; and Istituto di Medicina Interna,† Università di Milano, Milan, Italy

Abstract An apolipoprotein A-I gene promoter polymorphism, due to an adenine (A) to guanine (G) transition 78 base pairs upstream from the transcription initiation site, was studied by amplification of the corresponding region of the apoA-I gene, DNA sequencing, and allele-specific oligonucleotide hybridization. The frequency of the polymorphism was studied on female and male individuals classified into three groups according to the high density lipoprotein (HDL) cholesterol concentration. The allelic frequencies for the A polymorphism were 0.10, 0.14, 0.27 in women and 0.08, 0.17, 0.14 in men in the lowest, in the intermediate (between 10th and 90th percentile), and the highest decile of HDL cholesterol levels, respectively. Statistical analysis showed a significant difference of allelic frequencies between the highest and the lowest deciles ($P < 0.006$) and between the highest and the intermediate deciles of HDL cholesterol in women ($P < 0.04$) but not in men. As the sequences surrounding the polymorphism are known to be involved in transcription modulation, it is possible that the A–G transition polymorphism may have an influence on apoA-I synthesis and, in consequence, on the HDL cholesterol levels in women. —**Pagani, F., A. Sidoli, G. A. Giudici, L. Barenghi, C. Vergani, and F. E. Baralle.** Human apolipoprotein A-I gene promoter polymorphism: association with hyperalphalipoproteinemia. *J. Lipid Res.* 1990. **31:** 1371–1377.

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Apolipoprotein A-I (apoA-I) is the major protein constituent of high density lipoproteins (HDL) and plays a crucial role in lipid transport and metabolism. Several epidemiological studies have reported that HDL cholesterol (HDL-C) levels are inversely related to the incidence of coronary heart disease (CHD) (1, 2). HDL promotes cholesterol efflux from cells and this process may protect against atherosclerosis (3). ApoA-I, C-III, and A-IV genes are clustered on human chromosome 11 in a 15 Kb region (4). A structural variation in the A-I–C-III gene complex has been described in some patients with premature atherosclerosis, leading to combined apoA-I, apoC-III, and HDL deficiency (5, 6).

Several studies have analyzed the possible involvement of genetic variations in the A-I–C-III locus in determining plasma lipid phenotypes and CHD (see 7 for review). The methodology more widely used was the association of restriction fragment length polymorphisms (RFLPs) with lipids, lipoprotein, and apoprotein levels, and CHD. Unfortunately, some controversial results have been reported (7–14), and the reason for this variability may be due to different factors. The vast majority of the RFLPs are due to neutral mutations that probably do not directly affect the structure of the mature protein or the regulation of the neighbor apoprotein genes. In addition, the different frequency of minor alleles between populations, as we have previously reported for the SstI polymorphism in the 3' noncoding region of apoC-III (13), may account for some of the controversial results obtained in association studies (15).

So, when found, the significance of the association of these RFLPs with dyslipidemias and/or CHD may be due to the fact that these variations constitute a marker for a mutation(s) present within that locus either in structural genes or in their regulatory elements (16–19). The more interesting types of polymorphisms and the most difficult to pinpoint are those found in the regulatory regions of the genes. Recent reports have identified several *cis*-acting DNA elements that control expression of the human and rat apoA-I genes in different cell types (20, 21). The DNA segment located between nucleotides −256 and −41 upstream from the transcription start site of the human apoA-I gene is necessary and sufficient for maximal ex-

Abbreviations: apo, apolipoprotein; HDL, high density lipoprotein; TC, total cholesterol; TG, triglyceride; CHD, coronary heart disease; A, adenine; G, guanine; RFLP, restriction fragment length polymorphism.

[1]To whom correspondence should be addressed at: Fondazione Rivetti, Laboratorio di Biochimica e Biologia Molecolare, Viale Monte Nero 32, 20136 Milano, Italy.

pression levels in a hepatocellular carcinoma cell line (HepG2) (20).

Amplification of the promoter region of apoA-I followed by allele-specific oligonucleotide hybridization allowed us to study the distribution of a polymorphism due to an adenine (A) to guanine (G) transition, 78 base pairs (bp) upstream from the transcription start site of the apoA-I gene. This mutation is located within a sequence that has been shown to be critical for normal function in other promoters (19, 22). We have examined the frequency of this polymorphism in a well-selected group of subjects participating in an epidemiological survey of atherosclerosis risk factors to determine the extent to which genetic variation at the apoA-I gene promoter region affects HDL-C and apoA-I concentrations.

MATERIALS AND METHODS

Selection of subjects and statistical analysis

The subjects, 136 women (W) and 108 men (M), were selected from the candidates in an epidemiological study on atherosclerosis risk factors (The Rivetti Heart Study) that takes into account a working population from the Turin area (Italy). The criteria of exclusion were as follows: total cholesterol (TC) or triglycerides (TG) above the 90th percentile of our distributions according to age and sex; body mass index (kg/m^2) greater than 26 and 27 for women and men, respectively; cigarette smoking more than 5 per day. Menopausal women and subjects with conditions such as excessive alcohol consumption, strenuous physical activity, use of oral contraceptives and drugs known to affect HDL-C levels were also excluded. The selected subjects were divided into three groups in relation to the lowest decile (42 mg/dl and 35 mg/dl for women and men, respectively) and to the highest decile (75 mg/dl and 56 mg/dl for women and men, respectively) of HDL-C distribution. The intermediate group included the subjects with HDL-C levels between the 10th and 90th percentiles. The TC, TG, HDL-C, apoA-I and apoB levels are shown in **Table 1**. Differences between allele numbers in the various groups were tested for significance by the chi-square test with Yates correction and G-test in a 2 × 2 contingency table.

Lipid and lipoprotein analysis

All of the assays were performed using fresh serum. An automatic apparatus EPOS 5060 (Eppendorf GmbH, Hamburg, FR Germany) was used for lipid measurements. Serum cholesterol and TG were determined enzymatically (Boehringer Biochemica, Mannheim, FRG). HDL-C was quantified after precipitation of apoB-containing lipoproteins by a magnesium chloride-phosphotungstic acid method (23). ApoA-I and apoB were determined by single radial immunodiffusion (Daiichi Pure Chemicals Co. Ltd., Tokyo, Japan). Quality control data showed accuracy of results within the limits suggested by the National Cholesterol Education Program (24). The overall coefficients of variation were 4% for TC, TG, and HDL-C, and 7% for apoA-I and apoB.

General procedures

Purification of DNA, ligation reactions, restriction enzyme digestions, gel electrophoresis, and nick translation were performed according to established procedures as described by Maniatis, Fritsch, and Sambrook (25). Genomic DNA from peripheral lymphocytes was extracted with the method of Kunkel et al. (26).

DNA amplification and genotyping analysis with allele specific oligonucleotide hybridization

The following oligonucleotides were synthesized on an Applied Biosystem 308A DNA Synthesizer and purified on 15% polyacrylamide 7M urea gel.

TABLE 1. Serum total cholesterol (TC), triglyceride (TG), HDL cholesterol (HDL-C), apoA-I, and apoB concentrations in different groups stratified by HDL cholesterol levels

Percentile of HDL-C	n[a]	Age	TC	TG	HDL-C	ApoA-I	ApoB
		yr		*mg/dl, mean* ± SD			
Women							
<10th	40	37 ± 5	166 ± 27	74 ± 23	37 ± 4	127 ± 11	107 ± 23
10th-90th	45	39 ± 7	190 ± 27	65 ± 16	58 ± 9	152 ± 15	104 ± 22
>90th	51	40 ± 8	211 ± 29	61 ± 18	86 ± 10	179 ± 19	99 ± 20
Men							
<10th	42	39 ± 12	169 ± 35	99 ± 31	33 ± 5	129 ± 19	114 ± 31
10th-90th	32	41 ± 10	193 ± 30	80 ± 18	46 ± 3	142 ± 9	117 ± 27
>90th	34	41 ± 10	194 ± 22	73 ± 26	63 ± 8	162 ± 17	101 ± 23

[a]Number of subjects

PCR AI.1 5'-CACCCGGGAGACCTGCAAGC-3' 20mer; 5' end at −259
PCR AI.2 5'-TCTAAGCAGCCAGCTCTTGCA-3' 21mer; 5' end at −1
A Probe 5'-CAACAGGGCCAGGGCTG-3' 17mer; 5' end at −88
G Probe 5'-CAACAGGGCCGGGGCTG-3' 17mer; 5' end at −88

The coordinates above refer to a previously published sequence where the transcription start site (+ 1) corresponds to position 471 (27). The localization of oligonucleotides in the 5' flanking region of apoA-I is shown in **Fig. 1**.

Amplification with the Taq polymerase was performed under conditions recommended by the manufacturers (Anglian, Biotec Ltd., England) with PCR AI.1 and PCR AI.2 oligonucleotides as primers. Three μg of genomic DNA template was added to 67 mM Tris (pH 8.8 at 25°C), 6.7 mM magnesium chloride, 16.6 mM ammonium sulfate, 10% DMSO, 10 mM 2-mercaptoethanol, 6.7 μM EDTA, 200 μM of each dNTP, and 50 pM of each oligonucleotide in a 100 μl volume. The DNA was denatured at 100°C for 15 min followed by successive cycles of annealing at 48°C for 30 sec, extension at 63°C for 1 min, and denaturation at 93°C for 1 min. Two units of Taq DNA polymerase were added after the first denaturation step, and every tenth cycle thereafter until 30 cycles were completed.

Ten μl of the amplification reaction was electrophoresed in 1.5% agarose gel at 2 V/cm for 4 h and then transferred to Zeta Probe Nylon membranes (Bio-Rad, Richmond, CA) by Southern blotting followed by UV irradiation. The filters were incubated in 5 × SSPE, 0.5% SDS, 2 × Denhardt's solution, and 100 μg/ml of herring sperm DNA for 2–5 h. For hybridization experiments, oligomers A probe and G probe were end-labeled to a specific activity of 10^8 dpm/μg using gamma ^{32}P-ATP (Amersham, England) and T4 polynucleotide kinase (Boehringer, Mannheim FRG) (25). Hybridization was performed overnight in the same solution containing 1 × 10^6 cpm/ml of end-labeled oligonucleotides. Hybridization temperatures for A probe and G probe were 54°C and 56°C, respectively. Filters were rinsed in 6 × SSC, 0.5% SDS (2 × 15 min) and 3 × SSC, 0.5% SDS (2 × 15 min) at 54°C and 56°C, respectively, and autoradiographed at −70°C, for 5 h.

DNA sequencing

The 258 bp amplified fragment was purified by agarose gel electrophoresis (25) and subjected to PstI digestion. The fragments obtained between positions −36 to −188 were subcloned in M13 mp18 for sequencing. Sequencing experiments were performed on single-strand DNA templates with the dideoxynucleotide chain termination method of Sanger, Nicklen, and Coulson (28).

RESULTS

ApoA-I gene promoter polymorphism

Nucleotide variations in the 5' flanking region of the apoA-I gene were identified by comparison of previously reported apoA-I gene sequences (27, 29). DNA preparations from ten normolipidemic subjects were amplified with the two PCR AI.1 and PCR AI.2 primers and the resulting fragments were cloned and sequenced. Sequence analysis revealed the adenine to guanine transition in position −78 from the initiation transcription site of the apoA-I gene (**Fig. 2**). The detection of the mutation in the population sample was originally performed by digestion with MspI (if G is present, a new MspI site is created). However, this technique proved to be unreliable as often partial cleavage was observed also in the presence of an excess of enzyme. Hence hybridization with two allele-specific oligonucleotides, probe A and probe G, was the method of choice. Three genotypes were detected: homozygote for the A allele (**Fig. 3**, lane 1), homozygote for the G allele (Fig. 3, lanes 2 and 3) and heterozygote for both alleles (Fig. 3, lane 4).

Frequencies of apoA-I gene promoter polymorphism as a function of HDL-C and apoA-I levels

A total of 244 individuals made up after the selection of subjects enrolled in the epidemiological study (see Materials and Methods) was examined for possible associa-

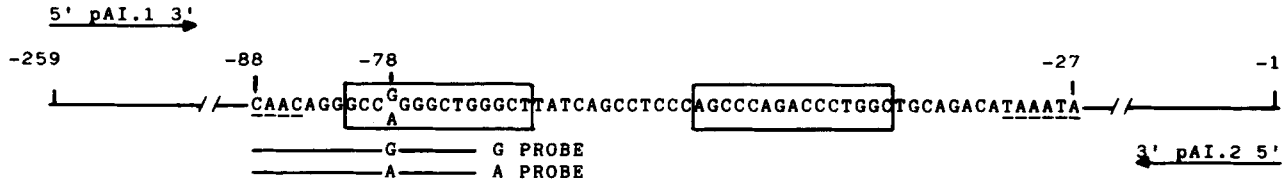

Fig. 1. The 5' flanking region of apoA-I gene. DNA sequence of the region expanding the "CAAT" and "TATA" boxes, amplification and allele specific hybridization strategy. The oligonucleotide amplification primers pA1.1 and pA1.2 are described in Materials and Methods. Numbers indicate position of nucleotides from transcription start site. The presumed "CAAT"- and "TATA"- like boxes are underlined. The A–G variation is shown in position −78 along with the two oligonucleotides, probe A and probe G, used in allele specific hybridization. The 14/15 bp inverted repeat sequences are boxed.

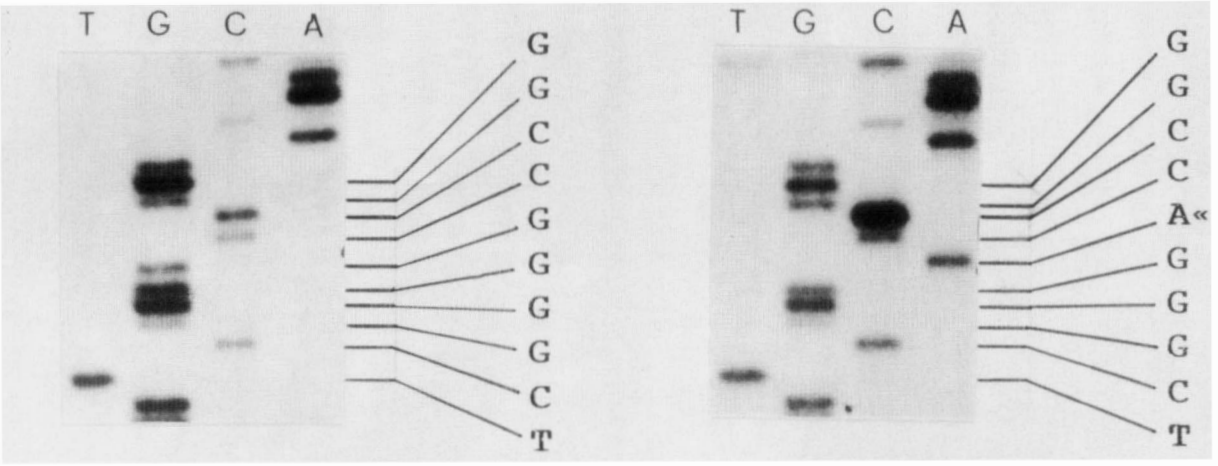

Fig. 2. DNA sequencing gel showing the A to G transition in position -78 of the apoA-I promoter. The polymorphic nucleotide is marked with (◄).

tion of the A and G alleles at promoter region of apoA-I gene with HDL-C concentration.

Table 2 presents genotype and allelic frequencies of the polymorphism in the different groups analyzed. The allelic frequencies for the A and G polymorphism were not statistically different between men and women and the distribution of the different apoA-I genotypes was not statistically different from the expected Hardy-Weinberg equilibrium. The allelic frequencies for the A allele were 0.10, 0.14, and 0.27 in women and 0.08, 0.17, and 0.14 in

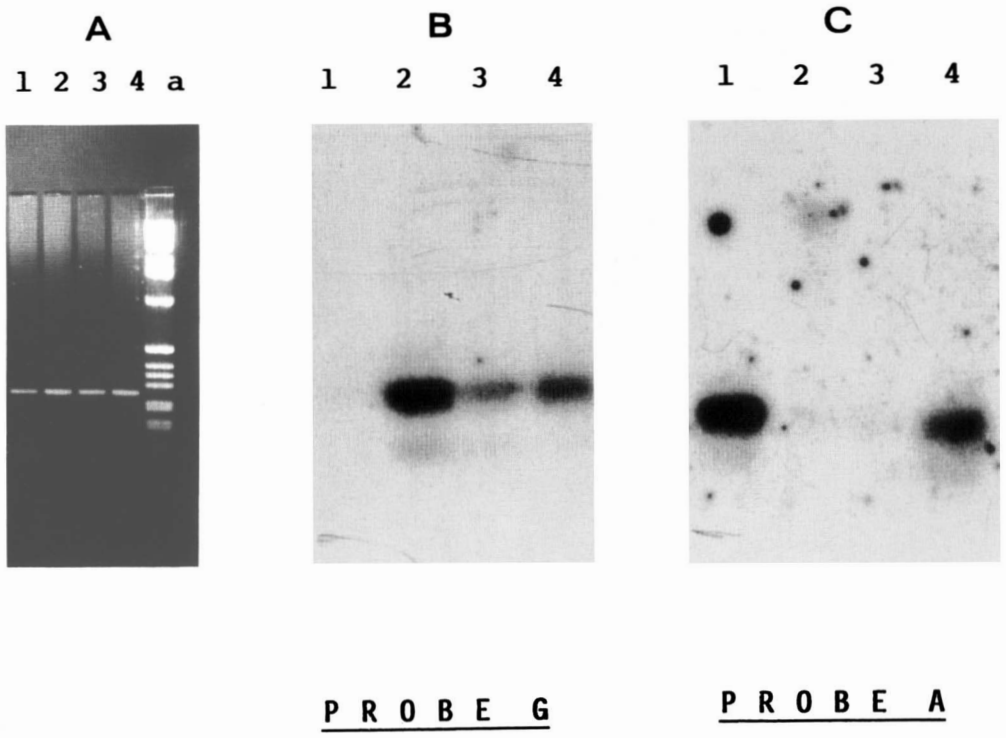

Fig. 3. Genotyping analysis on amplified DNA with allele specific oligonucleotide hybridization. Amplified 258 bp fragment corresponding to the apoA-I promoter is indicated by an arrow. A: Agarose gel, stained with ethidium bromide; B and C: Southern blots probed with allele specific oligonucleotides probe G and probe A, respectively. Lanes 1-4: amplified DNA preparation with different genotypes, 1 = A/A, 2 = G/G, 3 = G/G, 4 = A/G; lane A: 1Kb ladder size marker.

TABLE 2. Genotype and allelic distribution for the A-G polymorphism in different groups stratified by HDL cholesterol (HDL-C) levels

Percentile of HDL-C	n[a]	Genotype			Number of Alleles		A-Allele Frequency
		GG	AG	AA	G	A	A
Women							
<10th	40	32	8		72	8	0.10
10th-90th	45	33	11	1	77	13	0.14
>90th	51	27	20	4	74	28[b]	0.27
Total	136	92	39	5	223	49	0.18
Men							
<10th	42	36	5	1	77	7	0.08
10th-90th	32	21	11		53	11	0.17
>90th	34	26	7	1	59	9	0.14
Total	108	83	23	2	189	27	0.13

[a]Number of subjects
[b]Highest decile versus lowest decile, chi-square = 7.54, $P < 0.006$; G = 9.07, $P < 0.005$. Highest decile versus 10th-90th percentile, chi-square = 4.07, $P < 0.04$; G = 4.96, $P < 0.04$.

men for the lowest decile, intermediate group, and the highest decile of HDL-C levels, respectively. A statistically significant difference in allelic frequencies was observed in women between the highest and lowest decile of HDL-C levels (chi-square = 7.54, $P < 0.006$; G = 9.07, $P < 0.005$), and between the highest decile and intermediate group of HDL-C levels (chi-square = 4.07, $P < 0.04$; G = 4.96, $P < 0.04$). No statistically significant difference was observed between the other group in women and between the various groups in men.

DISCUSSION

A number of studies have shown RFLPs in the apoA-I-C-III-A-IV gene locus that associate in patient groups with dyslipidemia and/or atherosclerosis (7). In particular, several reports suggest that polymorphisms of that locus are associated with low HDL-C levels (11, 14, 30). We have focused our attention on the 5' flanking region of the apoA-I gene and we report here an apolipoprotein A-I gene promoter polymorphism due to an adenine to guanine transition, located in position −78 from the transcription start site, 51 bp upstream from the "TATA" box and 7 bp downstream from the "CAAT"-like sequence 5'-CAACA-3' or 30 bp downstream from the other proposed putative "CAAT"-like sequence 5'-CCACAT-3' (20).

Amplification of the promoter region of apoA-I followed by allele-specific oligonucleotide hybridization allow us to analyze the frequency of the two alleles in the population. The distribution of the polymorphism was studied in three different groups of subjects of each sex selected from an epidemiological survey on atherosclerosis risk factors as a function of HDL-C concentration (Table 2).

In women, the group with high HDL-C levels (>75 mg/dl corresponding to the highest decile) showed a statistically significant difference at the 4% level with the intermediate HDL-C concentration group (42-75 mg/dl) and at the 6‰ level with the lower HDL-C concentration group (<42 mg/dl corresponding to the lowest decile). In men, the distribution of allelic frequencies of the A-G polymorphism did not associate with HDL-C levels. No statistically significant difference was observed in the distribution of allelic and genotype frequencies for the A and G polymorphism between men and women as a whole and also the genotype distribution was in Hardy-Weinberg equilibrium for both sexes. Thus, the different distribution of allelic frequencies as a function of HDL-C concentration between men and women is not due to a selection effect, and other genetic or environmental factors may account for this difference.

There are two possible explanations for the association of the A allele with high HDL-C and apoA-I levels in women but not not in men. It is possible that the allele A is in linkage disequilibrium with a different mutation in a nearby gene, which in turn may influence HDL steady state levels. However, because of its location and homologies with other genes (18, 31-34), the polymorphic site may be in a sequence of the apoA-I gene promoter that regulates its expression in response to hormonal stimulation. The 5' flanking regions of the genes are known to be involved in transcriptional modulation of gene expression (19). The DNA region surrounding the polymorphism is a 51 bp fragment that is G-C rich and contains an inverted repeat composed of two 14/15 bp elements (Fig. 1).

The homology and self complementarity of the inverted repeats is disrupted when G is present instead of A in position −78. Both direct and inverted repeat sequences with peculiar base composition within 5' flanking regions have been involved in the regulation of gene expression (18, 31, 32). Point mutations within these repeated elements either naturally occurring, such as in the human beta globin gene (33, 34), or produced by in vitro mutation, such as in the herpes virus thymidine kinase gene (18) or in the mouse beta globin gene (22), have been shown to reduce transcriptional activity in vitro. Of particular interest is a mutation that alters the last A of the sequence 5' (−81) AGGGCCA (−75) 3' in the mouse beta globin gene (22) thus dramatically reducing the rate of transcription. The A to G transition at position −78 in the apoA-I gene promoter occurs in a DNA element of identical sequence.

In order to clearly define the relationship between this promoter polymorphism and HDL-C and apoA-I levels, we are currently performing in vitro studies on the expression of the two different promoters with the A and G substitution and following their segregation in the kindreds of the hyperalphalipoproteinemic subjects.

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