A Polymorphism (G → A Transition) in the −78 Position of the Apolipoprotein A-I Promoter Increases Transcription Efficiency*

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A G → A transition at −78 base pairs from the transcription start site of the apolipoprotein A-I (apoA-I) gene has been associated with increased apoA-I serum levels in humans. We report here that this mutation (G → A) increases significantly (5–7-fold) the expression of a reporter gene fused to the apoA-I promoter in human liver and intestine cells. In addition, the presence of A at −78 base pairs from the transcription start site of the gene significantly decreases the binding affinity of a nuclear factor present in liver and intestine cells. We suggest that the reduced affinity of this factor increases the transcription efficiency of the promoter and explains why individuals carrying the A allele have high serum apoA-I levels.

Apolipoprotein A-I (apoA-I) is the major protein component of high density lipoproteins (HDL). Several clinical and epidemiological studies have demonstrated that plasma levels of apoA-I and HDL cholesterol (HDL-C) are negatively correlated with the risk of developing coronary artery disease (1–3). It has been suggested that HDL exerts this antiatherogenic effect by mediating the "reverse cholesterol transport" from peripheral tissues to the liver where the cholesterol is excreted (4).

In humans HDL is present in two major forms, one containing both apoA-I and apolipoprotein A-II (A-I/A-II HDL) and another composed exclusively of apoA-I (A-I HDL). The antiatherogenic role of apoA-I has been stressed in a recent study, which indicates that the protein composition of HDL modulates its antiatherogenic effect, since A-I HDL is more effective in preventing atherosclerotic lesions than A-I/A-II HDL (5).

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This DNA region has been extensively analyzed. Several restriction fragment length polymorphisms (RFLP) have been identified, and the association of some RFLPs in this region with coronary artery disease and other lipid abnormalities has been demonstrated in different populations (9-15). A RFLP located in the 5'-flanking region of the apoA-I gene at −78 base pairs from the transcription start site has been described. This polymorphic trait (present in 15–20% of normal individuals) consists of a G → A transition that eliminates a cleavage site for the restriction endonuclease MspI (16, 17). In a previous study (14), we analyzed the allelic and genotypic frequencies of the MspI polymorphism in a sample of normal individuals from southern Italy. Haplotype analysis suggested a link between serum levels of HDL-C and the A allele. The association of the A allele with high plasma levels of apoA-I and HDL-C has also been reported in other populations (15–17). These studies, however, gave no indication as to the possible mechanism underlying this association.

The expression of apoA-I is restricted to the liver and intestine (18), and some of the cis-acting elements within the apoA-I promoter have been characterized (19-21). A specific DNA segment (−220 to −110 bp from the transcription start site) that directs the hepatic-specific transcription of apoA-I has been identified (22). Recently, several trans-acting factors have been characterized, all of which bind to the liver enhancer region (22-24). The −100 to −70 region has not been extensively studied, and so far there has been no clear experimental evidence linking the G → A mutation at −78 bp to apoA-I gene expression.

Here we report that the G → A transition at −78 bp from the transcription start site of apolipoprotein A-I gene (A allele) increases the basal transcription activity of the apoA-I promoter. This change parallels the decrease in the binding affinity of a 90-kDa nuclear protein to the promoter. Taken together, these data indicate that the apoA-I gene carrying A in position −78 is expressed more efficiently than G allele, and this may represent the ultimate cause of higher apoA-I levels in individuals carrying the A allele.

EXPERIMENTAL PROCEDURES

Plasmids—Genomic DNA from peripheral leucocytes was prepared as previously described (14). DNA (0.5 μg) was used as template in a polymerase chain reaction (PCR) (26) to amplify the region spanning nucleotides −375 to +29 from the transcription start site of the apoA-I gene. The PCR was performed using the GeneAmp kit and a thermal cycler (Perkin-Elmer Corp.). The reaction mixture (50 μl) included 0.2 μM of each deoxyribonucleotide triphosphate (dNTP), 50 mM KC1, 10 mM Tris-HCl, pH 8.3, 1.5 mM MgCl2, 0.8 unit of Taq DNA polymerase, and 30 pmol of following oligonucleotides: 5'-TGAGGAATTCGTCGTCGATCT-3' (5' end at -375) and 5'-GCAAGAGAGACGAGAGAAGCCTCCTCCTCCG-3' (5' end at +29) (19). After denaturation (5 min at 94 °C), the reaction was carried out for 30 cycles, each consisting of incubations at 94 °C for 1 min (denaturation), at 55 °C for 1 min (annealing), and at 72 °C for 1 min (extension). The last extension step was prolonged for 10 min.

G and A alleles were detected by digestion of amplified DNA with MspI. The A allele generated two fragments (119 and 286 bp), while the presence of three fragments (119, 178, and 107 bp) marked the G allele. 1 μg of amplified DNA (A or G allele) was filled in with 1 unit of T4 DNA polymerase (Boehringer Mannheim) and 0.2 μM of each dNTP. The
DNA was then phosphorylated with 10 units of T4 polynucleotide kinase (Boehringer Mannheim) and 10 mM ATP. The reaction was carried out for 30 min at 37 °C; then, an additional 10 units of T4 polynucleotide kinase were added, and the incubation was continued for another 30 min. After phenol-chloroform extraction and ethanol precipitation, the DNA was dissolved in 10 mM sodium phosphate buffer (pH 7.4), 2 mM glutamine (Sigma), 2 mM phenylmethylsulfonyl fluoride (Sigma), 100 units ml−1 filter penicillin, and 100 mg ml−1 of 15% glycerol in 1 ml of saline: 280 mM NaCl, 10 mM KCl, 4.3 mM Na2HPO4, 1.5 mM NaH2PO4, 1.47 mM KH2PO4. 48 h later, the cells were collected by scraping in PBS containing 1.47 mM KH2PO4, pH 7.2, and washed with PBS containing 1.47 mM KH2PO4, pH 7.2.

RESULTS AND DISCUSSION

The G → A Transition at −78 bp Increases Transcription Efficiency of ApoA-I Promoter—Since the apoA-I promoter is efficiently expressed in liver and intestine cells (19), for transfection experiments Hep G2 and CaCo2 cells, derived from human hepatoma and colon carcinoma, respectively, were used (see “Experimental Procedures”). To evaluate the role of the G → A transition in the expression of apoA-I gene, Hep G2 cells were transiently transfected with four different plasmids containing the bacterial CAT gene fused to the promoter region of the apoA-I gene. Two plasmids contained the 5'-flanking region, from −375 to +29 of the apoA-I gene, and differed in having A or G at the −78 position (S-A-CAT and S-G-CAT, respectively). The other CAT fusion plasmids contained the −2500 to +29 promoter region in the G or A configuration (L-G-CAT or L-A-CAT, respectively, see “Experimental Procedures”). To normalize for the transfection efficiency, these plasmids were cotransfected with a reference plasmid containing the Escherichia coli lacZ gene under control of the long terminal repeat of Rous sarcoma virus (RSV-lacZ). The CAT enzyme produced was measured in extracts of transfected cells, either immunologically or by chromatographic assay of CAT enzyme activity. The CAT fusions containing A at −78 bp consistently expressed more CAT activity than their G counterparts. In addition, the longer promoter fusions were more active than the shorter ones (Fig. 1). We also tested other fusions containing an extended 5'-intragenic segment (up to +397 bp). These A configuration fusions expressed consistently more CAT than the fusions carrying G in position −78 (data not shown). To minimize the effects of mutations occurring during PCR reactions, we tested plasmid fusions derived from independent amplifications (see legend of Fig. 1).

The shorter promoter fusions were not expressed in intestine-derived CaCo2 cells, since they lacked the apoA-I promoter sequences required for maximal levels of expression in intestine (19). In contrast, the long promoter fusions were efficiently expressed in these cells. As in the hepatoma cells the promoter carrying A in position −78 was more active (5-6 fold) than the G variant (data not shown). On the basis of these data, we conclude that the presence of A at position −78 increases the transcriptional efficiency of apoA-I promoter.

The G → A Transition at −78 bp Decreases the Affinity of the 90-kDa Binding Protein to the Promoter—To determine the mechanism responsible for the improved promoter efficiency of the A versus the G allele, we analyzed the nuclear proteins that bind in the −78 region of apoA-I promoter.

Nuclear Extracts and Gel Retardation Assay—The preparation of Hep G2 and CaCo2 nuclear extracts and the analysis of DNA-protein complexes were carried out as described (31, 32). Two synthetic oligonucleotides (30-mer) containing the region from −93 to −63 from the apoA-I transcription start site with the A or G at position −78 were used as probes for gel retardation assay. A second set of probes included a fragment spanning the −105 to −6 region in the A or G configuration. These fragments were prepared by AvaII-DdeI digestion of the 404-bp fragment obtained by PCR, as described above. The double-strand fragments were dephosphorylated and 5'-end-labeled with T4 polynucleotide kinase and [γ-32P]ATP. Nuclear extracts were preincubated for 5 min at 37 °C with 1.5 mM NaCl, 7 mM MgCl2, 0.1 mM EDTA, 5 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 5% glycerol, 2 µg of poly(dI-dC), and 4 µg of salmon sperm DNA. When unlabeled DNA fragments were added as competitors, the reaction mix was preincubated for 10 min at room temperature before adding the labeled DNA.

UV Cross-linking—Labeled 30-mer oligonucleotides were incubated with Hep G2 nuclear extracts in the same conditions described for gel retardation assay. The reaction mixture was UV-irradiated for 10 min at a wavelength of 254 nm and loaded on a 6% polyacrylamide gel. The retarded bands corresponding to the DNA-protein complexes were excised from the gel and loaded on a 10% SDS-polyacrylamide gel.
Fig. 1. G → A transition at −78 bp of apoA-I gene increases promoter activity. Expression of CAT gene under the control of apoA-I promoter. At top left are shown the plasmid fusions used in this study. The short and long constructs correspond to S-A-CAT or S-G-CAT and to L-A-CAT or L-G-CAT, respectively (see "Experimental Procedures"). The letter indicates the A or G configuration. The coordinates of the constructs are −375 to +29 or −2500 to +29 from the transcription start site. The values indicated at top of figure are quantitative independent experiments normalized for lacZ activity. Each value represents the average of 2 independent determinations. The standard deviations in each experiment are the following: 58.5 ± 6, 8.9 ± 2.3, 29.5 ± 5.4, 5.6 ± 1.2, 16 ± 2.2, 3.3 ± 1.1, 112.8 ± 20, 12.0 ± 2.5, 154 ± 17.6, 8.4 ± 1.1, 100 (± 15), and 20 ± 2.5. The CAT ELISA was standardized as suggested by the manufacturer. † indicates -fold increase of CAT protein versus A configuraion. The coordinates of the figure is shown a representative CAT assay by thin layer chromatography of *4C-labeled acetylated chloramphenicol. A and G promoters are shown the plasmid fusions used in this study.

were carried out with nuclear extracts from Hep G2 cells (Fig. 2B). As in this case of the CaCo2 extracts, the G-containing oligonucleotide formed DNA-protein complexes that were more efficiently competed by oligonucleotide G than by A (Fig. 2B). The concentration of the putative proteins binding the −78 region appears to be comparable in intestine and liver extracts (Fig. 2B). We noted, however, that the fast moving complex was not efficiently competed by the homologous oligonucleotide in some experiments, suggesting that an aspecific component comigrates with a specific retarded complex.

Inspection of the sequence present in the region of apoA-I promoter between the TATAA and CAAT boxes (−27 to −88 bp) reveals the presence of an inverted repeat of 14/15 bases (17), which may form a potential palindrome. The formation of a stem-loop structure in this region is thermodynamically more stable when A is present rather than G at −78 position. Since the oligonucleotide used in gel retardation experiments shown in Fig. 2 contains only half of the palindrome, we carried out gel retardation and cross-competition experiments with a longer DNA fragment, spanning the region between −105 and −6 bp from the transcription start site of the apoA-I gene. The results, shown in Fig. 3A, confirm the data of Fig. 2. The 100-bp fragment detected three retarded bands, with the same mobility as the DNA-protein complexes seen with the 30-bp oligonucleotide centered on the −78 position. This suggests that the −93 to −63 region is indeed the main binding site in the region −105 to −6 from the transcription start site. The fragment containing the G at −78 bp formed DNA-protein complexes that were less efficiently competed than the complexes formed by A fragment (Fig. 3A, compare lanes G/A with lanes A/G). Quantitative analysis of competition experiments with the 30-mer oligonucleotides or with the 100-bp fragments indicates that the difference in the affinity between oligonucleotide A versus G is ~3.5-fold for either band 1 or band 3 (Fig. 3B).
To identify the protein(s) that bind this area of the apoA-I promoter, we UV cross-linked the DNA-protein complexes formed with labeled oligonucleotide G. The retarded bands were excised from the neutral gel and analyzed on an SDS-polyacrylamide gel electrophoresis gel. Fig. 3B shows that the three retarded complexes (1, 2, and 3 in lane G) were formed by a single molecular species of protein of molecular mass ~90 kDa.

We conclude that the higher activity of apoA-I promoter containing A at ~78 bp parallels the lower affinity of the 90-kDa binding factor.

Epidemiological studies indicate that the A allele of apoA-I is associated with higher apoA-I serum protein levels than the G allele. The G allele will need the G allele is negative and is needed for repression of apoA-I gene transcription. Any conclusion as to the mechanism responsible for the increased transcription of apoA-I promoter lies in a GC-rich sequence (5′-GCC(A/G)GGG-3′). GC-rich boxes are ubiquitous elements and are found in the promoter regions of many housekeeping genes (25). The promoters of the calcium-dependent protease or epidermal growth factor receptor contain a sequence element with a consensus (5′-GC-CGGGG-3′) similar to the apoA-I ~78 region. The transcription of these promoters has been shown to be negatively regulated by this GC box (25). A GC binding factor has been cloned and shown to repress the transcription of promoters carrying this sequence element (25). This 90-kDa factor could mediate the repression of transcription observed in the G allele. The apoA-I promoter contains a sequence element with a consensus (5′-GC-box). This 90-kDa factor could mediate the repression of transcription observed in the G allele. The G allele will need the G allele is negative and is needed for repression of apoA-I gene transcription.

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REFERENCES