

Allelic polymorphisms in the transcriptional regulatory region of apolipoprotein E gene

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Received 3 December 1997

Abstract In this work, we explored the existence of genetic variants within the apolipoprotein E gene transcriptional regulatory region, using a denaturing gradient gel electrophoresis screening of a region comprising nucleotides –1017 to +406. Upon a population study, three new polymorphic sites (–491, –427 and –219) and two mutations were found. Functional effects of the polymorphisms, assayed by transient transfection and electrophoretic mobility shift assays in a human hepatoma cell line, showed that polymorphisms at sites –491 and –219 of the *APOE* promoter produce variations in the transcriptional activity of the gene, most probably through differential binding of nuclear proteins.

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Key words: Apolipoprotein E; Promoter; Polymorphism; Denaturing gradient gel electrophoresis; Transcription; Nuclear protein binding

1. Introduction

Apolipoprotein E (ApoE = protein; *APOE* = gene) is a structural component of several lipoprotein species and plays a central role in lipid metabolism through cellular uptake of lipoprotein particles by lipoprotein receptors in the liver and other tissues [1,2]. ApoE is a polymorphic protein, with three common isoforms (ApoE2, ApoE3, and ApoE4), encoded by three alleles (ϵ 2, ϵ 3, and ϵ 4) of a single gene on chromosome 19q13.2 [3]. This allelic variation contributes to susceptibility to atherosclerotic cardiovascular disease [4] and Alzheimer's disease [5]. The *APOE* promoter region is very complex, hosting numerous regulatory elements located in the proximal 5' flanking region and in the first intron of the human gene [6–9]. Thus, polymorphisms within this region could have functional repercussions mediated by the regulation of *APOE* transcription. To test this possibility, we have developed a methodology based on denaturing gradient gel electrophoresis (DGGE), which allowed the screening of the 5' proximal region of the gene. Here we report the existence of three new polymorphic sites in this region, two of which are associated with differential promoter activity and binding to nuclear proteins in a human hepatoma cell line.

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Abbreviations: *APOE*, apolipoprotein E gene; ApoE, apolipoprotein E; DGGE, denaturing gradient gel electrophoresis; AD, Alzheimer's disease

2. Materials and methods

For DGGE analysis, six overlapping fragments covering the region from –1017 to +406 relative to the transcriptional start site [7] of the *APOE* gene were obtained by PCR from genomic DNA and subjected to DGGE followed by ethidium bromide staining. Parallel DGGE experiments were performed as previously described [10,11] with minor modifications. Briefly, PCR products spanning nucleotides –1017 to –717; –786 to –507; –585 to –285; –363 to –147; –228 to +154; and +93 to +406, and with a 40 bp GCclamp at one end were subjected to DGGE using conditions specific for each fragment (Bullido et al., in preparation). PCR products showing abnormal migrating patterns were subjected to direct sequencing and analyzed in an ALF DNA Sequencer (Pharmacia LKB).

Restriction fragment length polymorphism (RFLP) analysis of –491A/T and –427T/C polymorphisms was performed by digestion of a PCR product spanning the region –512 to –285 with *DraI* and *AhaI*, respectively (primers: 5'-TGTTGGCCAGGCTGGTTTAA-3' (mismatched); 5'-CTTCCTTTCCTGACCCTGTCC-3'). For –219G/T polymorphism analysis, a PCR product spanning –240 to –147 was digested with *TaqI* (primers: 5'-CAGAATGGAGGAGGTG-TCTC-3' (mismatched) and 5'-GGAGGTGGGGCATAGAGGT-CT-3'). The +113G/C polymorphism was analysed by *NlaIV* digestion of a PCR product spanning +93 to +154 (primers: 5'-AAGAGCTGGGACCCTGGGAA-3' and 5'-CGACCCCGAGTAGCTCTCCTGA-3'). For ApoE genotyping, *HhaI* digestion of a 244 bp PCR amplified fragment containing the two polymorphic sites of exon 4 of the *APOE* gene was performed as described [12].

Transient transfection of human hepatoma (HepG2) cells was performed as described [9]. Briefly, *APOE* region –1017 to +406 and containing –491A/–427T/–219T allelic form was amplified by PCR, and site-directed mutagenesis was used to introduce the corresponding mutations at positions –491 (A to T), –427 (T to C) or –219 (T to G). The constructs were cloned into the luciferase expression vector pXP2 and transfected into HepG2 cells, together with a β -galactosidase expression vector. Luciferase and β -galactosidase activities were determined 2 days after transfection.

For electrophoretic mobility shift assays (EMSA), nuclear extracts were prepared according to a described method [13]. Single stranded oligonucleotides (5'-GCTGGTCTCAA(A/T)CTCCTGACCTTAA-3', 5'-ACAGGCGTGAGC(T/C)ACCGCCCCAGC-3' and 5'-GGAGGAGGTGTCTG(T/G)ATTACTGGGCG-3' for –491, –427 and –219 sites, respectively) were end-labeled with [γ -³²P]ATP, annealed to their complements and mixed with nuclear extracts in 15 mM Tris-HCl pH 7.6 containing 15% glycerol, 50 mM NaCl, 3.75 mM MgCl₂, 1.5 mM EDTA, 0.75 μ M DTT and 0.1 μ g/ μ l poly(dI-dC). Following a 20 min incubation at room temperature, the complexes were separated in a 4% non-denaturing polyacrylamide gel, which was dried and exposed to X-ray film.

3. Results and discussion

For the screening of polymorphisms in the *APOE* transcriptional regulatory region, a set of PCR products spanning the entire *APOE* promoter region (nucleotides from –1017 to +406 relative to the transcriptional start site of the gene) from 75 unrelated individuals were analyzed by DGGE. Frag-

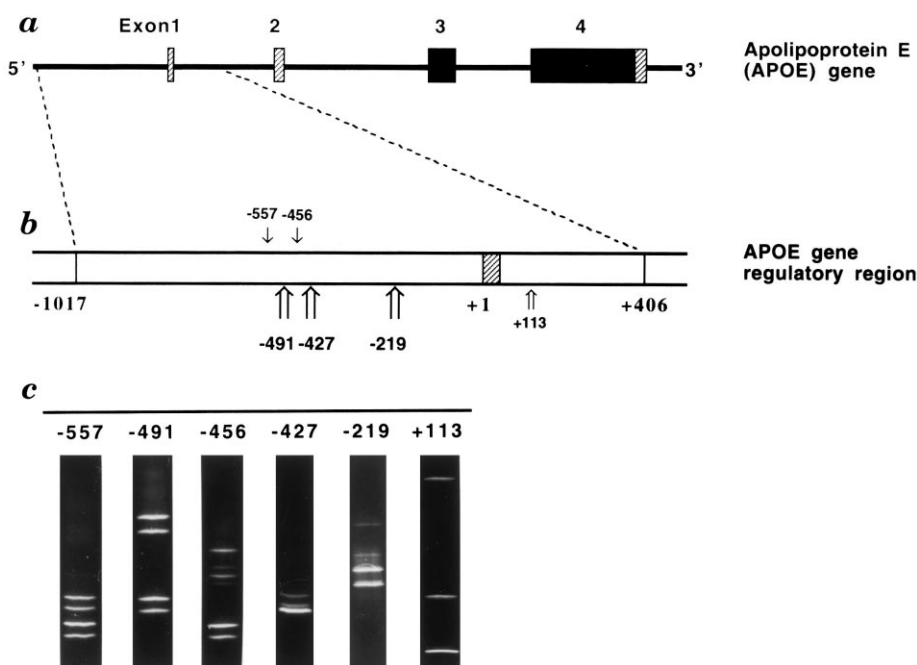


Fig. 1. Identification of polymorphic sites in the *APOE* promoter region. a: *APOE* gene scheme showing introns and 5' and 3' flanking regions (line) and exons (hatched boxes, non-coding regions; full boxes, coding regions). b: *APOE* promoter region showing the sites where polymorphisms (double arrow) or mutations (single arrow) have been detected. c: Parallel DGGE analysis of *APOE* promoter region. A sample of a heterozygous individual corresponding to each of the polymorphic sites is shown.

ments exhibiting altered migration patterns were subsequently sequenced to determine the molecular nature of the variation. We identified an A/T transversion at nucleotide -491, a T/C transition at nucleotide -427, and a T/G transversion at nucleotide -219 (Fig. 1). A C/G transversion at nucleotide +113 in intron 1 was also found, as has been recently described [14]. Two C/T changes at nucleotides -557 and -456, respectively, were also identified. No further variants were detected by DGGE screening. To explore the possibility that polymorphisms not detected by DGGE might exist, we sequenced in 10 randomly chosen individuals the region -1017 to +406 of the *APOE* gene. No polymorphisms were found other than those shown in Fig. 1.

RFLP was used to determine the frequency of the *APOE* promoter polymorphisms in a population of 215 healthy in-

dividuals aged 1–85 years. The genotype and allele frequencies in this population are shown in Table 1A. The population was also genotyped for the major *APOE* polymorphisms at exon 4 (Table 1B). The distribution of alleles into genotypes was consistent with the Hardy-Weinberg equilibrium (χ^2 test, $P > 0.2$). Analysis of the allelic frequencies of each site between the genotypes corresponding to the rest of the polymorphic sites revealed a strong association between -427C and ApoE2 alleles (χ^2 test; $P = 10^{-8}$) and between -219G and +113G alleles ($P < 10^{-10}$). According to previously reported data [10], we found an increased frequency of +113G allele in apoE4 individuals (χ^2 test; $P = 0.003$). No other significant associations between *APOE* polymorphic sites were detected in our population.

The effect of polymorphism in the transcriptional activity of

Table 1
APOE promoter genotype and allele distribution (A) and ApoE allele distribution (B)

Position	Genotype			Allele	
A: <i>APOE</i> nucleotide					
−557	CC 214 (0.99)	CT 1 (< 0.01)	TT 0	C 429 (0.99)	T 1 (< 0.01)
−491	AA 125 (0.58)	AT 81 (0.38)	TT 9 (0.04)	A 331 (0.77)	T 99 (0.23)
−456	CC 214 (0.99)	CT 1 (< 0.01)	TT 0	C 429 (0.99)	T 1 (< 0.01)
−427	TT 193 (0.90)	TC 21 (0.10)	CC 1 (< 0.01)	T 407 (0.95)	C 23 (0.05)
−219	GG 57 (0.28)	GT 105 (0.51)	TT 44 (0.21)	G 219 (0.53)	T 193 (0.47)
+113	GG 79 (0.37)	GC 100 (0.47)	CC 35 (0.16)	G 258 (0.60)	C 170 (0.40)
B: ApoE allele					
	4 34 (0.08)	3 372 (0.87)	2 24 (0.06)		

Figures are numbers and, in parentheses, frequencies.

APOE was analyzed by transient transfection assays. Since the hepatocytes constitute the main cell type synthesizing ApoE, the human cell line HepG2 (hepatoma) was used for transfection. Taking as reference the activity of the construct containing nucleotides –491A, –427T and –219T, which corresponds to the ‘wild type’ haplotype, it was found that a single A to T base substitution at nucleotide –491 caused a significant decrease in *APOE* promoter activity (63% of control, $P=0.002$ by Student’s *t*-test), whereas a T to G substitution at nucleotide –219 provoked an increase in promoter activity (169% of control, $P=0.001$) (Fig. 2). By contrast, T to C substitution at nucleotide –427 had no significant effect on promoter activity in these cells. These results suggest that sequences located around nucleotides –491 and –219 are involved in the regulation of *APOE* transcription in hepatoma cells.

In order to investigate if the differential promoter activity may be related to differences in protein binding, EMSAs were carried out using freshly prepared nuclear extracts from HepG2 cells and oligonucleotide probes corresponding to each of the allelic forms of the promoter. When the –219 site was studied, a specific band was detected which was more intense with the –219T probe than with the –219G probe (Fig. 3, arrow). A non-specific band of higher mobility was also observed (Fig. 3, asterisk). Similarly, we found a differential binding of oligonucleotides –491A and –491T to HepG2 nuclear extracts (Bullido et al., *Nature Genet.*, in press). We were unable to detect differences in the binding pattern of nuclear proteins to the –427T and –427C probes (not shown). The parallel behavior of the allelic forms in the reporter gene expression and EMSA strongly suggests that allelic differences in *APOE* promoter activity in the hepatoma cell line may be a consequence of differential binding of nuclear proteins present in these cells.

It is well documented that single nucleotide changes within a promoter region may dramatically affect transcriptional activity mediated by transcription factors [15,16], even when the nucleotide change does not alter directly a DNA binding protein sequence motif and may be related to changes in the secondary structure of DNA affecting the access of transcriptional factors [17]. Our findings identify an important source

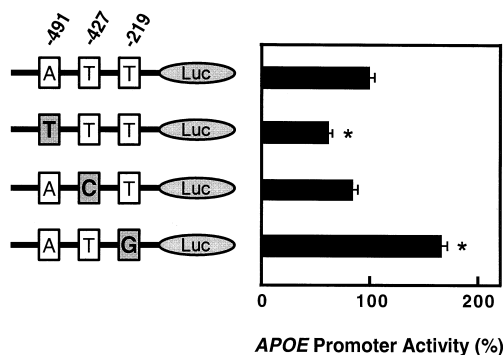


Fig. 2. Transcriptional activity of the allelic forms of the *APOE* promoter. Bars represent luciferase/ β -galactosidase activity ratios for the *APOE* constructs schematized on the left, transfected into HepG2 cells together with a β -galactosidase expression vector. Data are expressed as percent of activity of the upper construct. Data are the mean \pm S.E.M. of six determinations, and are representative of five independent experiments. *Statistically significant; $P<0.01$ by Student’s *t*-test.

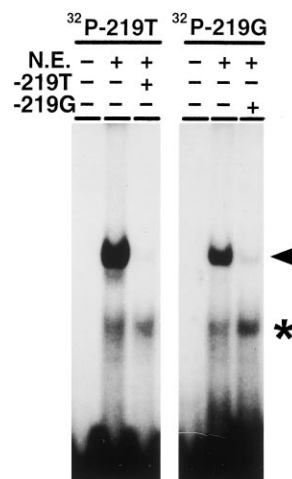


Fig. 3. Analysis of allelic-specific differences in DNA binding proteins of *APOE* promoter. EMSA was performed using oligonucleotide probes corresponding to –491A/T and –219T/G allelic forms in the presence of HepG2 nuclear extracts. ³²P-labeled probes were incubated in the absence (–) or the presence (+) of nuclear extract (N.E.). Competition experiments were performed by preincubating the nuclear extract in the absence (–) or the presence (+) of a 50-fold excess of unlabeled oligonucleotides, before addition of the probes. Results shown are representative of at least four independent experiments.

of *APOE* gene diversity, and raise the possibility that such regulatory region polymorphisms may confer in vivo allelic differences in expression, inducibility, and/or tissue specificity of human *APOE*, and in the susceptibility to pathologies in which ApoE is involved, such as atherosclerotic cardiovascular disease and Alzheimer’s disease. In this connection, we have recently found that one of the polymorphisms described here (–491A/T) is associated with an increased risk of AD (Bullido et al., *Nature Genet.*, in press).

Acknowledgements: This work was supported by Boehringer Ingelheim España and Fondo de Investigación Sanitaria (Grant 95-0022). An institutional grant from Fundación Ramón Areces to CBMSO is acknowledged. M.J.A. is the recipient of a fellowship from the Fondo de Investigación Sanitaria. M.A.G. is the recipient of a fellowship from the Ministerio de Educación y Ciencia. J.A. is the recipient of a fellowship from the Comunidad Autónoma de Madrid. We thank Prof. F. Mayor for his continuous encouragement and help. We thank Dr. Magdalena Ugarte, Dr. Anna Frank, Dr. Paloma Alonso and Margarita Revuelta for their kind collaboration in providing the samples, and Dr. Jesús Avila for his critical reading of the manuscript. We thank the individuals who collaborated in this work as blood donors.

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