

## DIFFERENTIAL METHYLATION OF THE HUMAN APOLIPOPROTEIN AI (APO AI) GENE IN LIVER AND LEUCOCYTE CELL DNA

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### ABSTRACT

The DNA methylation pattern within the transcribed region of the human Apolipoprotein AI (Apo AI) gene was analyzed in DNA sample preparations of uncultured liver and leucocyte cells from five unrelated Caucasian patients. While, CCGG sites were fully methylated in leucocyte DNA, liver cell DNA showed demethylation in three specific sites within the gene. These results support previous data from cultured cells, showing that demethylation within the Apo AI gene is correlated with gene expression.

### INTRODUCTION

Apolipoprotein AI (Apo AI) is the major protein component of the High Density Lipoproteins (HDL). Plasma levels of HDL appear to be inversely correlated with the incidence of arterial and cardiovascular heart disease (Miller *et al.*, 1977). Moreover, epidemiological and genetic studies have shown that low levels of Apo AI protein in plasma are frequently associated with premature atherosclerosis and myocardial infarction risk (Norum *et al.*, 1982). The isolation of Apo AI cDNA probes (Breslow *et al.*, 1982) permitted determination of the structure of the Apo AI gene and detection of gene rearrangements, in some cases showing low levels of HDL in the plasma (Karathanasis *et al.*, 1983a). These findings indicate that expression of Apo AI plays an important role in controlling the levels of blood HDL.

In mammals, the Apo AI gene is expressed primarily in the liver and small intestine cells and remains silent in all other tissues (Zannis *et al.*, 1985). The mechanism and factors that control such differential activity of this gene are still poorly understood. Cytosine methylation is a frequent event that occurs mainly in the dinucleotide sequence CpG of vertebrate DNA. There is overwhelming evidence indicating a correlation between gene expression and levels of DNA methylation (see review in Doerfler, 1983). In particular, some human cell lines have been shown to exhibit a correlation between hypomethylation and hypermethylation of transcribed and silent Apo AI genes, respectively (Ruiz-Opazo and Zannis, 1988). However, these observations have not been confirmed in uncultured human cells. Moreover, there is no data indicating that a change in the pattern of gene methylation will result in changes in Apo AI gene expression. We investigated the methylation levels of human Apo AI gene in DNA samples from uncultured liver cells and peripheral leucocytes by comparing their respective patterns of cleavage caused by MspI/HpaII methylation sensitive restriction enzymes.

## MATERIAL AND METHODS

Hepatic samples were obtained from five unrelated Caucasian patients, who underwent liver surgery. The samples were dropped into cold RPMI-1640 medium and frozen until DNA isolation. DNA was extracted from normal tissue sections, as confirmed by histology. Ten milliliter samples of peripheral blood were drawn with ACD solution B (Becton Dickinson) as anticoagulant (Gustafson *et al.*, 1987). For both samples, DNA was released from the nuclei by lysis with SDS and Proteinase K (pH 8.0) at 65°C, and isolated by phenol and phenol/chloroform extractions. The methylation pattern of the Apo AI gene was investigated by digesting 10 µg of DNA with the MspI in single digestion or HpaII in combination with PstI or EcoRI. MspI and HpaII are isoschizomers for CCGG site. The latter enzyme is inhibited by methylation of both the external and internal C of the CCGG site. On the other hand, MspI restriction is blocked only when the external C is methylated (Nelson and McClelland, 1989). PstI and EcoRI in combination with HpaII were used in order to localize HpaII sites within specific segments of the gene. PstI and EcoRI were also used in single digestion experiments.

All DNA fragments were electrophoresed in 1% agarose gels in Tris-Borate-EDTA buffer and blotted to nitrocellulose filters (Sambrook *et al.*, 1989); HindIII lambda DNA was used as a molecular weight marker. The full-length human Apo AI cDNA insert from clone 6 (Sharpe *et al.*, 1984) (kindly provided by F. Baralle) was labeled by random priming with  $[\alpha]^{32}\text{P}$  dCTP to a specific activity of  $1.4 \times 10^9$  cpm/µg, and used as a probe. Hybridizations were carried out in  $4 \times \text{SSC}$  at 65°C (SSC = NaCl 0.15 mol/l and trisodium citrate 0.015 mol/l at pH 7.6). The two final washes of the filters

were performed in 0.1 x SSC, 0.1% SDS at 60°C during 15 min each. Autoradiograms were exposed at -70°C, with enhancing screens, for four to eight days.

## RESULTS

The restriction enzyme digestion patterns of liver and leucocyte DNAs are shown in Figures 1 and 2. Regardless of cell source, the *Msp*I digestions produced a Southern pattern, characterized by three fragments of 1.7, 1.0 and 0.7 kb in two cases (Figure 1, lanes 2, 4, 7 and 9), whereas the other three cases showed only two of the

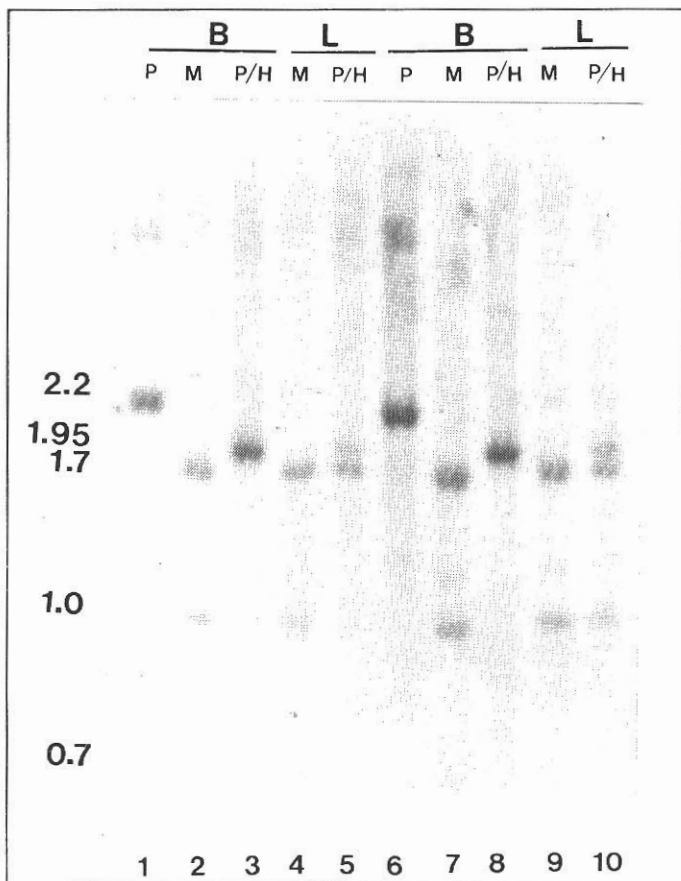


Figure 1 - Southern blot of blood (B) and liver (L) DNA samples from two patients, digested with *Pst*I (P, lanes 1 and 6), *Msp*I (M, lanes 2, 4, 7 and 9) and *Pst*I/*Hpa*II (P/H, lanes 3, 5, 8 and 10), and hybridized with [ $\alpha$ ] $^{32}$ P Apo AI cDNA probe.

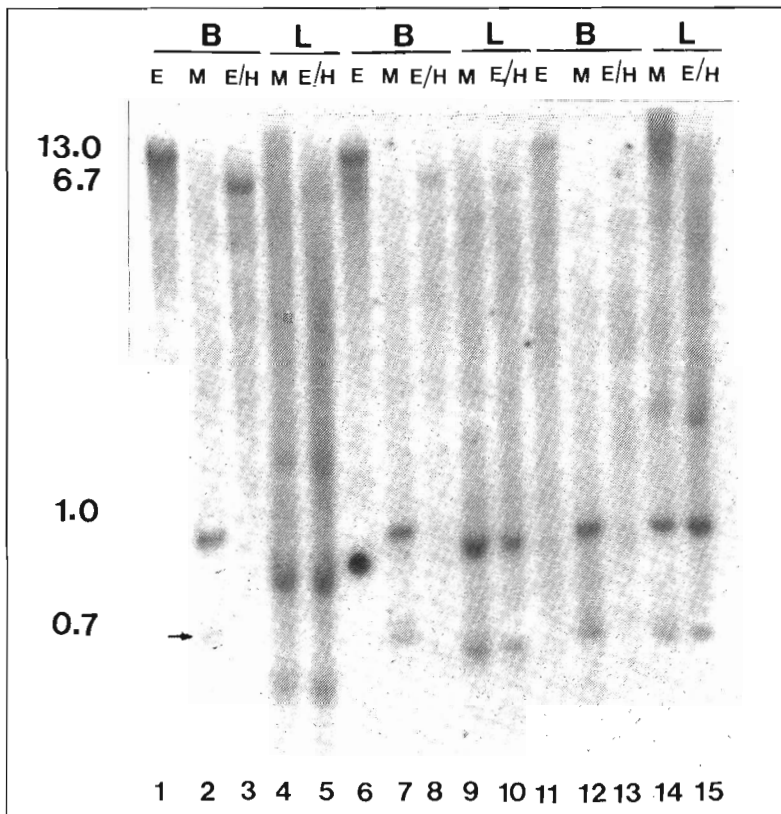


Figure 2 - Southern blot analysis of blood (B) and liver (L) DNA samples from three patients digested with EcoRI (E, lanes 1, 6 and 11), MspI (M, lanes 2, 4, 7, 9, 12 and 14) and EcoRI/HpaII (E/H, lanes 3, 5, 8, 10, 13 and 15).

fragments, i.e. 1.0 and 0.7 kb (Figure 2, lanes 2, 4, 7, 9, 12 and 14). These bands have been reported previously (Ferns and Galton, 1986). The 0.7 and 1.0 kb fragments result from cleavage of  $M_2/M_3$  and  $M_3/M_4$  sites, respectively (Figure 3). The 1.7 kb fragment results from a lack of an  $M_3$  site and restriction at  $M_2/M_4$  sites. Accordingly, the samples in Figure 1 are heterozygous for the lack of  $M_3$  ( $M_3+/M_3-$ ). On the other hand, the presence of only 1.0 kb and 0.7 kb fragments indicate that the individuals in Figure 2 are homozygous for this character ( $M_3+/M_3+$ ).

The 5' and 3' ends of the Apo A1 gene are flanked by two PstI sites located 2.2 kb apart (Figure 1, lanes 1 and 6; Figure 3) and by two EcoRI sites that produce a 13.0 kb fragment encompassing the gene (Figure 2, lanes 1, 6 and 11; Figure 3).

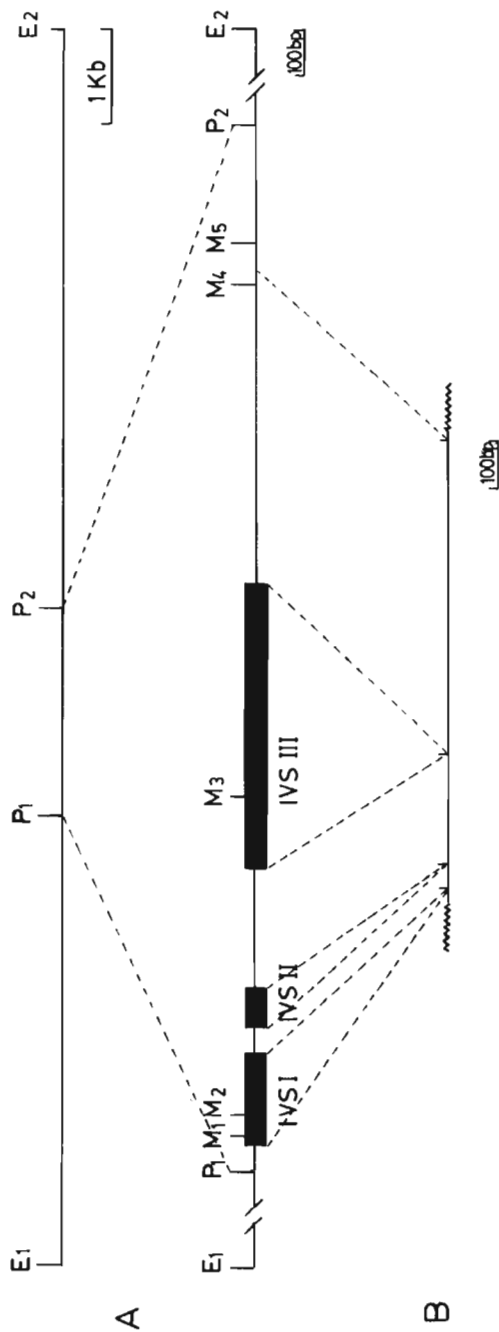


Figure 3 - A: Map of the human Apo A1 gene restriction enzyme sites EcoRI=E, PstI=P, MspI or HpaII=M. Positions were obtained from the nucleotide sequence of the gene (Shoulders *et al.*, 1983, Karathanasis *et al.*, 1983a) and confirmed by Southern blotting and hybridization with the Apo A1 cDNA probe. Restriction sites are numbered from the 5' to 3' end on the gene. IVS = Introns. B: Apo A1 cDNA probe used.

PstI/HpaII double digestions of blood cell DNA samples gave rise to a single 1.95 kb band (Figure 1, lanes 3 and 8). This fragment results from methylation of M<sub>1-4</sub> sites and cleavage at P<sub>1</sub> and M<sub>5</sub> sites (Figure 3). Double digestions of leucocyte DNA with EcoRI/HpaII generate a 6.7 kb segment due to restriction at E<sub>1</sub> and M<sub>5</sub> and methylation blockage of all intermediate M sites (Figure 2, lanes 3, 8 and 13; Figure 3). Demethylation of M<sub>5</sub> was further confirmed by EcoRI/SmaI digestions (not shown). This combination produced one 6.7 kb band, similar to that found with the EcoRI/HpaII double digestion of blood DNA (not shown).

Liver cell DNA samples double digested with PstI/HpaII (Figure 1, lanes 5 and 10) and EcoRI/HpaII (Figure 2, lanes 5, 10 and 15) produced a banding pattern similar to that induced by MspI single digestion. This indicates demethylation of all M sites (Figure 3). Weak bands at 1.95 kb (Figure 1, lanes 5 and 10) and 6.7 kb positions (Figure 2, lanes 5, 10 and 15) were also observed in PstI/HpaII and EcoRI/HpaII double digestions, respectively. These fragments probably derive from a small cell fraction (fibroblasts and endothelial cells, or a hepatocyte subpopulation), with methylation of M<sub>1-4</sub> sites.

## DISCUSSION

It has been demonstrated that most housekeeping genes have a CpG-rich island, commonly located upstream or in the 5' region of the gene (Bird, 1986). On the other hand, tissue-specific genes usually lack these islands and their level of expression is associated with methylation of CpG sites within the boundaries of the gene (see review in Cooper, 1983).

An analysis of the nucleotide sequence of a 2.3 kb long DNA domain comprising the Apo AI gene (Shoulders *et al.*, 1983, Karathanasis *et al.*, 1983b) did not show any region with characteristics of a CpG-rich island. Moreover, since this gene is primarily expressed in liver cells it should be considered tissue-specific.

We analysed the methylation levels of human Apo AI genes in liver tissue and leucocyte cell samples and we found that the liver genes are demethylated, while leucocyte genes are fully methylated. These results suggest that demethylation and Apo AI expression may be associated events. This assumption is in agreement with previous work done with cultured cells by Ruiz-Opazo and Zannis (1988). These authors analyzed the level of expression of human Apo AI genes transfected into a rat myogenic cell line, and found that exogenous human Apo AI genes were expressed and hypomethylated, while endogenous rat Apo AI genes were silent and fully methylated. Moreover, in these as well as in other Apo AI-active cells, i.e. HepG2 (human hepatoma cell line) they found an HpaII 0.7 kb band, indicating hypomethylation of the Apo AI transcription unit (Ruiz-Opazo and Zannis, 1988). Our data from uncultured cells are coincident with these

findings: DNA from liver (Apo AI-producing tissue) showed 0.7 kb and 1.0 kb fragments. Analysis of the Apo AI gene sequence (Shoulders *et al.*, 1983; Karathanasis *et al.*, 1983b) allows us to conclude that these bands may result from HpaII digestion of CCGG sequences at M<sub>2</sub>, M<sub>3</sub> and M<sub>4</sub> sites (Figure 3), provided they are demethylated. Therefore, demethylation of specific sites within the Apo AI gene domain correlates with its activity. In contrast, leucocyte DNA (Apo AI-non producing cell) does not show any of these bands upon digestion with HpaII. These findings further support the conclusion that the inactivity of the Apo AI gene in these cells is associated with extensive methylation of the Apo AI gene domain.

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### RESUMO

O padrão de metilação do DNA dentro da região transcrita do gene humano Apolipoproteína AI (ApoAI) foi analisado em uma preparação de DNA de células de fígado não cultivadas e de células de leucócito de cinco pacientes caucasianos sem parentesco. Enquanto os sítios CCGG foram totalmente metilados em DNA de leucócitos, DNA da célula de fígado mostrou demetilação em três sítios específicos dentro do gene. Estes resultados confirmam dados prévios de células cultivadas mostrando que demetilação dentro do gene Apo AI correlaciona com a expressão do gene.

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