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Differences in the accessibility of methylated and unmethylated DNA to DNase I

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ABSTRACT

DNase I binds in the minor groove of DNA and is used as an enzymatic tool to investigate the interaction of proteins with DNA. Here we show that the major groove located 5-methyldeoxycytidine can enhance or inhibit the cleavage rates of DNA by DNase I. This effect may be caused in part by changes in DNA structure affecting the accessibility of the minor groove of DNA to DNase I.

INTRODUCTION

Bovine DNase I has been used as an enzymatic tool to analyze DNA–protein interactions both *in vitro* and *in vivo* (1, 2). DNase I binds to the minor groove of the B form of DNA and establishes contacts with phosphodiester bonds in both compartments of double-stranded DNA (3, 4). The accessibility of DNA to DNase I is largely determined by the width of the minor groove (5), and by the flexibility or stiffness of DNA (6). Upon binding of DNase I in the minor groove of the DNA template, the DNA bends towards the major groove and opens up the minor groove (6).

In nuclear extracts from HeLa cells or from primary human lymphocytes, the cell-free transcription of several RNA polymerase III-activated genes is reduced by enzymatic CpG methylation of the control regions of these genes (7, 8). When investigating the molecular basis for this transcriptional inactivation by *in vitro* footprinting experiments, we observed that DNA templates, which had been either CpG- or mock-methylated by the CpG DNA methyltransferase (M.SssI) (9), were differently cleaved by DNase I.

In this report, we show that DNase I degradation of DNA can be either locally enhanced or inhibited by deoxycytidine methylation.

MATERIALS AND METHODS

Plasmids

The following plasmids were used as templates in the DNase I degradation experiments: (i) The plasmid pAngio2 contains a 1686 bp PvuII–BglII fragment of the upstream region of the human angiogenin gene (10) cloned into the Bluescript KS+ vector (8). (ii) The plasmid pVAI+II contains a 759 bp fragment of adenovirus type 2 (Ad2) DNA carrying the VAI and VAII genes cloned into the pUC18 vector (7).

DNA methylation and radioactive labeling

Linearized plasmid DNA was methylated *in vitro* by the 5′-CG-3′-specific DNA methyltransferase from *Spiroplasma species*, M.SssI (9), as described earlier (7). In mock-methylation experiments, S-adenosylmethionine as the methyl group donor was omitted from the reaction mixture. The templates were radioactively labeled at their 5′ termini by using γ-[^{32}P] ATP and T4-polynucleotide kinase (11) or were labeled at their 3′ termini by using α-[^{32}P] dNTPs and the Klenow fragment (12) of *E.coli* DNA polymerase I (13) in fill-in reactions. Subsequently, the templates were cleaved with a second restriction endonuclease in order to generate end-labeled fragments. The labeled fragments were purified by gel electrophoresis. In detail, pAngio2 DNA was labeled at a NcoI site (Fig. 1) and was then cleaved with SacI within the polylinker of the vector. The pVAI+II DNA was labeled at a HindIII site (Fig. 2) and then cleaved with KpnI. The completeness of the enzymatic DNA methylation was ascertained by direct nucleotide sequencing (14) using the hydrazine reaction, as described elsewhere (15).

DNase I treatment of methylated and mock-methylated DNA

An amount of 30,000 cpm of end-labeled template was incubated on ice for 2 min in a total volume of 20 μl 10 mM Hepes, pH 7.9, 20 mM KCl, 5 mM $MgCl_2$, 2.5 mM $CaCl_2$, 400 ng of pUC18 DNA with 0.4, 2 or 10 ng of DNase I (Serva). The reaction was stopped by adding 100 μl of 1% SDS, 20 mM EDTA, 250 mM NaCl, 5 μg of tRNA, 2 μg of proteinase K, and the mixture was incubated for 30 min at 37°C, followed by purification of the DNA with phenol/chloroform and by ethanol precipitation.

The DNase I-generated fragments were separated on 5% polyacrylamide sequencing gels, containing 7 M urea, together with C- and G-sequencing ladders (14) of the same templates.

RESULTS AND DISCUSSION

The scheme in Fig. 1a presents the upstream region of the human angiogenin gene (8, 10) containing a very CpG-rich Alu element. The end-labeled CpG- or mock-methylated DNAs were treated with different concentrations of DNase I (for details see Materials and Methods). The reaction products were then analyzed on denaturing sequencing gels. In Fig. 1b the results of one

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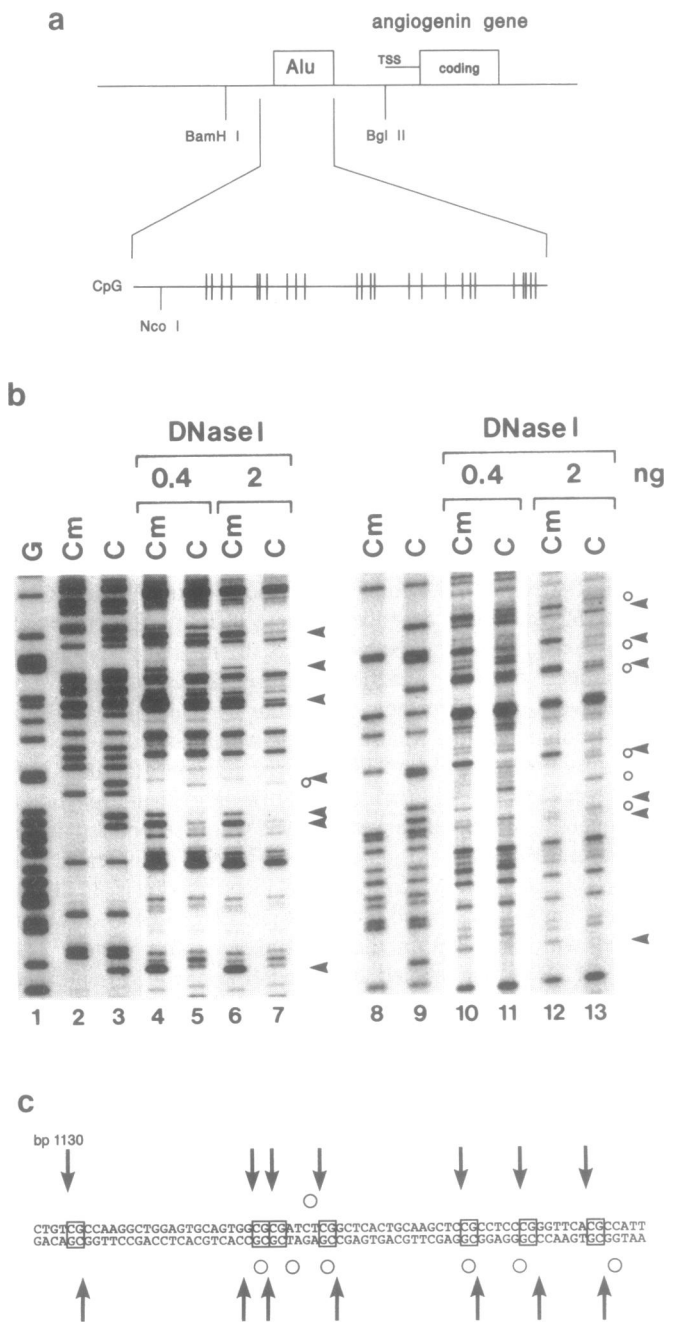

Figure 1. a) Map of the CpG rich Alu element located upstream of the angiogenin gene. b) Differential accessibility of the methylated or unmethylated Alu sequence to DNase I. Lanes 1–7: top strand; lanes 8–13: bottom strand. Lane 1: G (DMS = dimethyl sulfate) reaction; lanes 2 and 8: C (hydrazine) reaction of the CpG methylated templates. Lanes 3 and 9: C reactions of the mock-methylated template. Lanes 4, 6, 10, 12: DNase I cleavage of the CpG methylated templates; lanes 5, 7, 11, 13: DNase I cleavage of the mock-methylated templates. The DNase I concentrations were indicated. Enhanced cleavage of the 5′-CG-3′ methylated templates was indicated by arrows, reduced cleavage by circles. c) Nucleotide sequence of part of the Alu element. The location of the additional or enhanced DNase I cleavage sites in the CpG methylated template were marked by arrows, inhibition of DNase I cleavage by circles.

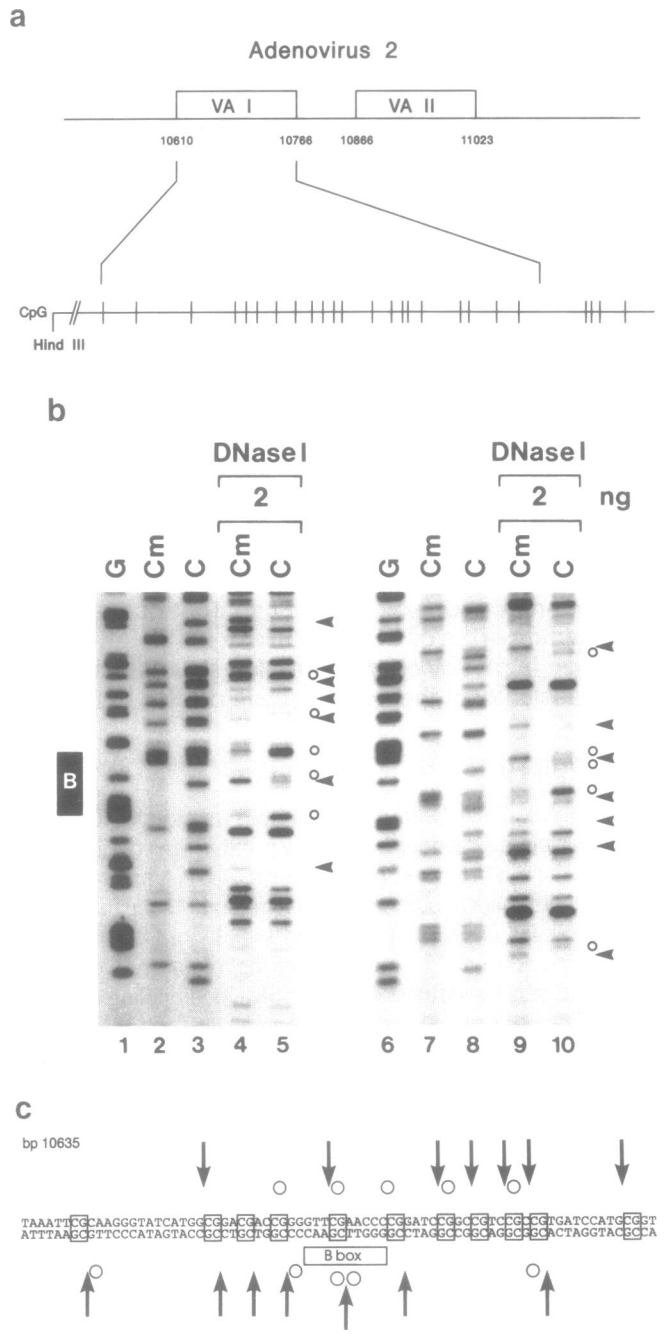

Figure 2. a) Map of the VAI and VAII genes of Ad2. b) Differential accessibility of the methylated or unmethylated DNA to DNase I. Lanes 1–5: top strand; lanes 6–10: bottom strand. Lanes 1 and 6: G (DMS) reaction; lanes 2 and 7: C (hydrazine) reaction of the CpG methylated DNA; lanes 3 and 8: C reaction of the mock-methylated DNA; lanes 4 and 9: DNase I cleavage of the CpG methylated templates; lanes 5 and 10: DNase I cleavage of the mock-methylated templates. Enhanced cleavage of the 5′-CG-3′ methylated templates was indicated by arrows, inhibition of cleavage by DNase I by circles. c) Nucleotide sequence of part of the VAI gene. The locations of the additional or enhanced DNase I cleavage sites in the CpG methylated templates were designated by arrows, inhibition of DNase I cleavage by circles.

experiment are shown for the top (lanes 1–7) and the bottom strands (lanes 8–13). Differences in the cleavage patterns exist between CpG-methylated and mock-methylated DNA templates. The results were reproduced in many experiments and were independent of the DNase I concentrations used. In comparison to the cleavage patterns in the unmethylated DNA template (lanes 5, 7, 11, 13), many additional or strongly enhanced bands were observed which represent DNA fragments cleaved immediately

5′ of methylated deoxycytidine residues (lanes 4, 6, 10, 12). These bands were designated by arrows. In contrast, at several positions the cleavage of the DNA template by DNase I was inhibited by the presence of 5-methyldeoxycytidine, as indicated by circles.

In Fig. 1c, a part of the nucleotide sequence of the Alu element located upstream of the angiogenin gene was reproduced. The additional or enhanced DNase I cleavage sites on both DNA strands of the 5′-CG-3′ methylated template were designated by arrows, the inhibition of DNase I cleavage by methylation by circles. It was apparent that the additional or enhanced cleavage sites lay immediately 5′ of nearly all methylated deoxycytidine residues in both DNA complements. The locations of reduced DNase I sensitivity by 5′-CG-3′ methylation failed to show a consistent distance to the next methylated cytidine residue.

In Fig. 2a, a scheme of the VAI gene of Ad2 DNA is depicted (15). The end-labeled CpG-methylated or mock-methylated template was incubated with DNase I. The reaction products were analyzed on denaturing sequencing gels. Again, strong differences between CpG-methylated (lanes 4, 9) and mock-methylated (lanes 5, 10) DNA templates were observed (Fig. 2b). In the methylated templates either additional or enhanced signals could be detected at most of the CpG dinucleotides. In contrast, at several positions the attack of the template DNA by DNase I was strongly inhibited by the presence of 5-methyldeoxycytidine (circles in Fig. 2b). The enhancing or inhibiting effect of CpG methylation on DNase I cleavage was most prominent in the region of the polymerase III internal control element B of the VAI gene. In Fig. 2c, the sequence of part of the VAI gene was presented. Additional or enhanced DNase I cleavage sites in both DNA strands of the methylated template were indicated by arrows; the sites of inhibition of DNase I cleavage by CpG methylation were marked by circles.

Individual DNase I-sensitive sites were not affected to the same extent, as demonstrated in Fig. 1b, c, 2b, c. However, a quantitative analysis of the data was not undertaken because the differences between methylated and unmethylated sequences were reproduced several times.

Very similar results were obtained with three additional DNA templates containing Alu elements located in the ACTH gene, 3′ of the alpha-1 globin gene or in the tPA gene (8), with both enhancement or inhibition of DNase I cleavage by CpG methylation (data not shown).

Methylation of the central deoxycytidine residue in the DNA sequence 5′-GCGC-3′ by the HhaI DNA methyltransferase resulted in enhanced susceptibility to cleavage by DNase I immediately 5′ of the methylated cytidine (16). Minor alterations in the orientation of the phosphodiester bond on the 5′ side of the 5-methyldeoxycytidine residue in the GMeCGC sequence were implicated to cause selective cleavage. Here we confirmed and extended these results. Enhanced cleavage by DNase I immediately 5′ of the methylated cytidine was observed for all four DNA nucleotides preceding the 5-methyldeoxycytidine of the 5′-MeCG-3′ sequence (Fig. 1c, 2c). In addition, at certain positions a decrease of the susceptibility to DNase I cleavage in 5′-CG-3′ methylated DNA compared to unmethylated DNA was reproducibly observed (Figs. 1c, 2c). The decrease of DNase I cleavage in the methylated versus the unmethylated template was found at different distances from the next methylated 5′-CG-3′ sequence in the same or the complementary DNA strand. This finding suggested structural changes in the DNA as the basis for the observed effect. DNA methylation as a signal in the long-term transcriptional inactivation of gene expression was extensively studied (for reviews, 17, 18). The mechanism of transcriptional inactivation of genes by DNA methylation might involve the direct interference of the methylated cytidine with the binding of DNA binding proteins to DNA (17). Alternatively, the preferential binding of methylated DNA binding proteins to the major groove of the methylated DNA (19) could interfere with transcription. The two possibilities are not mutually exclusive. Here we suggest that methylated cytidine residues might affect the interaction of proteins with the minor groove of DNA as observed for DNase I (3, 4), possibly by inducing structural changes in DNA.

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