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Changes in chromatin structure due to hypomethylation induced with 5-azacytidine or DL-ethionine

Jiří Fajkus, Boris Vyskot and Milan Bezděk

Institute of Biophysics, Czechoslovak Academy of Sciences, CS-61265 Brno, Czechoslovakia

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Changes in chromatin structure of the HRS60 family of repetitive sequences in tobacco DNA were studied after hypomethylation induced with 5-azacytidine or DL-ethionine. The *Taq*I site in the HRS60 units lies in nucleosomal core regions and its cleavage is enhanced in the hypomethylated chromatin. In contrast, the cleavage of the *Sau*3A1 site located in linker DNA does not depend on the level of methylation of DNA.

DNA methylation; Nucleosome positioning; Repetitive DNA sequence; Tobacco tissue culture

1. INTRODUCTION

DNA methylation provides a mechanism for a heritable alteration of a gene and affects the formation of active chromatin. A number of experimental results have shown an inverse relationship between DNA methylation and gene expression [1–3]. For example, de-methylation of tissue-specific genes is correlated with their transition into an active state [4]. This transition appears to consist of a decreased compacting of chromatin, which turns from the solenoid into the 'beads on a string' configuration. In the latter structure nucleosomes are depleted of histone H1 and contain histones H3 and H4 in the acetylated form.

Up to now the methylation/de-methylation processes have been investigated predominantly in coding sequences and their flanking regions. The experiments described here were designed to monitor changes in chromatin structure of repetitive DNA sequences which result from their drug-induced hypomethylation. We analyzed the HRS60 family of DNA repeats of *Nicotiana tabacum* defined by the cloned sequences HRS60.1 (EMBL X12489), HRS60.3 (X12490), HRS60.5 (X12491) and HRS60.dim1 (X15068). HRS60 monomeric units (182–184 bp) are organized tandemly and comprise about 2% of the tobacco nuclear genome [5,6]. We have shown that these sequences are methylated in CpG dinucleotides and CpCpG trinucleotides, and that the methylation is inhibited by 5-azacytidine (AzaC) [7].

Correspondence address: M. Bezděk, Institute of Biophysics, Czechoslovak Academy of Sciences, Královopolská 135, CS-61265 Brno, Czechoslovakia. Fax: (42) (5) 744 581.

Abbreviations: AzaC, 5-azacytidine; EtBr, ethidium bromide; Ethi, DL-ethionine; MNase, micrococcal nuclease; TE, 10 mM Tris-HCl, 1 mM EDTA, pH 8.

In some experiments the chromatin structure of the R8.1 family of dispersed repetitive sequences, defined by the cloned 186 bp fragment, R8.134 (X59606), was also analyzed [8]. In this work, digestion of nuclei with micrococcal and restriction endonucleases were used to investigate nucleosome spacing and positioning, and methylation-dependent protein shielding of certain DNA sites within repetitive sequences.

2. MATERIALS AND METHODS

2.1. *Plant material and treatment with drugs*

Hypomethylation of the DNA was induced by culturing *Nicotiana tabacum* calli in the presence of either 5-AzaC or DL-ethionine (Ethi) as described previously [9]. Cell nuclei were isolated from calli according to Bedbrook [10].

2.2. *Micrococcal nuclease (MNase) digestion*

Nuclei suspended in a medium containing 0.3 M sucrose, 100 mM NaCl, 10 mM Tris-HCl (pH 7.6), 5 mM $MgCl_2$ and 1 mM $CaCl_2$, were digested with MNase (Serva), 1 U per μg DNA, for 5, 15, 45 and 90 min at 37°C. The reaction was terminated by adding 0.5 M EDTA (pH 8) to a 10 mM final concentration and chilling on ice.

2.3. *Digestions with restriction endonucleases*

Nuclei or extracted DNA were digested with 10 U of either *Sau*3AI or *Taq*I per μg of DNA for 2 h.

2.4. *Extraction of DNA and Southern blot hybridization*

After digestion with MNase or restriction endonuclease the nuclei were treated by proteinase K (50 μg/ml, 1 h at 52°C) in the presence of NaCl and SDS (1 mol/l and 0.5%, respectively). The DNA was extracted with phenol, precipitated with isopropanol, diluted in TE, and separated either on 2% agarose or on a 7% polyacrylamide gel. The gels were blotted onto Hybond N membrane and hybridized with [α-^{32}P]dCTP-labeled probes at the highest stringency.

3. RESULTS AND DISCUSSION

3.1. *MNase assay of nucleosome spacing*

Nuclei from AzaC-treated calli, which were shown to

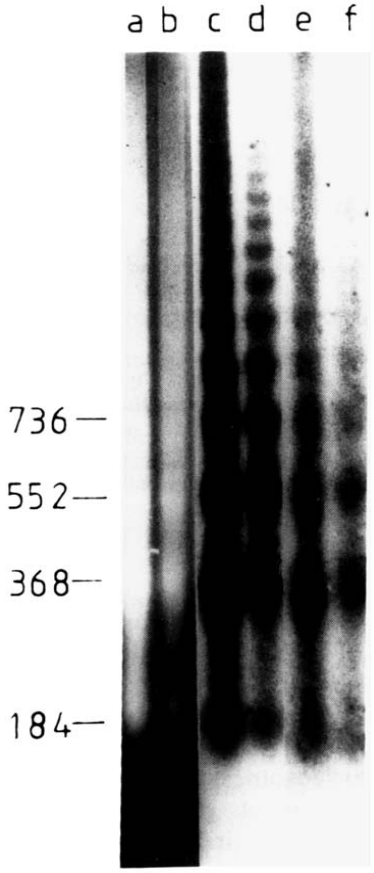

Fig. 1. MNase digestion of nuclei isolated from AzaC-treated (lanes a,c,e) and control (lanes b,d,f) calli. Couples of patterns are shown which were obtained after 45 min incubation with MNase, extraction of DNA, agarose gel electrophoresis and staining with EtBr (a,b), blotting and hybridization with the HRS60.1 probe (c,d) and rehybridization with the 8.134 probe (e,f).

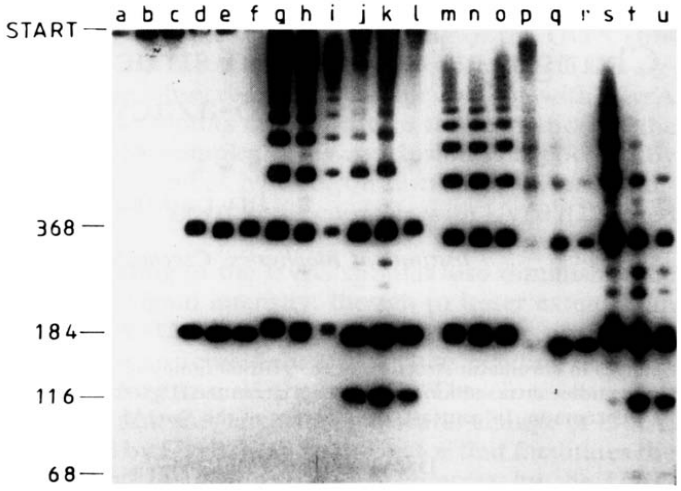

Fig. 2. Hybridization patterns of DNA restriction fragments from the control, AzaC-, and Ethi-treated tobacco tissue cultures obtained after PAGE (7% gel), blotting, and hybridization with the HRS60.1 probe: DNA extracted from undigested nuclei (a,b,c), and subsequently digested with *Taq*I (d,e,f); nuclei digested with *Sau*3AI (g,h,i) and DNA extracted from them subsequently digested with *Taq*I (j,k,l); DNA as in lanes a,b,c digested with *Sau*3AI (m,n,o); nuclei digested with *Taq*I (p,q,r) and DNA extracted from them subsequently digested with *Sau*3AI (s,t,u).

contain hypomethylated DNA [7], were first digested with MNase to analyze eventual changes in nucleosome spacing. The results showed that DNA hypomethylation did not influence the nucleosomal repeat length (180–184 bp). Fig. 1 shows a ladder of fragments corresponding to oligomers of nucleosomes in the AzaC-treated cells and in the control. The ladder visualized after staining the gel with EtBr, and the hybridization patterns obtained with HRS60 and R8.134 probes, reveal the same spacing of nucleosomes. These results are consistent with recent findings of Weih et al. [11] who showed that the nucleosomal structure of the promoter region of the tyrosine aminotransferase gene remained unchanged after de-methylation with AzaC. Normal nucleosome spacing was also observed in chromatin at CpG islands [12]. We would like to emphasize that our findings are pertinent to arrays of non-transcribed repeated sequences, expected to be located mostly in heterochromatic regions. Here we assume that chromatin at repeated sequences represents a suitable object for analyzing direct effects of DNA de-methylation independent of an intervention of DNA binding proteins involved in the control of transcription.

3.2. *Restriction endonuclease protection assay*

We expected that the cleavage of chromatin from AzaC- or Ethi-treated cells [9] with suitable restriction enzymes would allow the detection of subtle changes in nucleosome structure or in protein shielding due to

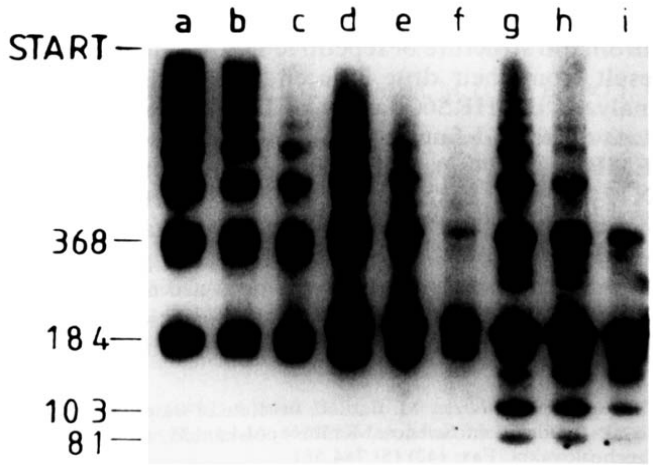

Fig. 3. Mapping of position of the nucleosomal core in HRS60 chromatin. DNA extracted from MNase-digested chromatin (5 min) of non-treated, AzaC-, and Ethi-treated cells (lanes a,b,c) were subsequently digested with *Sau*3AI (lanes d,e,f) or *Taq*I (lanes g,h,i), separated on PAGE, blotted and hybridized with the HRS60.1 probe.

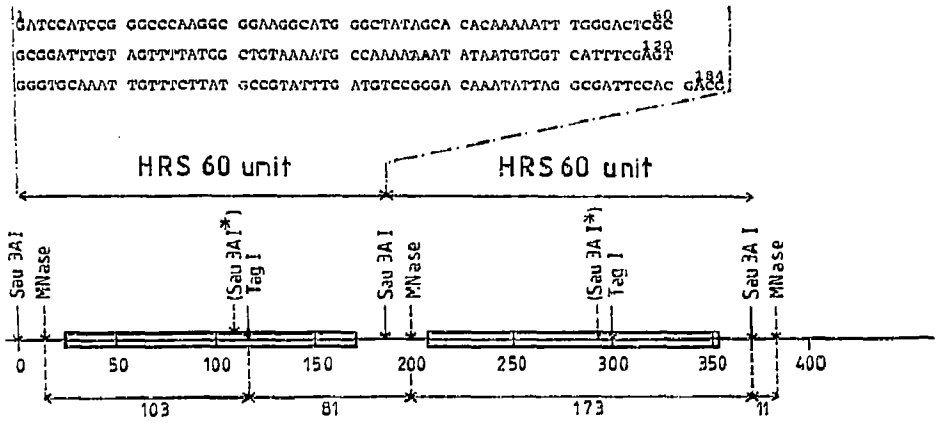

Fig. 4. Positioning of the nucleosomal core in HRS60 chromatin. Asterisk indicates the rarely occurring *Sau*3AI site. Boxes indicate regions covered with core histones.

changes in the DNA methylation status. We used *Sau*3AI and *Taq*I to analyze chromatin at DNA sequences of the family HRS60. *Sau*3AI, a non-CpG enzyme, has its cleavage site at the border of the *Bam*HI monomeric unit of HRS60 (nucleotides 1–4). *Taq*I, a CpG enzyme, cleaves the *Bam*HI monomeric unit of HRS60 near its centre (nucleotides 115–118) in the proximity of a 13 bp track AAAAAAATATAA. Fig. 2 shows an autoradiogram of a Southern blot hybridized with the HRS60.1 probe: nuclei from non-treated, AzaC-, and Ethi-treated cells were digested either with *Sau*3AI (lanes g, h, i, respectively), or *Taq*I (lanes p, q, r, respectively) in parallel. After the digestion DNA was extracted and aliquots digested with the other enzyme (lanes j, k, l and s, t, u). Free DNA was cleaved in parallel (lanes d, e, f and m, n, o). The comparison of these cleavage patterns shows that *Sau*3AI sites are equally accessible in nuclei of the control, AzaC-, and Ethi-treated cells and that the accessibility is approximately the same as in free DNA. In contrast, the accessibility of the *Taq*I recognition site was strongly limited in control nuclei (lane p) and increased significantly after treatment with AzaC or Ethi, almost to the level of free DNA (lanes q, r). If it were due to a shift of nucleosomes along the DNA molecule, the increase in accessibility for *Taq*I digestion should be accompanied by a simultaneous decrease in accessibility for *Sau*3AI.

It should also be noticed that the *Taq*I cleavage of free DNA produced, predominantly, monomers and dimers of HRS60 (lanes d, e, f), while *Sau*3AI cleaved free DNA to a ladder of oligomers (lanes m, n, o). The latter case, which is typical for tandemly repeated multicopy sequences, reflects a random accumulation of point mutations within the cleavage site. Therefore the *Taq*I site is much more conservative in the members of the HRS60 family, compared with the *Sau*3AI target sequence. This may be due to the location of the *Taq*I site within the nucleosome positioning site recognized by the $(H3–H4)_2$ tetramer [13].

3.3. *Positioning of HRS60 in nucleosome fibre*

To determine the positions of the *Sau*3AI and *Taq*I cleavage sites with respect to the nucleosome core, MNase digestion of nuclei was monitored by digesting extracted DNA with either *Sau*3AI or *Taq*I. This experiment revealed that MNase and *Sau*3AI cleavage sites coincide at HRS60 chromatin units irrespective of their methylation status. *Taq*I splits MNase fragments into 103 bp and 81 bp parts (Figs. 3, 4) as expected. To summarize, the observed resistance of methylated chromatin in standard nuclei towards *Taq*I cannot result from the protection of DNA by core histones. At least the other two proteins which bind to DNA (depending on the level of its methylation), H1 histone [14] or MeCP-1-like protein [15], might be responsible for this effect.

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