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Communication

DNA Methylation is Reduced in DNaseI-Sensitive Regions of Plant Chromatin[1]

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ABSTRACT

Pea, barley, and corn chromatin was isolated and subjected to limited digestion with DNaseI. The preferentially degraded, small molecular weight fraction, presumably consisting of sequences in an active state of expression, was isolated and its 5-methylcytosine content determined. The DNaseI-sensitive chromatin fraction from all three plant species investigated contained a markedly reduced level of DNA methylation compared to total DNA.

The DNA of higher animals and plants contains the modified base m^5C[4] in the sequence CG and, in plants, CNG (N = any base). Only a fraction of the cytosines occurring in these sequences are methylated (13), and the distribution of cytosine methylation is not random. High levels of m^5C are found in unexpressed regions of genomes, such as centromeric and heterochromatic areas of animal chromosomes (20), several types of satellite DNA (6), and inactive mammalian X-chromosomes (14). A number of studies have demonstrated that certain genes are undermethylated in tissues where they are actively expressed, but highly methylated in tissues (or different developmental stages of the same tissue) where the genes are silent (8). Thus, DNA methylation is postulated to be a determinant of gene expression.

Chromatin containing actively expressed genes (or genes that are poised for expression) is maintained in a structure that renders it more susceptible to nuclease attack, and presumably more accessible to transcription factors, than chromatin containing inactive genes (30). This nuclease-sensitive fraction of animal chromatin has been shown to have a reduced level of methylation (11, 21). In addition, the nuclease-sensitive fraction of animal chromatin is enriched in HMG-proteins and depleted in histone HI (4, 31).

Plant genomes are methylated to a far greater extent than animal genomes. Up to 40% of the cytosine residues are modified in certain plant species, compared to 4 to 6% in mammals (1). The role, however, of DNA methylation in the plant genome is not well understood. The relationship between plant DNA methylation and gene expression has been primarily examined for specific T-DNA (2, 12, 15, 23) and transposon insertions (9, 25). In all of these cases, expression has been found to correlate with reduced methylation. In addition, the methylation state of a maize seed storage protein gene was recently found to correlate with its expression at different developmental stages (7). Although a relationship between chromatin structure and gene activity has been found for a number of plant genes (17 and references therein), and HMG-like proteins have been implicated in the determination of plant chromatin structure (26), little is known about the relationship between DNA methylation and plant chromatin structure. In a study of T-DNA expression in tobacco cells, Reid *et al.* (24) found a correlation between nuclease-sensitive chromatin, gene expression, and reduced methylation of the T-DNA insertion. A similar correlation was found for pea rRNA genes (29). However, it has not always been possible to correlate patterns of DNA methylation with expression of specific genes or with nuclease accessibility of chromatin in plants (10, 22, 28).

In this study, we examined the relationship between chromatin structure and DNA methylation on a genome-wide level. We found that a DNaseI-sensitive chromatin configuration is associated with a reduced level of DNA methylation. These results suggest that chromatin around active genes is generally characterized by a reduced level of DNA methylation in plants.

MATERIALS AND METHODS

Plants

Pea *(Pisum sativum* L., var No.7 early perfection), barley *(Hordeum vulgare* L., var morex), and corn *(Zea mays* L.) were germinated for 5, 6, and 8 d, respectively, in the dark on vermiculite at 21°C with 100% humidity.

Isolation of Nuclei and Chromatin

Nuclei from pea shoots were isolated as described (24) and digested with 0.25 units of DNaseI/mL (Worthington, Free-

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[4] Abbreviation: m^5C, 5-methylcytosine.

hold, NJ) for 3 min at 25°C in 250 mM sucrose, 60 mM KCl, 10 mM NaCl, 5 mM $MgCl_2$, 20 mM Mes, pH 6.5/KOH. Because of the high endogenous nuclease content, chromatin from barley and corn shoots was isolated according to the procedure of Steinmüller and Apel (27), which yielded chromatin containing high mol wt DNA. The chromatin was digested for 3 min at 25°C with 0.4 units DNaseI/mL in 20 mM Tris/HCl (pH 7.5), 250 mM sucrose, 5 mM KCl, 5 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.1% β-mercaptoethanol. Purified DNA was digested for 3 min at 25°C in the same buffer with 0.05 units DNaseI/mL. The digests were stopped and the DNA purified as described (24).

DNA Fractionation

DNAs (200–250 μg) were size-fractionated by electrophoresis through 0.7% agarose gels in TBE buffer as described (19). After separation, the gel was sliced into five size fractions, and the DNA was electroeluted into 8 M ammonium acetate. The DNA was then extracted once with phenol, once with chloroform/isobutanol (24:1), and ethanol precipitated. The DNA was resuspended in 10 mM Tris/HCl (pH 8.0), 1 mM EDTA, and further purified by spermine precipitation as described (16).

Determination of the m^5C Levels by HPLC Analysis

DNAs (10–20 μg) were suspended in 400 μL 88% of formic acid, then hydrolyzed to individual bases by incubation at 120°C for 90 min. The bases were separated on a Beckman-Ultrasphere IP C18 column (4.6 × 150 mm) in 10 mM potassium phosphate (pH 5.6), 5 mM hexanesulfonic acid, 0.7% methanol at a flow rate of 1 mL/min. An example of the resolution achieved in this system is given in Figure 1. The column was washed with 2 mL 20% methanol in running buffer between runs. The bases were detected at 280 nm, and the $\%m^5C$ was calculated as $(m^5C/[m^5C + C]) \times 100$. The ratio of the effective molar extinction coefficients of C and m^5C under these conditions was determined by separation of equimolar C and m^5C standards and integration of the corresponding peaks. The peaks were identified by coelution with base standards (Sigma), absorbtion patterns of the different peaks at other wavelengths, and comigration with standards in other HPLC solvent systems. All hydrolyzates were analyzed a minimum of two times. The standard deviation between different runs of the same hydrolyzate was less than 0.6% m^5C.

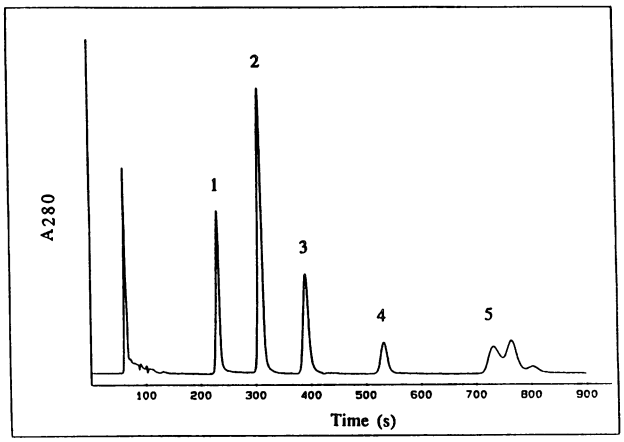

Figure 1. HPLC chromatogram of a DNA hydrolyzate from a high mol wt fraction of barley chromatin, gel-eluted and purified as described in "Materials and Methods." A full scale signal corresponds to 0.05 absorbance units at 280 nm. The DNA bases elute in the order; 1) cytosine, 2) guanine, 3) thymine, 4) 5-methylcytosine, 5) adenine, together with a partially resolved unidentified peak.

RESULTS AND DISCUSSION

Pea nuclei and chromatin from barley and corn were isolated and partially digested with DNaseI. The DNA from this chromatin was purified and then size-fractionated by agarose gel electrophoresis. Under these experimental conditions, the most nuclease-sensitive domains of the chromatin, which are presumed to represent active genes (26, 30), are recovered in the low mol wt fractions. The DNA from these fractions was purified and its m^5C level determined by HPLC analysis (Table I).

The m^5C content in all of the lowest mol wt DNA fractions of the nuclease-treated chromatin samples was markedly reduced compared to that of total DNA from each plant species (Table I). The lowest mol wt fraction from pea chromatin contained DNA with 13.2% m^5C, whereas total pea DNA has a methylation level of 26.2% m^5C. The lowest mol wt fraction from barley chromatin had a methylation level of 13.9% m^5C, compared to 25.7% in total barley DNA. The lowest mol wt fraction from corn chromatin contained 16.3% m^5C, while total corn DNA contained 27.2% m^5C. These lowest mol wt fractions represent between 2 and 4% of the total genome, as

Table I. *Methylation Levels of Different Size Fractions of DNaseI-Digested Chromatin and Protein-Free DNA of Pea, Barley, and Corn*

Plant	m^5C[a] in Total DNA	Chromatin			DNA	
		Size[b]	m^5C	of digest[c]	Size	m^5C
	%		%			%
Pea	26.2	0.20–0.54	13.2	2	0.20–0.68	24.1
		0.54–1.10	17.3	4	0.68–2.60	25.0
		1.1–2.8	21.4	9	2.6–6.0	26.4
		2.8–9.0	24.7	14		
		9.0–>30	28.0	71		
Barley	25.7	0.12–0.48	13.9	2	0.12–0.37	21.2
		0.48–1.05	16.3	3	0.37–1.50	23.4
		1.05–2.2	19.5	6	1.5–4.6	25.3
		2.2–6.7	24.1	11		
		6.7–>30	25.7	78		
Corn	27.2	0.12–0.71	16.3	4	0.12–0.58	24.1
		0.71–1.40	22.7	10	0.58–1.80	26.4
		1.4–3.7	26.0	15	1.8–5.0	28.0
		3.7–16	30.2	31		
		16–>30	31.8	39		

[a] The methylation levels are % m^5C of total C plus m^5C. [b] Size expressed as number of kilobasepairs (kb) as estimated by agarose gel electrophoresis and comparison to size standards. [c] The % DNA of the total digest in each fraction was estimated from the intensity of base absorption during HPLC runs and from separation of aliquots on ethidium-bromide stained agarose gels.

estimated from the quantity of bases detected by the HPLC analysis of each size fraction.

Although the reduction in methylation is most pronounced in the lowest mol wt fraction, the methylation levels are reduced in the three lowest mol wt DNA fractions (ranging from below 200 bp to greater than 2 kbp) that were analyzed (Table I). For the pea nuclei digest, these size classes together comprise about 15% of the total genome. This correlates well with estimates of the number of actively expressed genes in the similarly sized tobacco genome (18). The two highest mol wt fractions contained unchanged or slightly increased levels of methylation, as would be expected after selective removal of a hypomethylated fraction of the chromatin.

Our results suggest that the plant genome is organized into domains that differ in the level of DNA methylation and nuclease sensitivity. Domains of nuclease sensitivity are likely to result from the presence or absence of specific chromatin proteins in these regions (31). However, an alternative explanation of our results is that DNA methylation, or some other associated DNA modification, directly interferes with the ability of DNaseI to cleave certain regions of plant DNA. Therefore, to demonstrate that the association of reduced DNA methylation with a nuclease-sensitive chromatin conformation depends upon chromatin proteins, we purified DNA from undigested chromatin, digested this DNA with DNaseI to a similar extent as that from chromatin and nuclei samples, and analyzed the m^5C content of the resulting low mol wt fractions (Table I). Although the low mol wt fractions from the control digests of purified DNA show a slight reduction in m^5C content compared to total DNA, this reduction is considerably smaller, and extends over a smaller size range, than that found in the chromatin digests. Thus, we conclude that the correlation between nuclease-sensitive chromatin and reduced DNA methylation is protein dependent. The minor reduction in the methylation level of the most nuclease-sensitive fraction of the digests of purified DNA may be due to endogenous nucleases acting during the chromatin isolation. After random cleavage of purified DNA by DNaseI, those regions of DNA in chromatin that contained preexisting cuts from endogenous nucleases would accumulate in the small mol wt fraction. It is also possible that DNaseI cleavage is slightly inhibited by DNA methylation.

We have demonstrated an association between a nuclease-sensitive chromatin structure and a reduced level of DNA methylation in experiments with a dicotyledonous and two monocotyledonous plants, using two different chromatin isolation protocols. Thus, we believe this association will be a general feature of the genomes of higher plants that contain high levels of DNA methylation. Our results suggest that these plant genomes are organized into domains of nuclease-accessible chromatin containing reduced levels of DNA methylation and nuclease-resistant regions that are more heavily methylated. Recently, the presence of a hypomethylated fraction of plant DNA was also detected by other methods (3). In previous studies of animal and plant chromatin, increased nuclease sensitivity has been associated with expressed genes as well as with genes poised for expression (17, 26, 30).

Because reduced methylation of DNA in nuclease-sensitive regions of the genome is a property of intact chromatin, the observed correlation must be mediated by factors such as proteins bound to the DNA. Since DNA methylation can alter specific DNA/protein interactions in eukaryotic systems (5), it is possible DNA methylation patterns are involved in the organization and maintenance of active and inactive regions of plant chromatin.

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