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Large-scale methylation patterns in the nuclear genomes of plants

(DNA; isochores)

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SUMMARY

Methylation was investigated in compositional fractions of nuclear DNA preparations (50–100 kb in size) from five plants (onion, maize, rye, pea and tobacco), and was found to increase from GC-poor to GC-rich fractions. This methylation gradient showed different patterns in different plants and appears, therefore, to represent a novel, characteristic genome feature which concerns the noncoding, intergenic sequences that make up the bulk of the plant genomes investigated and mainly consist of repetitive sequences. The structural and functional implications of these results are discussed.

INTRODUCTION

Vertebrate genomes are mosaics of isochores, large (>300 kb) DNA segments that are remarkably homogeneous in base composition and belong to a small number of families characterized by different GC levels (Bernardi et al., 1985; Bernardi, 1989). The compositional range and distribution of isochores, as well as those of coding sequences (and of their different codon positions), characterize compositional patterns, which are very different for cold- and warm-blooded vertebrates; smaller differences have been observed for vertebrates belonging to either one of those two broad classes.

In the past five years we have investigated the compositional organization of plant genomes and we have found that they also are mosaics of isochores (Salinas et al., 1988; Matassi et al., 1989; Montero et al., 1990). The compositional patterns of plants were found to be very different in some Gramineae compared to the other mono- and dicotyledonous angiosperms studied.

In this work, we have investigated the correlation between DNA methylation and compositional patterns of nuclear DNAs from three monocotyledons (onion, maize and rye) and two dicotyledons (pea and tobacco). The experimental approach used was to analyze and compare the methylation levels of compositional fractions of nuclear DNA preparations 50–100 kb in size. Plant genomes are generally characterized by high methylation levels (see Adams and Burdon, 1985, for a review), methylation occurring on CpG doublets and CpNpG triplets (Gruenbaum et al., 1981).

The plant genomes investigated here were chosen because they had been previously characterized in their iso-

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Abbreviations: BAMD, bis(acetato-mercuri-methyl)dioxane; bp, base pair(s); C, deoxycytosine (unmethylated); GC, % of guanine + deoxycytosine + 5-mC; HPLC, high-performance liquid chromatography; kb, kilobase(s) or 1000 bp; mC, 5-methyldeoxycytosine; N, any nucleoside; nt, nucleotide(s); r_f, BAMD/nt molar ratio.

chore organization (Salinas et al., 1988; Matassi et al., 1989; Montero et al., 1990), and because their GC contents covered the whole GC range observed so far for angiosperms. In addition, they covered a fairly large genome size range, from 7.8 pg of DNA per haploid nucleus in tobacco to 33.5 pg in onion (Bennett and Smith, 1976). In all species investigated, we found that 5-mC levels increased from GC-poor to GC-rich DNA fractions. This methylation gradient showed different patterns in different plants and appears, therefore, to represent a novel, characteristic feature which essentially concerns the noncoding, intergenic sequences that make up the bulk of the plant genomes investigated.

The key results of this work were presented at the NATO Workshop on Genome Organization and Evolution held in Spetsai, Greece, September 15–19, 1990. The present article was part of the 'Doctorat d'Université' Thesis of G. Matassi (1992).

RESULTS AND DISCUSSION

(a) Base compositions and methylcytosine levels of the DNAs investigated

Table I displays the GC levels, the mC levels (over total bases) and the mC/mC+C ratios of DNAs extracted from different tissues of the five plants investigated in the present work. The GC levels cover a very extended range, from 35.0 to 47.7%, whereas the mC levels are all comprised in a narrow range between 6.0 and 7.5%. In fact, the methylation level differences among species are barely significant, except for the highest values which were found for tobacco. It should be noted that this lack of methylation differences is just a coincidence, since an analysis of recent data on plant methylation (concerning a total of 22 species) shows quite a variability in mC levels, from 1.1 to 9.4% (Wagner and Capesius, 1981; Leutwiler et al., 1984; Scott et al., 1984; Morrish and Vasil, 1989; Palmgren et al., 1990; and present work).

Differences among DNAs from different tissues of the same plant also were barely significant. This is at variance with the differences in DNA methylation levels reported in various tissues and cell types of *Daucus carota* (Palmgren et al., 1991).

Finally, the mC/mC+C ratios showed a relatively broad range in different plants, from 27 to 37%; this result is not surprising in view of the narrow range of mC and the wide range of GC values.

(b) Genome fractionation and methylcytosine levels of DNA fractions

Fig. 1 shows the relative amounts of DNA, and the methylation levels of the fractions obtained by centrifugation of the nuclear DNAs from the five plants investigated in Cs_2SO_4/BAMD density gradients. In all experiments, an increase of DNA methylation was observed in subsequent DNA fractions which are characterized by increasing GC levels (see section **c**). At least in three cases, however, the last fractions showed an apparently deviating behaviour. In onion DNA, fraction 12 showed an mC level remarkably

TABLE I

GC, mC and mC/mC+C, levels are listed for the different species and tissues investigated[a]

Species and tissues	GC, %	mC, %	mC/mC+C, %
Onion (*Allium cepa*)			
bulb	35.0	6.4	36.3
Pea (*Pisum sativum* cv. Alaska)			
etiolated seedlings	39.0	6.3	32.1
leaves	38.8	6.0	30.5
roots	39.2	6.6	33.2
Tobacco (*Nicotiana tabacum* cv. Xanthi XHFD8)			
etiolated seedlings	40.0	7.1	35.3
leaves	40.2	7.5	37.3
Rye (*Secale cereale*)			
etiolated seedlings	46.8	6.5	27.6
leaves	47.2	6.8	28.4
roots	47.3	6.4	26.9
Maize (*Zea mays* W64A)			
etiolated seedlings	47.5	6.7	28.1
leaves	47.2	6.8	28.6
roots	47.7	7.0	29.0

[a] Nuclear DNAs were prepared according to Jofuku and Goldberg (1988), with minor changes. Species are listed in order of increasing genome GC. Nuclear DNAs were digested to deoxyribonucleosides that were analyzed using a modification of the reverse-phase liquid chromatography (RPLC) procedure developed for the analysis of ribonucleosides by Gehrke and Kuo (1990). In this modified procedure, 1–10-μg samples of nuclear DNAs were dissolved in 100 μl of water, heated for 2 min in a boiling water bath, and cooled rapidly in ice water; 5 μl of 10 mM $ZnSO_4$ and 10 μl of nuclease P1 (Boehringer Mannheim Biochemicals, Indianapolis, IN; 200 units/ml in 30 mM Na-acetate pH 5.4) were then added, and mixtures were incubated for 16 h at 37°C 0.5 M Tris (10 μl pH 8.3) and 10 μl of bacterial alkaline phosphatase (Sigma Chemical Co, St. Louis, MO, 100 units/ml in 2.5 M ammonium sulfate) were added, and mixtures were incubated for an additional 2 h at 37°C. After centrifugation to remove suspended protein, aliquots of the hydrolysates were injected onto a Supelcosil C-18S column (150 × 4.6 mm; Supelco Chemical CO., Bellefonte, PA 16823-0048) and separated using a completely automated HPLC instrument (Hewlett Packard HP 1090 M., Avondale, PA 19311). Elution was carried out at a flow-rate of 0.1 ml per min for 0–3 min with Buffer A (2.5% methanol in 0.050 M KH_2PO_4 pH 4.5), 3–12 min with a linear gradient from Buffer A to Buffer B (20% methanol in 0.050 M KH_2PO_4 pH 4.0), 12–17 min with Buffer B, and 17–19 min with a linear gradient from Buffer B to Buffer A. Control samples of *E. coli* and calf thymus DNA were hydrolyzed and analyzed in parallel. The relative standard deviation (RSD; defined as standard deviation/mean value), was less than 0.5% for the major bases and less than 3% for mC. The mC/mC+C ratios were calculated from the experimental mC and C values. In some cases, these ratios were lower (about 1%) than the ones calculated from the GC, % column. This is due to slight deviations from base parity.

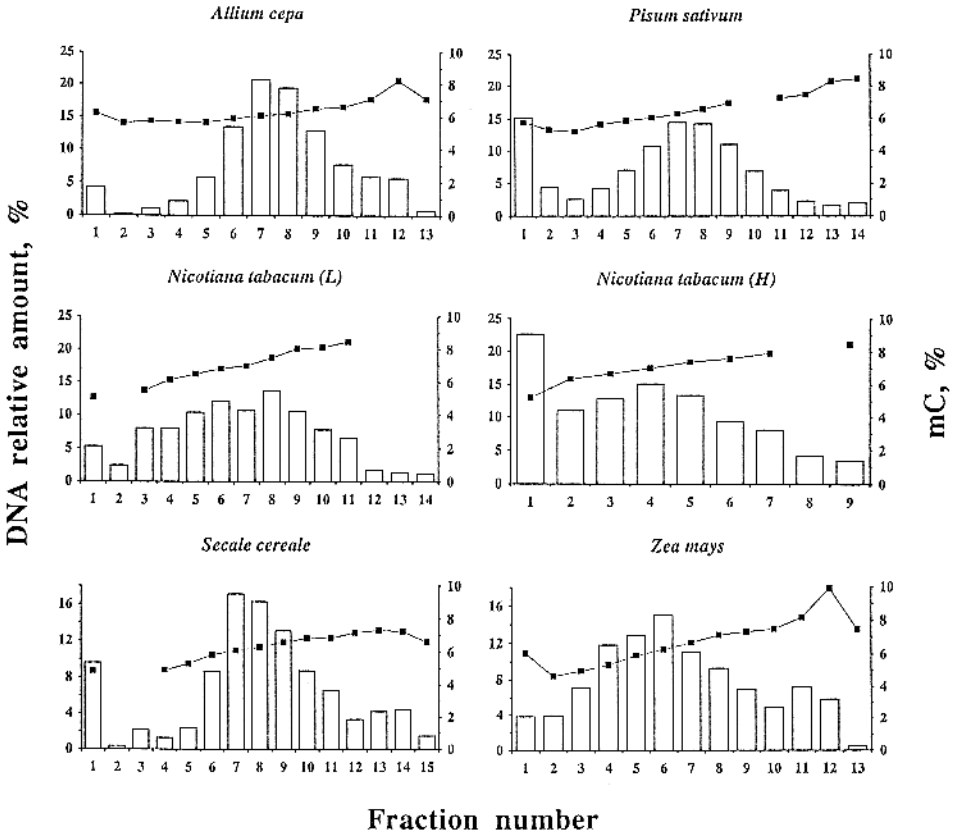

Fig. 1. Relative DNA amounts (bars) and mC levels (squares) of DNA fractions as obtained by preparative centrifugation in Cs_2SO_4/BAMD density gradients from the plants investigated in this work. Two experiments run at two different actual r_f values are shown for tobacco. Missing points correspond to samples which were not available for analysis. In most cases, pelleted DNA (Fraction 1) is characterized by slightly higher GC and methylation levels compared with the immediately following fractions. This is due to a slight contamination with DNA from the upper part of the gradient. This DNA, the last to be taken out of the centrifuge tube (the aspiration needle being close to the bottom of the tube) is the highest in both GC and methylation levels. Fractionation of DNA preparations (50–100 kb in size) by preparative centrifugation in Cs_2SO_4 density gradient in the presence of BAMD and analytical ultracentrifugation of the corresponding DNA fractions in CsCl density gradients were performed as previously described (Cortadas et al., 1977; Salinas et al., 1988). A nominal r_f value of 0.10 was used for DNAs from all plants, except for tobacco, in which case the two experiments reported were done at a nominal r_f of 0.14, but, in fact, at slightly different actual r_f values (as indicated by the different amounts of pellets). The DNAs which were fractionated were extracted from bulb (onion), etiolated seedlings (pea, maize and rye) and mature leaves (tobacco). HPLC analyses of DNA fractions were performed as described in Table I, footnote a.

higher than those of the preceding and following fractions. By examining the analytical CsCl profiles, which were obtained for each DNA fraction (not shown), and by hybridization with an appropriate probe (not shown), we detected in fraction 12 a previously described GC-rich satellite (Barnes et al., 1985) making up about 4% of total nuclear DNA, which was, in all likelihood, responsible for the higher mC content of this fraction. A similarly deviating situation was also found in fraction 13 from maize; in this case, the overmethylated fraction was followed by an undermethylated one. Finally, the last two fractions of rye showed a lower methylation level than the preceding ones.

(c) Correlations between mC and GC in DNA fractions

The relationship between methylation level and DNA base composition was studied by plotting mC vs. GC for DNA fractions (Fig. 2A). Positive linear relationship were found in all cases with correlation coefficients ranging from 0.95 to 0.99; slopes covered an approx. twofold range, the lowest and highest values being found for onion and tobacco, respectively (Table II). Differences in slopes among different plant DNAs were accompanied by differences in intercepts (see Fig. 2A) and in methylation ranges (Table II).

The relationship between methylation and GC levels was also studied by plotting the mC/mC+C ratio against the GC content of the corresponding DNA fractions (Fig. 2B). Positive linear relationships, with correlation coefficients ranging from 0.88 in rye to 0.98 in pea, were found in all cases. In all genomes investigated, a gradient of increasing mC and mC/mC+C from GC-poor to GC-rich DNA fractions was observed.

The features of the methylation gradients are reported in Table II. Onion DNA exhibited a gradient which had the

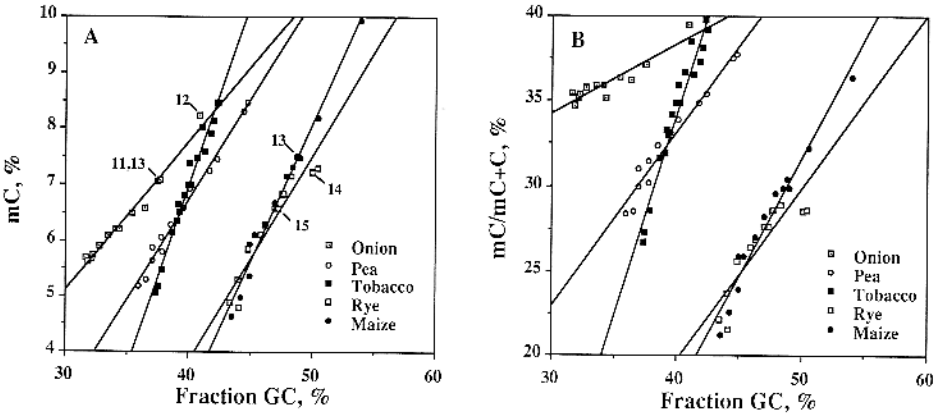

Fig. 2. Plots of mC (**A**) and mC/mC+C (**B**) against the GC levels of the corresponding Cs_2SO_4/BAMD DNA fractions In the case of tobacco, the data from the two fractionation experiments shown in Fig. 1 were pooled together. In **A**, points from onion, maize and rye which are not in the expected order are indicated. For instance, the rightmost point of onion, corresponding to fraction 12, is remarkably higher than those of the preceding and following fractions 11 and 13.

TABLE II

Methylation gradients in plant genomes

Species[a]	GC range[b]	mC vs. GC		mC/mC+C vs. GC	
		Slope[c]	mC range	Slope[d]	mC/mC+C range
Onion	31.8–40.9	0.27	5.6–8.2	0.41	34.7–39.5
Pea	35.9–44.7	0.36	5.2–8.5	1.02	28.4–37.7
Tobacco[e]	37.4–42.4	0.65	5.1–8.5	2.40	26.7–39.8
Rye	43.4–50.4	0.37	4.8–7.3	1.01	21.5–28.9
Maize	43.5–53.9	0.49	4.7–9.9	1.40	21.2–36.4

[a] Species are listed as in Table I.
[b] GC range in Cs_2SO_4-BAMD DNA fractions.
[c] Slopes were calculated from Fig. 2A.
[d] Slopes were calculated from Fig. 2B.
[e] Results were obtained by pooling together the data from the two fractionation experiments performed at lower (L) and higher (H) actual BAMD/nt molar ratio shown in Fig. 1.

lowest slope. The other methylation gradients showed increasing slopes from pea to rye and maize. The highest slope was that of tobacco.

The relative contribution of methylcytosine and unmethylated cytosine to the increase in GC content across the fractions from the preparative gradient was examined in plots of mC and C against mC+C (Fig. 3). In the case of onion, C and mC appeared to increase in parallel. In maize, the increase of C + mC from GC-poor to GC-rich DNA fractions was almost exclusively due to increments in mC. In pea and rye, the contribution of mC to the increase in total cytosines was still largely predominant. Finally, tobacco was characterized by an increase in mC and a decrease of C.

(d) The methylation gradient in plant genomes

Fig. 2,A and B establish the existence of an excellent linear correlation between methylation and GC levels of compositional fractions within all the plant genomes explored. This correlation may seem to be not surprising in view of the fact that DNA methylation takes place on CpG doublets and on CpXpG triplets and that these sequences statistically increase in frequency with increasing GC. Indeed, a modest correlation between mC or mC/mC+C and GC is found (Fig. 4) in the case of unfractionated plant DNAs, even if the *Arabidopsis* genome, which has an exceptionally low level of methylation, is included.

What is remarkable, however (Fig. 2,A and B; Table II) is that the features of the methylation gradients appear to be specific to the species examined and that such methylation gradients could certainly not be predicted within any plant genome on the basis of the data in Fig. 4. This conclusion, which is emphasized by the fact that the average methylation level of the genomes investigated here were practically the same, is strongly supported by the results shown in Fig. 3, namely by the species-specific contributions of mC to the increase in total cytosines from GC-poor to GC-rich DNA. The methylation gradient appears, therefore, to represent a novel, characteristic genome feature of plants.

To understand how this situation can have arisen, one should recall (*i*) that DNA methylation, as studied here, essentially concerns intergenic non-coding sequences, because genes represent such a low percentage of the plant genomes investigated; and (*ii*) that intergenic sequences are essentially made up of repeated sequences which differ from plant to plant. It is then conceivable that methylation may have different levels and different gradients in different plants according to the frequency of sites which are methylated in the interspersed repeats. This point can be understood by considering the methylation levels exhibited by satellite DNAs, in which case the frequency of short sequences strikingly changes from case to case. In satellite

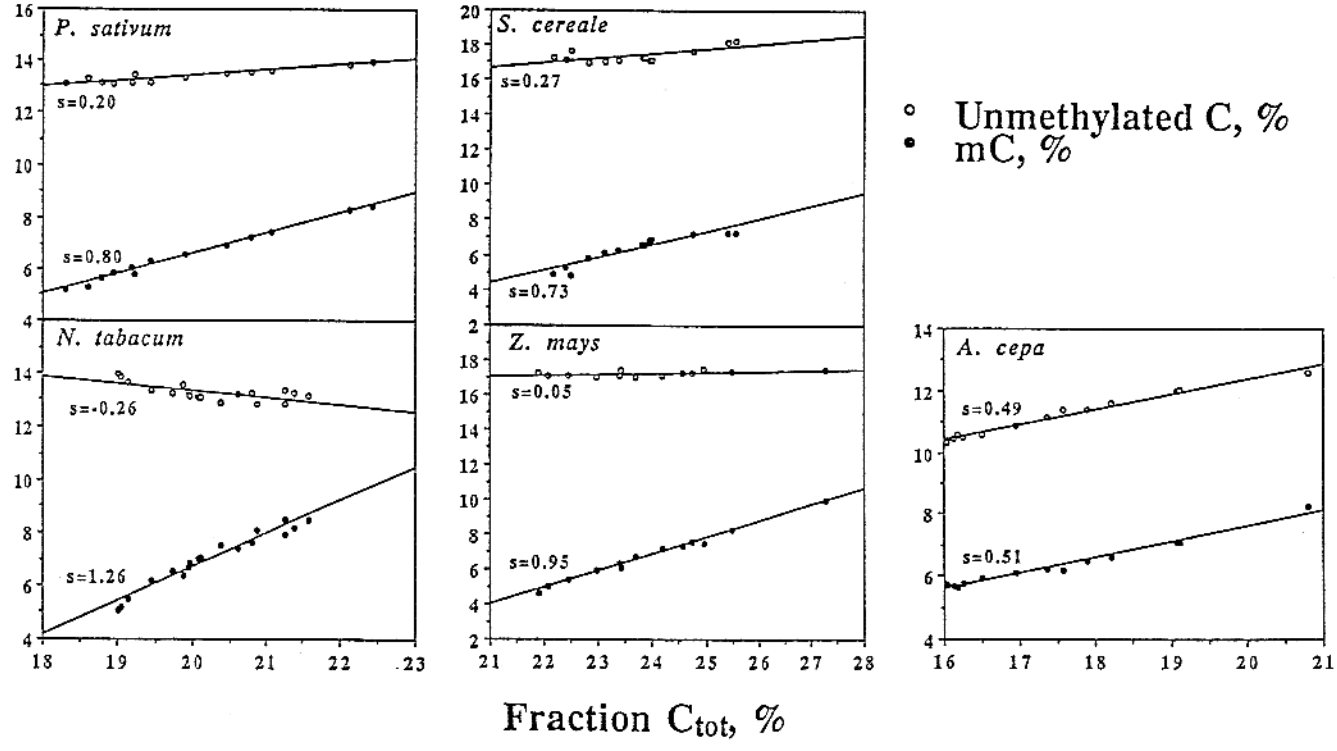

Fig. 3. Plots of mC and C against mC+C of the corresponding Cs_2SO_4/BAMD DNA fractions from five plants.

DNAs, methylation levels may be equal to those of non-satellite sequences exhibiting the same GC levels, but may also be lower or higher. These situations are exemplified by the variable methylation levels of the last fractions of Fig. 1, which largely correspond to satellite DNAs (Montero et al., 1990; Matassi et al., 1991).

An example of differences in methylation gradients is given by the comparison of the pea and tobacco genomes. These genomes have similar average GC levels, similar compositional (GC) ranges, similar average methylation levels, and yet their methylation gradients are characterized by significantly different slopes (Table II). In fact, even if the slopes in Fig. 2,A and B were identical among the species analyzed, the observed shift along the abscissa would have been sufficient to support the species-specificity of the methylation gradient. For instance, a relative methylation level (mC/mC+C) of 35% (Fig. 3) is found in DNA fragments having a GC content of about 32% in onion, 39% in tobacco, 41% in pea and 54% in maize. Conversely, a DNA fragment of a given GC content, say 43% (Fig. 2B), will show different methylation levels, for example in tobacco and maize. Incidentally, this DNA fragment will be located in the GC-richest fraction of the tobacco genome, most likely made up of satellite DNA sequences

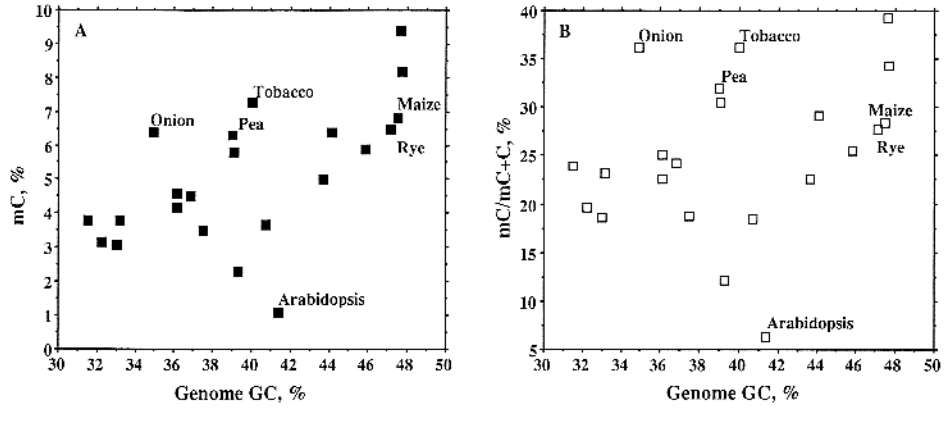

Fig. 4. Plot of mC (**A**) and mC/mC+C (**B**) against the GC levels of plant DNAs. Plants investigated in this work are indicated as well as *Arabidopsis*. Data are from: Wagner and Capesius (1981) Leutwiler et al. (1984) Scott et al. (1984) Morrish and Vasil (1989) Palmgren et al. (1990) and present work. Correlation coefficients were 0.61 ($p = 0.0028$) and 0.32 ($p = 0.1485$) in plots **A** and **B**, respectively.

(see above), and in a GC-poor one of the maize genome, where coding sequences are present. Even more striking would have been the case of *Arabidopsis*. This genome, while having an overall GC content of 41.4% (Leutwiler et al., 1984), similar to those of pea and tobacco (Table I), has a much lower methylation level (1.1% mC; Leutwiler et al., 1984). Its methylation gradient would, therefore, be shifted in any case towards very low ordinate values in the plots of Fig. 2,A and B. Thus, the methylation level of isochores does not merely depend on their GC levels but rather on the sequences making up the bulk of isochores.

(e) Conclusions; the structural and functional implications of methylation gradients

Several comments may be made here as far as the biological meaning of the methylation gradients in plant genomes is concerned.

(*1*) The results reported in the present work concern overall methylation levels, as seen at a DNA size level (50–100 kb) which was not explored so far, and essentially apply to the vast expanses of intergenic sequences (see also next paragraph). There is no contradiction, therefore, between the present results and previous studies (Klaas and Amasino, 1989) which showed the existence of undermethylated DNA segments in DNaseI-sensitive regions (0.1–1.0 kb in size) of pea, barley and corn chromatin , which presumably consist of DNA sequences in an active state of expression. Unmethylated CpG islands have been described to be associated with genes in higher plant DNAs (Antequera and Bird, 1988); in maize, such islands (which were detected because of their richness in unmethylated *Hpa*II and *Hin*pI sites in 0.1–0.5 kb fragments) represent about 2.5% of the genome (Antequera and Bird, 1988).

(*2*) DNA methylation, as already mentioned, essentially concerns intergenic sequences. For this reason, the unmethylated or undermethylated sequences comprising genes and their immediate flanking sequences, including CpG islands (see above), stand out of chromosomal regions characterized by a higher methylation level. This level is obviously different for genes located in high GC and highly methylated regions and for genes located in GC-poor regions which have a lower methylation. This difference in the genomic methylation context of different genes (as well as that in their compositional context) may conceivably have a functional relevance.

(*3*) GC level is a factor which stabilizes DNA not only in dilute solution, but also in chromosomes, as indicated by the fact that the most denaturation-resistant regions of human metaphase chromosomes are the T-bands (Dutrillaux, 1973), and the coincident chromomycin A3-positive, DAPI-negative bands (Ambros and Sumner, 1987), which correspond to the highest GC levels of the genome (Gardiner et al., 1990; De Sario et al., 1991; Saccone et al., 1992). It is, therefore, conceivable that DNA methylation, which also stabilizes DNA in solution, may do so, too, in chromosomes. If such is the case, the correlation between methylation and GC level would indicate that two stabilizing factors increase in concert with each other. Since genome stability in vertebrates is achieved by GC increases only, this would also indicate that the much higher methylation of plant DNAs compared to vertebrate DNAs is an additional stabilizing factor which might be required by plant genomes.

On May 4, 1992, this paper was submitted for publication in *Nucleic Acids Research*, which rejected it on July 27, 1992. In the meantime, *Nucleic Acids Research* published another paper on the same subject (Montero et al., 1992).

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