

# Higher Order Chromatin Structures in Maize and Arabidopsis

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We are investigating the nature of plant genome domain organization by using DNase I- and topoisomerase II-mediated cleavage to produce domains reflecting higher order chromatin structures. Limited digestion of nuclei with DNase I results in the conversion of the >800 kb genomic DNA to an accumulation of fragments that represents a collection of individual domains of the genome created by preferential cleavage at super-hypersensitive regions. The median size of these fragments is ~45 kb in maize and ~25 kb in Arabidopsis. Hybridization analyses with specific gene probes revealed that individual genes occupy discrete domains within the distribution created by DNase I. The maize alcohol dehydrogenase *Adh1* gene occupies a domain of 90 kb, and the maize general regulatory factor *GRF1* gene occupies a domain of 100 kb in length. Arabidopsis *Adh* was found within two distinct domains of 8.3 and 6.1 kb, whereas an Arabidopsis *GRF* gene occupies a single domain of 27 kb. The domains created by topoisomerase II-mediated cleavage are identical in size to those created by DNase I. These results imply that the genome is not packaged by means of a random gathering of the genome into domains of indiscriminate length but rather that the genome is gathered into specific domains and that a gene consistently occupies a discrete physical section of the genome. Our proposed model is that these large organizational domains represent the fundamental structural loop domains created by attachment of chromatin to the nuclear matrix at loop basements. These loop domains may be distinct from the domains created by the matrix attachment regions that typically flank smaller, often functionally distinct sections of the genome.

## INTRODUCTION

The genome of eukaryotes is thought to be organized into topologically independent, supercoiled loop domains of chromatin anchored by attachments to a protein matrix in the nucleus (Cook and Brazell, 1976; Paulson and Laemmli, 1977; Mirkovitch et al., 1986; Loc and Stratling, 1988; Jackson et al., 1990; von Kries et al., 1991; Freeman and Garrard, 1992; Zlatanova and van Holde, 1992; Luderus et al., 1994; Davie, 1995; Jupe et al., 1995; Razin and Gromova, 1995). The sites where the chromatin fiber is attached are referred to as matrix attachment regions (MARs) or scaffold attachment regions (SARs), although this latter term now tends to be reserved for attachments within the protein scaffold of individual mitotic chromosomes (Gasser et al., 1989; Laemmli et al., 1992). MARs often coincide with chromatin features associated with gene function, such as nuclease hypersensitive sites, non-B-DNA structures, origins of replication, and gene regulatory elements (Villeponteau et al., 1984; Cockerill and Garrard, 1986; Rowe et al., 1986; Bustos et al., 1989; Jackson et al., 1990; Bode et al., 1992; Avramova and Bennetzen, 1993; Paul and Ferl, 1993; Targa et al., 1994). In addition, transgenic analyses with both animals and plants have demonstrated that MARs can contribute to the normal-

ization of transgene expression, further suggesting that MARs play a functional role in creating or maintaining transcriptional domains in vivo (Stief et al., 1989; Slatter et al., 1991; Allen et al., 1993, 1996; Breyne et al., 1994; Phi-Van and Stratling, 1996).

The term "matrix association/attachment region" or MAR was coined to refer to the chromosomal loop attachments characterized in interphase nuclei (Cockerill and Garrard, 1986a; Blasquez et al., 1989). Although the class of sequences through which the attachment is made appears to be evolutionarily conserved, no clear consensus sequence can be defined for an entire MAR other than a propensity for AT richness and a few conserved motifs embedded in the region (such as topoisomerase II sites). There were early concerns that such attachments were artifactual in nature. Jackson et al. (1990) addressed some of the discrepancies among preparation techniques. However, among their findings was an indication that loops prepared under "physiological" conditions from HeLa cells (which averaged 86 kb in length) remained a constant structural feature throughout the cell cycle.

Although it is widely accepted that the eukaryotic genome is organized into looped domains that are anchored by attachment to the nuclear matrix, there is not yet a consensus regarding the frequency of MAR distribution in the genome and the in vivo role of MARs in genome organization. This

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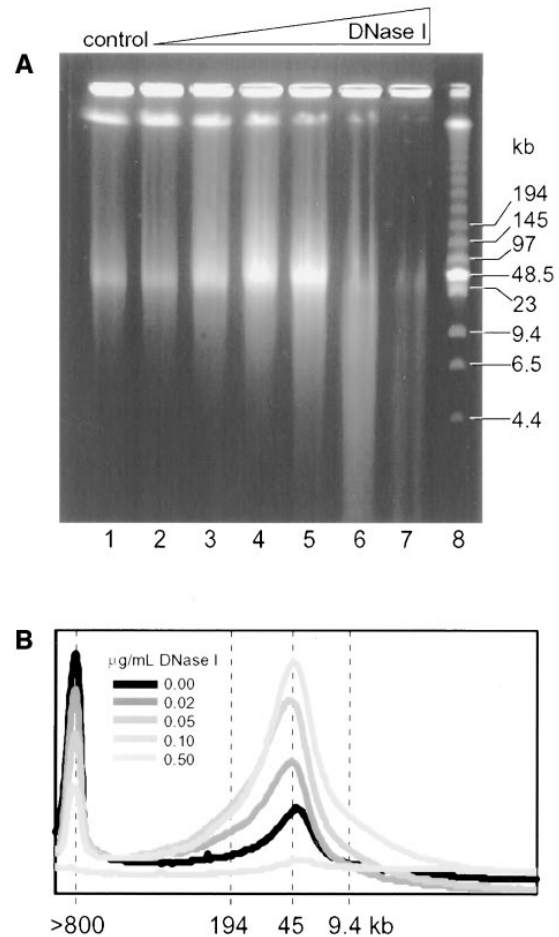
contraposition is partly due to the fact that the type of MAR isolation method used for *in vitro* assays may influence the estimated number, placement, and type of MAR identified for a given system (e.g., Jackson et al., 1990). The two most commonly used methods for preparing nuclear matrices employ either a high-salt extraction (Cockerill and Garrard, 1986b) or a detergent extraction (Mirkovitch et al., 1984) procedure to remove histones and soluble nuclear components. Razin and Gromova (1995) reviewed current models in relation to the many problems relevant to elucidating the nature of MARs; however, questions about whether any *in vitro* method of matrix characterization actually reflects the organization of the genome *in vivo* remain unanswered. For this reason, we have avoided matrix preparations and applied an approach that is most likely to reflect the configuration of the nucleus *in vivo*. Our approach is modeled after Gromova et al. (1995a) and incorporates *in situ* treatments of nuclei and *in vivo* treatments of cells to release domains, with direct transfer and hybridization of specific single-copy genes. It has been demonstrated in several animal systems that a chromatin loop basement can be cleaved by nucleases (Filipski et al., 1990; Targa et al., 1994; Gromova et al., 1995a; Lagarkova et al., 1995b) and through the action of cytotoxic drugs that interact with the topoisomerase II component of the nuclear matrix (Cockerill and Garrard, 1986b; Rowe et al., 1986; Kas and Laemmli, 1992; Razin et al., 1993; Gromova et al., 1995b; Iarovaia et al., 1996). However, information about the organization of plant genomes at this scale is extremely limited (Espinosa and Carballo, 1993). In the experiments presented in this study, we use DNase I and the cytotoxic drugs VM26 and genistein to address the organization of the genomes of maize and *Arabidopsis*.

## RESULTS

Figure 1 shows an ethidium bromide-stained contour-clamped homogeneous electric field (CHEF) gel of limited DNase I digestion of maize nuclei. The pulsed field electrophoresis parameters for the CHEF gel shown in Figure 1A created a region of compression that migrates behind the 800-kb limit of the size markers (the "parent" genomic DNA band; this band represents the intact, unfragmented genome that is >800 kb in length) and allowed for resolution of fragments ranging from ~400 to 5 kb. Increasing the concentration of DNase I converted the parent band into an accumulation of a specific set of subgenomic fragments. It can be seen from the stained gel that the parent band was completely converted to a set of fragments that have a median size of 45 kb, without first creating a larger sized set at lower DNase I concentrations and without creating smaller sets of fragments at higher concentrations of DNase I.

Figure 1B shows a series of densitometric scans of selected lanes from the gel in Figure 1A. The black line is the 0.0  $\mu\text{g/mL}$  DNase I control. The gray lines illustrate how the

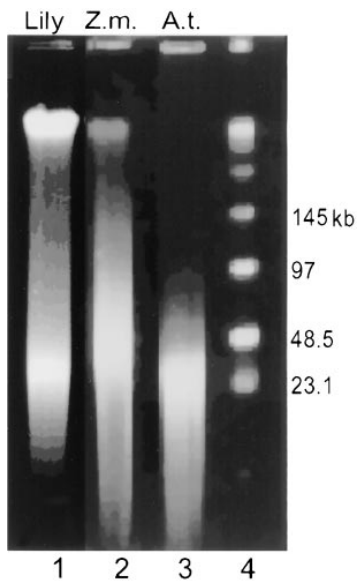
parent band is converted to the domain-sized fragments with an increase in DNase I concentration. Note that the peak, centered at ~45 kb, increases in proportion to the decrease of the peak centered over the compressed parent band at >800 kb, and no other peaks are formed. The control lanes in Figure 1A (the first two lanes are the untreated control and the 0.0  $\mu\text{g/mL}$  DNase I control, respectively) and



**Figure 1.** Limited DNase I Digestion of Maize Nuclei Converts the Genome into a Set of Fragments with a Median Length of 45 kb.

**(A)** Maize nuclei incubated with increasing concentrations of DNase I. Lane 1 is the control, no treatment; lanes 2 to 7 contain 0.0, 0.02, 0.05, 0.1, 0.2, and 0.5  $\mu\text{g/mL}$  DNase I, respectively. Lane 8 contains molecular markers labeled in kilobases.

**(B)** Densitometric scans of selected lanes from **(A)**. The black line is the DNase I control. The gray lines illustrate how the parent band is converted to the domain-sized fragments with an increase in DNase I concentration: increasingly lighter gray indicates increasing concentrations of DNase I. The legend denotes shades for concentrations of 0.02, 0.05, 0.1, and 0.5  $\mu\text{g/mL}$  DNase I. The positions of key molecular length markers (dotted lines) are indicated beneath the graph.



**Figure 2.** The Median Size of Chromatin Domains Varies among Three Species of Plants.

Lane 1, lily; lane 2, maize (Z.m.); lane 3, Arabidopsis (A.t.); and lane 4, molecular markers. Each lane was digested with 0.01  $\mu\text{g}/\text{mL}$  DNase I. CHEF gel running parameters are designed to expand the resolution in the 5- to 100-kb range.

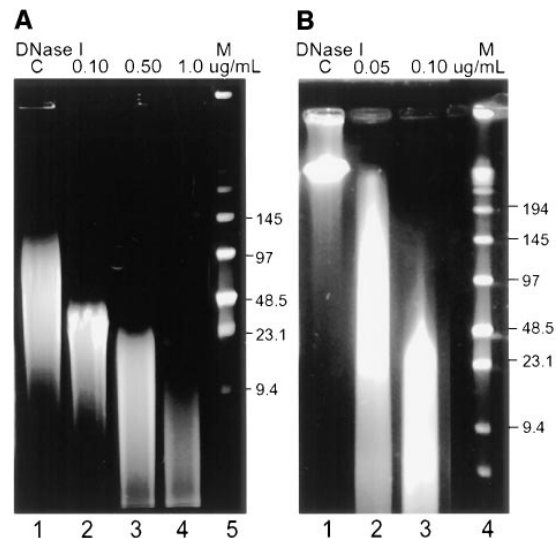
the black line in Figure 1B (0.0  $\mu\text{g}/\text{mL}$  DNase I) indicate the levels of endogenous nuclease activity in the nuclei. The faint bands produced by endogenous nucleases correspond in size with the bands generated by exogenous DNase I in the subsequent lanes.

The size distribution of domains among plant species is variable. However, a comparison of three species with widely different genome sizes indicates that genome size is not directly correlated with a median domain size. Figure 2 shows the domains created by DNase I digestion in lily, maize, and Arabidopsis by using CHEF gel running parameters designed to expand the resolution in the 10- to 100-kb range. Lily, with the largest genome size ( $\sim 10^{11}$  bp), shows a median domain size of 35 kb, whereas maize ( $10^9$  bp) has a median domain size of 45 kb. The median domain size for Arabidopsis is almost half that of maize at 25 kb, but the genome size ( $10^7$  bp) is close to 100 times smaller than that of maize. These apparent domain size differences also indirectly suggest that the accumulation of fragments at approximately a median size is not a CHEF gel artifact, because each plant generates a different size class distribution.

The digestion of naked (protein-free) DNA with DNase I and subsequent resolution of the digestion products on a CHEF gel also address the possibility of gel artifacts as well as whether the accumulation of fragments is mediated by chromatin. These data are shown in Figure 3. It is common

practice to generate naked DNA controls for in vivo and chromatin analyses (e.g., Paul et al., 1987); however, two obstacles exist for conducting naked DNA control digests for these experiments. First, conventional methods fragment genomic DNA, making intact DNA of megabase size essentially impossible to isolate. Second, although it is possible to prepare agarose-embedded high molecular weight genomic DNA (deproteinated embedded nuclei), some structural features of the genome that are imposed by nuclear proteins (e.g., bends, kinks, and nicks) may be retained and may possibly continue to influence the effects of nucleases on sensitive areas of the genome.

These problems were addressed by two different naked DNA digestions shown in Figure 3. For Arabidopsis, conventional bulk genomic DNA preparations yield DNA fragments greater than the 25-kb median domain generated with nuclear DNase I digestion. Direct DNase I digestion of purified Arabidopsis DNA with DNase I failed to show the accumulation of any specific size class of fragments (Figure 3A). The first lane in Figure 3A illustrates the degree to which genomic DNA is fragmented by a conventional isolation technique (CsCl density gradients; e.g., Paul and Ferl, 1991). The



**Figure 3.** Naked DNA Shows a Different DNase I Digestion Pattern than Do Nuclear Digests.

(A) The first lane (C) illustrates that genomic DNA prepared by direct lysis and CsCl density gradients is mechanically fragmented to lengths  $< 100$  kb. Lanes 2 to 4 contain 3  $\mu\text{g}$  of the control DNA incubated with 0.1, 0.5, and 1.0  $\mu\text{g}/\text{mL}$  DNase I, respectively. Lane 5 contains molecular length markers indicated in kilobases.

(B) The control lane (C) was derived from an excised parent-genomic DNA band resolved on a preparative CHEF gel of untreated nuclei (see Methods). Lanes 2 and 3 show the results of digesting a similar plug containing the parent-genomic DNA band with 0.05 and 0.1  $\mu\text{g}/\text{mL}$  DNase I, respectively. Lane 4 contains molecular length markers indicated in kilobases.

subsequent lanes show the effects of increasing concentrations of DNase I on the DNA represented in the control lane. The median lengths of fragments resulting from increasing concentrations of DNase I do not re-create the plateau effect seen in DNase digestions in nuclei (refer to Figure 1); rather, a gradual progression of size products proceed from the 50-kb median of undigested DNA through median lengths of ~30, 15, and 5 kb as the DNA is digested in vitro.

For maize, bulk genomic DNA preparations (such as the CsCl gradients used for the Arabidopsis DNA in Figure 3A) already are fragmented to a size range close to that of the nuclear domain size, so instead of using bulk DNA preparations, naked DNA digestions were performed on the parent-genomic band from untreated nuclei run on preparative CHEF gels. In this case, the control represents an agarose plug containing the parent-genomic DNA band excised from a preparative CHEF gel of untreated nuclei (see Methods) and rerun on the CHEF gel shown in Figure 3B. Lanes 2 and 3 in Figure 3B show the results of digesting a similar plug containing the parent genomic DNA band with 0.05 and 0.10  $\mu\text{g}/\text{mL}$  DNase I. Digestion of embedded naked DNA results in a more general smear of fragments than is seen with DNase I digestions of nuclei.

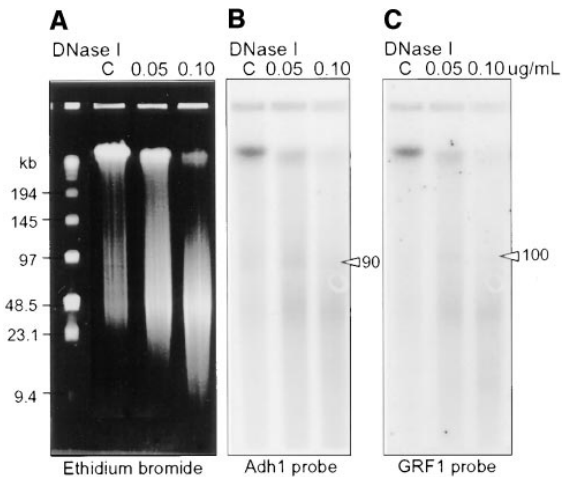
Hybridization with gene-specific probes from maize and Arabidopsis indicates that genes occupy discrete domains of defined length. The ethidium bromide-stained CHEF gel in Figure 4A shows the molecular length markers next to the lanes used for the subsequent hybridizations. Note that the CHEF gel running parameters for Figure 4A, and all subsequent CHEF gel figures, are such that the resolution between 5 and 100 kb is expanded significantly compared with the parameters used in the CHEF gels shown in Figure 1. The median domain fragment size is still 45 kb for maize, but these running parameters also illustrate that although most fragments ranged close to the median size, many were much larger or smaller than the median. The sizes of individual domains can be visualized by hybridizing with specific genes. Maize *alcohol dehydrogenase (Adh1)* was used as a probe in the blot shown in Figure 4B and hybridized with a 90-kb fragment. Stripping this blot and reprobing with the probe for maize *general regulatory factor 1 (GRF1)* (Figure 4C) illustrates that this gene resides on a 100-kb domain—a domain clearly distinct from the fragment occupied by *Adh1*. The size of individual gene domains is also apparent in Arabidopsis. Figure 5A shows the ethidium bromide-stained CHEF gel of domain fragments from the Arabidopsis genome used for the hybridization analyses. The Arabidopsis *Adh* gene was used as a probe in the blot shown in Figure 5B and shows hybridization with two bands—one of 8.3 kb and one of 6.1 kb. Reprobing this blot with the Arabidopsis *GRF4* gene shows that this gene resides on a domain fragment of 27 kb (Figure 5C).

Domains liberated by topoisomerase II-mediated cleavage correlated in length with domains liberated by DNase I. The cytotoxic drugs VM26 and genistein create cleavable complexes at topoisomerase II sites, but the creation of the

cleavable complex is an inefficient process. Nonetheless, drug-released domains were observed when VM26 and genistein were incubated with Arabidopsis protoplasts, and the resulting DNA was analyzed (Figure 6). The blot shown in Figure 6 was hybridized with Arabidopsis *Adh* and shows evidence of drug-dependent cleavage. VM26 and genistein both produced the same 8.3- and 6.1-kb bands that were generated by the DNase I treatments shown in Figure 5B.

Figure 7 demonstrates that the hybridizing bands illustrated in Figures 5 and 6 are the result of chromatin-associated effects in the genome. Arabidopsis genomic DNA (CsCl gradient purified) was digested with increasing concentrations of DNase I or VM26, resolved by using conventional electrophoresis (Figure 7A) and CHEF analysis (Figure 7B), transferred to nylon membranes, and hybridized with the same *Adh* probe used in Figures 5 and 6. No specific hybridizing bands were detected in naked genomic DNA after in vitro digestion with DNase I or treatment with VM26.

The hybridization data of Figures 4 and 5 are summarized in Figures 8A and 8B. Figures 8C and 8D address the presence of a wide, hybridizing band averaging 45 kb for maize *Adh1* and *GRF1* and a similar smear centered at ~25 kb in the blots with Arabidopsis DNA. Densitometric scans of hybridized lanes were compared with a lane from the ethidium

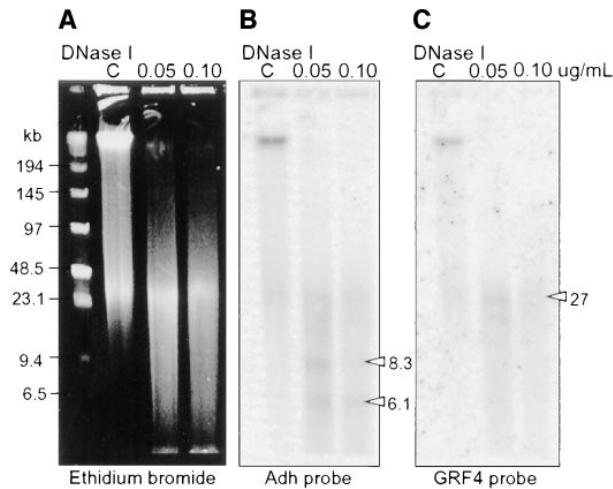


**Figure 4.** Maize *Adh1* and *GRF1* Genes Are Contained on Discrete Domains in the Genome.

CHEF gels transferred to nylon membranes and hybridized with maize *Adh1* created distinctly hybridizing bands.

(A) The ethidium bromide-stained CHEF gel before transfer is shown with molecular markers.

(B) and (C) The hybridized membranes show that *Adh1* occupies a 90-kb domain (B) and that *GRF1* occupies a 100-kb domain (C). The gene-specific bands are indicated with arrowheads. The broad band at 45 kb that hybridizes with both probes likely reflects nonspecific binding to the concentration of genomic DNA that migrates to this position in CHEF gels (see Figure 8).



**Figure 5.** The Arabidopsis *Adh* Gene and the *GRF4* Gene Are Contained on Discrete Domains in the Genome.

(A) The ethidium bromide-stained CHEF gel before transfer is shown with molecular markers.

(B) and (C) The hybridized membranes show that Arabidopsis *Adh* hybridizes with two domains, one of 8.3 kb and one of 6.1 kb (B), whereas *GRF4* occupies a single 27-kb domain (C). The gene-specific bands are indicated with arrowheads. Nonspecific hybridization is less of a problem with Arabidopsis, so the band corresponding to the accumulation of domain-sized fragments at  $\sim 25$  kb is a minor feature of these blots.

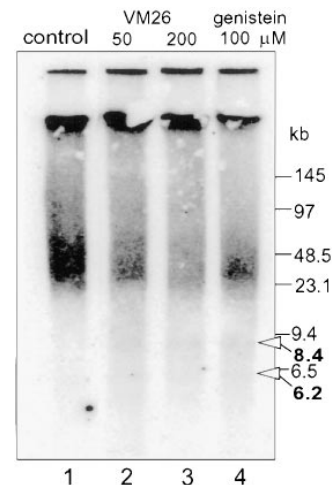
bromide-stained CHEF gel. Figure 8A shows the data for maize. The black line represents the densitometric scan of the bottom (ethidium bromide-stained) lane, the dark gray line represents the middle *Adh1* lane, and the light gray line represents the top *GRF1* lane. Note that each scan has a similar peak profile centered at  $\sim 45$  kb, suggesting that nonspecific hybridization contributes to this peak. Figure 8B shows similar scans of the data for Arabidopsis. The black line represents the ethidium-stained lane on the bottom, the dark gray line represents the middle *Adh* lane, and the light gray line represents the top *GRF4* lane. Again, the broad peak centered at  $\sim 25$  kb is seen in all scans, whereas gene-specific bands rise above this profile. However, for Arabidopsis, the gene-specific bands rise more fully above the background hybridization than is the case for maize.

An additional experiment was conducted to approximate the relative contribution of nonspecific hybridization to this broad band. Figures 8C and 8D compare the amount of background hybridization between the domain analysis of maize *Adh1* and a simple restriction digest of embedded maize nuclei, respectively. Both blots were probed with a section of the *Adh1* promoter. In Figure 8C, the region under the curve defining the hybridizing region of the scan profile was integrated (dark gray plus light gray) and divided into

the integrated value of the area under the specific peaks (light gray). This value was compared with a similar calculation conducted on BamHI-digested DNA that was resolved on a CHEF gel (Figure 8D). The two hybridizing BamHI fragments can be seen to rise above the background in a fashion similar to the DNase I-liberated hybridizing fragments. In the case of the restricted DNA, all of the background signal can be attributed to nonspecific hybridization to random, probably repetitive sequences in the genome. A comparison of the signal-to-background ratios for the fragments created by DNase I (Figure 8C) and the BamHI fragments (Figure 8D) suggests that at least 40% of the broad-band signal seen with the DNase I experiments can be attributed to nonspecific hybridization.

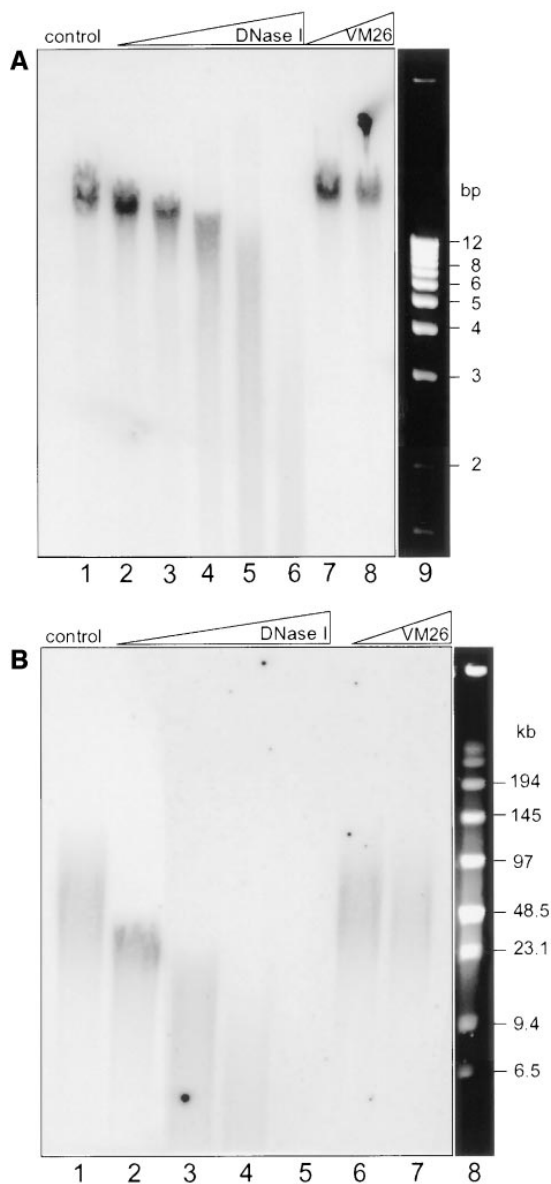
## DISCUSSION

The data presented here indicate that limited DNase I treatment of nuclei releases discrete, defined regions of chromatin that correlate with domains released by topoisomerase II poisons. This observation is consistent with observations from animal systems and with a model wherein domain release is due to super DNase I hypersensitivity at loop base-ment attachments. Extrapolation of this model suggests that



**Figure 6.** Topoisomerase II-Mediated Cleavage in Arabidopsis Produces Domains of the Same Size as Created by DNase I.

Lane 1 is the control; lane 2, 50  $\mu$ M VM26; lane 3, 200  $\mu$ M VM26; and lane 4, 100  $\mu$ M genistein. The positions of molecular markers are shown at right. The faint, drug-dependent bands showing hybridization to Arabidopsis *Adh* are indicated by arrowheads at 8.4 and 6.2 kb.



**Figure 7.** DNase I Digests and VM26 Treatments of Naked DNA Do Not Release Any Specific Domains to *Arabidopsis Adh*.

**(A)** Standard electrophoresis of *Arabidopsis* genomic DNA (purified on CsCl gradients) that was digested with increasing concentrations of DNase I or VM26 and hybridized with the *Adh* probe after transfer to a nylon membrane. Lane 1 is the undigested genomic DNA control. DNA in lanes 2 to 6 was treated with 0.1, 0.2, 0.5, 1.0, and 2.0  $\mu\text{g}/\text{mL}$  DNase I, respectively. Lanes 7 and 8 show DNA treated with 50 and 200 mM VM26, respectively. The corresponding molecular length markers from the agarose gel are shown in lane 9. No specific bands were detected.

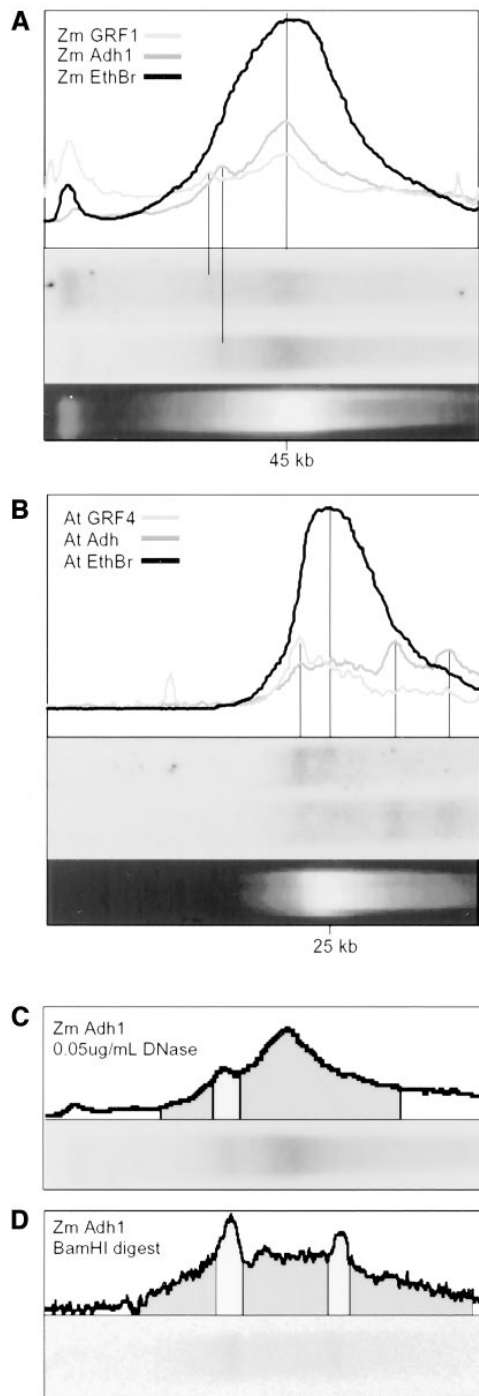
**(B)** CHEF analysis of some of the DNAs shown in **(A)**. Lane 1 is the control. DNA in lanes 2 to 5 was treated with 0.1, 0.5, 1.0, and 2.0  $\mu\text{g}/\text{mL}$  DNase I, respectively. DNA in lanes 6 and 7 was treated with 50 and 200 mM VM26, respectively. The corresponding molecular

length markers from the agarose gel are shown in lane 8. Again, no specific bands were detected in any of the naked DNA treatments.

plant genomes are organized into looped domains much like animal genomes. Moreover, these results show that specific genes can be localized to specific loop-sized fragments, suggesting that the organization imposed on the genome to facilitate condensation and packaging is not random. The idea of small-scale domains created by attachments to the matrix (in which specific MARs play a role in defining the functional domain of a gene) is well documented (e.g., Cockerill and Garrard, 1986a, 1986b; Reitman and Felsenfeld, 1990; Breyne et al., 1994; Chinn and Comai, 1996; Phi-Van and Stratling, 1996; Van Drunen et al., 1997). The extension of this idea to include the larger organizational domains that play a role in the global packaging of the genome has yet to be widely explored in other systems.

Much of the evidence contributing to the hypothesis that the eukaryotic genome is organized into looped domains is derived from experiments designed to create scissions at putative loop basements; the premise is that supercoiled structures (such as those envisioned for the loops) are torsionally stressed and contain sections of DNA that are extremely hypersensitive to enzymatic reagents, such as DNase I, S1 nuclease, and Bal 31 nuclease, in the sequences associated with the attachment region. However, the relationship between loop basements and MARs has yet to be established. Many of the MARs that define topologically discrete sections in the genome contain topoisomerase II recognition motifs and subsequently are vulnerable to the actions of cytotoxic drugs (such as VM26) that generate a cleavable complex with topoisomerase II (Liu et al., 1983; Chen et al., 1984) in addition to being hypersensitive to DNase I (e.g., Cockerill and Garrard, 1986b; Rowe et al., 1986; Kas and Laemmli, 1992; Razin et al., 1993; Gromova et al., 1995b; Iarovaia et al., 1996). Razin and co-workers have used several approaches to define chromatin domain loops (Targa et al., 1994; Gromova et al., 1995a, 1995b; Lagarkova et al., 1995b; Iarovaia et al., 1996). In a review of the data addressing genome organization and attachments to the matrix, they tendered the idea that there are at least two types, or classes, of MARs that organize the eukaryotic genome: those that are the result of a transient functional activity of the genome (such as those that contribute to creating transcriptionally competent chromatin—the classic MAR) and those that might represent more permanent associations of DNA to the nuclear matrix that partition the genome into larger structural loops (Razin and Gromova, 1995; Razin, 1996). This idea confers more flexibility in the definition of regions of matrix attachment and provides at least one explanation for why not all characterized MARs participate equally in the macroorganization of the genome.

length markers from the agarose gel are shown in lane 8. Again, no specific bands were detected in any of the naked DNA treatments.



**Figure 8.** Summary of the Hybridization Data in Figures 4 and 5.

(A) At bottom are three lanes from maize (Zm). The top lane was hybridized with *GRF1*, the middle lane was hybridized with *Adh1*, and the bottom lane is from the ethidium bromide-stained CHEF gel before transfer for DNA blot analysis. At top are densitometric scans from each of the lanes at bottom. Light gray indicates *GRF1*; dark gray, *Adh1*; black, ethidium bromide (EthBr) staining. The thin verti-

Razin's group has referred to attachment regions that serve to anchor sections of the genome into organizational loops as loop basements to distinguish them from MARs defined in the classic sense (Razin and Gromova, 1995; Razin, 1996). We have adopted this nomenclature and refer to the DNA sites that have been functionally defined by in vivo DNase I and VM26 cleavage as loop basement attachment regions or LBARs.

The current data from maize and Arabidopsis provide an opportunity to draw conclusions about higher order chromatin structure in plants in three areas: the contribution of looped domains in the global packaging of the genome, the domain loop size distribution among plants, and the gene distribution among individual loops. The first issue is addressed by the pattern of DNase I-mediated cleavage domain fragments described in Figure 1. These data suggest that the LBARs are intensely hypersensitive to DNase I and represent extreme hot spots of nuclease sensitivity in the genome that are more reactive than other hypersensitive sites, such as those in gene promoter regions that are not resolved under the conditions used in these experiments (Paul et al., 1987). An alternative explanation is that the loop basements are physically exposed to the exogenous nuclease prior to the nuclease accessing other regions of the genome. The latter explanation is consonant with the "channels model" of nuclear matrix structure. In this model, the nuclear matrix is contiguous with the nuclear pore, and interphase chromosomes are arranged in loops attached to the outer surface of channels formed from matrix material invaginated from the pore (Razin and Gromova, 1995).

Second, the median size of the chromatin loops that organize the genome appears to be variable among plants. This is an especially striking feature in the comparison of

cal lines highlight the bands with respect to the corresponding peaks in the scan.

(B) At bottom are three lanes from Arabidopsis (At). The top lane was hybridized with *GRF4*, the middle lane was hybridized with *Adh*, and the bottom lane is from the ethidium bromide-stained CHEF gel before transfer for DNA blot analysis. At top are densitometric scans from each of the lanes at bottom. Light gray indicates *GRF4*; dark gray, *Adh*; black, ethidium bromide staining. The thin vertical lines highlight the bands with respect to the corresponding peaks in the scan.

(C) At bottom is the lane that was hybridized with *Adh1* in (B). At top is a densitometric scan that has been partitioned into regions denoting background hybridization (dark gray) and specific hybridization (light gray).

(D) At bottom is a lane from a CHEF gel genomic blot of DNA restricted with BamHI and hybridized with *Adh1*. At top is a densitometric scan that has been partitioned into regions denoting background hybridization (dark gray) and specific hybridization (light gray).

maize and *Arabidopsis* because the median size of chromatin loops in these plants varies by almost twofold (45 and 25 kb, respectively). It is tempting to speculate that this difference reflects the disparity in the sizes and repetitive nature of the maize and *Arabidopsis* genomes; however, because a representative from the genus *Lilium* (whose members contain extremely repetitive genomes as large as  $10^{11}$  bp) was shown to be organized into loops averaging 35 kb (Figure 2), this generalization cannot be applied to all plants. There is (to our knowledge) only one other example of a characterization of chromatin loops in plants. Espinas and Carballo (1993) addressed the role of DNA methylation in chromatin folding and estimated that the maize genome was organized into loops averaging 50 kb in length, which is very similar to our observation that the median size of maize loops is 45 kb.

The third and most important implication from the work presented in this study is that the genome is not organized into random loops of chromatin but rather that a gene occupies an organizational loop of discrete and defined length within the genome. In maize, the *Adh1* and *GRF1* genes occupy loops of 90 and 100 kb, respectively. In *Arabidopsis*, the *Adh* gene is found within two distinct loops at 8.3 and 6.1 kb, whereas the *GRF4* gene occupies a single loop of 27 kb. If the loop sizes were random in the genomes of these plants, individual loops would not have been detected as hybridizing bands. This conclusion is supported by the fact that different genes occupy loops of different sizes. Thus far, there are no other examples of single-copy genes that have been identified with a discrete loop size *in vivo* with which these data can be compared. However, in an example of direct hybridization of CHEF-resolved loops from a human cell line, it was found that the *c-myc* amplicon fell within a range of fragments between 80 and 110 kb (Gromova et al., 1995b). The size of the DNase I-liberated loops occupied by *c-myc* is also consistent with the positions of MARs around the gene identified through the use of topoisomerase II inhibitors that created cleavable complexes at these sites (Gromova et al., 1995a, 1995b). A similar observation was made with nucleolar DNA from activated lymphocytes; individual rDNA repeats were found to be packaged in the nucleolus as looped structures that could be excised with either endogenous topoisomerase II-mediated cleavage or exogenous Bal 31 nuclease cleavage (Iarovaia et al., 1995).

LBARs, then, are areas of extreme hypersensitivity to DNase I that likely mark the boundaries of structural loops within the chromatin structure of the genome. This conclusion is supported by control digestions of naked genomic DNA, which fail to produce discrete domains. The potential co-residence at LBARs of DNase I hypersensitivity and topoisomerase poison cleavage suggests that LBARs are sites of direct attachment to the matrix and that this attachment forms the basis for loop domains. The discrete hybridization of genes to individual loop domains suggests that the LBAR-mediated attachments are fundamentally stable within the genome. However, not all of the gene-specific hybridization is

within the liberated specific domains. In these experiments, there is hybridization associated with the broad distribution of DNA characteristic of that species. This background hybridization may be artifactual, because a large portion of the broad-band signal can be attributed to nonspecific hybridization to high concentrations of DNA and because the broad-band signal is reduced in *Arabidopsis* compared with maize. It is also possible that the specific domains identified here represent only the most stable loop configuration within the population of cells and that a broad range of loop sizes also exists at lower frequencies.

These data extend the model that genomes are organized into anchored loops in eukaryotes in general. Thus far, however, the implication that the global packaging of the eukaryotic genome is not through random condensation and that genes occupy a loop of discrete and defined length within the genome is limited to these plant systems.

## METHODS

### Preparation of Nuclei

Nuclei were prepared (also described in Paul and Ferl, 1993) from cultured cell suspensions of maize (*Zea mays*) line P3377 (Duncan et al., 1985) or *Arabidopsis* (*Arabidopsis thaliana*; Ferl and Laughner, 1989). Between 3 and 5 g (fresh weight) of filtered cell suspension was ground in a prechilled mortar and pestle on ice with 3 to 5 mL of cold (4°C) nuclei isolation buffer (NIB; 50 mM Tris, pH 8.0, 0.30 M sucrose, 5 mM MgCl<sub>2</sub>, 0.05 mM β-mercaptoethanol, 0.01 mM phenylmethylsulfonyl fluoride [PMSF], and 0.1 mM EGTA). Extra buffer was added to bring the final volume to 15 to 25 mL, and the slurry was transferred to a 400-rpm motor-driven homogenizer (Eberbach Con Torque, Ann Arbor, MI). The cell debris was removed from the nuclear suspension by filtration through cheesecloth and a 105-μm mesh polypropylene screen. Nuclei were then pelleted by centrifugation at 1500g at 4°C for 10 min. The nuclear pellet was resuspended in 2 mL of NIB with a paintbrush.

### DNase I Treatments

Seven tubes were set up for each experiment. In each case, the control buffer or enzyme was added to the tube before adding the nuclear suspension as follows: (1) a no treatment control (20 μL of 100 mM EGTA and 50 mM EGTA); (2) DNase I control (1 μL of DNase I dilution buffer containing 20 mM sodium acetate, 5 mM CaCl<sub>2</sub>, and 0.1 mM PMSF in 50% glycerol); (3) 1 μL of 0.025 μg/mL DNase I; (4) 1 μL of 0.05 μg/mL DNase I; (5) 1 μL of 0.1 μg/mL DNase I; (6) 1 μL of 0.25 μg/mL DNase I; and (7) 1 μL of 0.5 μg/mL DNase I. A 200-μL aliquot of nuclei was then added to each tube and allowed to react at room temperature for 1 min. The reaction was stopped by the addition of 20 μL of 100 mM EGTA and 50 mM EGTA, and then the DNase I-treated nuclei were embedded by mixing the treated nuclei with an equal volume of 3% low-melting-temperature agarose (contour-clamped homogeneous electric field [CHEF] embedding agarose; Sigma) at 45°C in embedding buffer (50 mM Tris, 5 mM EDTA, and 0.1 mM EGTA) and applied to a mold to form plugs of embedded



nuclei. We found that we encountered less endogenous nuclease activity when the nuclei were treated with DNase I before embedding for pulsed field electrophoresis. The embedded nuclei were lysed and protease treated for 16 to 20 hr at 45°C in lysis buffer (0.1 M EDTA and 1% SDS containing 2 mg/mL proteinase K). After lysis, the plugs were stored in 20 mM EDTA and 10 mM EGTA storage buffer at 4°C.

#### Naked DNA Control for DNase I Digestions

Untreated nuclei were embedded in agarose, lysed, and deproteinized, as described above, and resolved on a CHEF gel. The parent-genomic band from lanes of untreated nuclei resolved on this preparative CHEF gel was excised and then digested with DNase I. The *in vitro* DNase I digests were conducted by incubating the parent-genomic plug (cut to roughly the same dimensions as the embedded nuclear plugs) in 500  $\mu$ L of Hepes-buffered saline (10 mM Hepes, pH 7.2, 150 mM NaCl, 5 mM CaCl<sub>2</sub>, and 0.2 M mannitol) plus DNase I (concentrations ranging from 0.0 to 0.1  $\mu$ g/mL) for 30 min at 30°C. The reactions were stopped by bringing the EDTA and EGTA concentrations to 20 and 10 mM, respectively. The single-strand nicks created *in vivo* and in nuclei by endogenous nucleases and the natural base-unpaired tracks inherent in the regions we are studying are present in the parent-genomic band; thus, subsequent digestion with DNase I still affects to some degree the patterning that is seen with DNase I digestions conducted *in vivo* and in nuclei.

An additional naked DNA control was conducted with CsCl density gradient-purified Arabidopsis genomic DNA. This method (previously described in Paul and Ferl, 1991) creates less endogenous nicks as intact cells are quick-frozen in liquid nitrogen and lysed in a detergent buffer before the DNA is purified on CsCl density gradients. The drawback of this method is the mechanical shearing that reduces the genome to a collection of fragments <100 kb in length. Naked genomic DNA prepared in this fashion was dispensed as 6- $\mu$ g aliquots (30  $\mu$ L), brought to 200  $\mu$ L with Hepes-buffered saline plus DNase I (concentrations ranging from 0.0 to 1.0  $\mu$ g/mL), and incubated for 10 min at 30°C. The reactions were stopped by bringing the EDTA and EGTA concentrations to 20 and 10 mM, respectively. The naked DNA treatments were resolved on CHEF gels or with conventional gel electrophoresis on 1% agarose gels.

#### Cytotoxic Drug Treatments

Arabidopsis cell cultures were harvested by vacuum filtration 3 days after their transfer to fresh media. The cells were then resuspended in 20 mL of cell wall digesting solution (0.05% cellulysin, 0.1% macer-ase, and 0.05% pectolyase in 10% mannitol and 0.1% CaCl<sub>2</sub>), transferred to Petri plates, and rotated gently overnight. The following day, a pipette was used to break up any clumps, and then the mixture was filtered through Miracloth (Calbiochem, La Jolla, CA) into conical screw-top tubes. The protoplasts were collected by centrifuging at 1000 rpm in a swinging bucket rotor for 10 min. The protoplasts were washed twice (respinning as given above) with Hepes-buffered saline. The final protoplast pellets were resuspended in 1.5 mL Hepes-buffered saline plus the nuclease inhibitor aurintricarboxylic acid to 100  $\mu$ M (Sigma) and preincubated for 1 hr at room temperature. The protoplasts were then divided into 200- $\mu$ L aliquots. One aliquot was reserved as the control for endogenous nuclease activity. The other aliquots were brought to the following concentrations of each drug:

VM26 (4,-demethylepipodophyllotoxinthenylidene- $\beta$ -D-glucoside; kindly provided by Bristol-Myers, New York, NY), 50 and 200  $\mu$ M; genistein (ICN, Costa Mesa, CA), 100  $\mu$ M. The reactions were allowed to incubate at room temperature for 30 min and then stopped by the addition of EDTA and EGTA to 20 and 10 mM, respectively. The protoplasts were then embedded and lysed as described above. Choices and concentrations of the various drugs and nuclease inhibitors are a compilation of methods (Espinosa and Carballo, 1993; Osheroff et al., 1994; Gromova et al., 1995a, 1995b; Iarovaia et al., 1996).

#### Pulsed-Field Electrophoresis Conditions

The plugs described in the preceding sections were cut into fourths to provide  $\sim$ 3  $\mu$ g of DNA per lane in a 10-well CHEF gel (Bio-Rad). The gels were run for either 20 hr at 150 V, with 10 initial and 60 final pulses per sec to resolve high molecular weight species (Figure 1), or for 12 to 14 hr at 175 V, with one initial and 12 final pulses per second to resolve lower molecular weight species (remaining CHEF gels). The gels were electrotransferred to Hybond Plus nylon membrane (Amersham, Arlington Heights, IL) with a Genie electroblotting apparatus (Idea Scientific, Minneapolis, MN), following the manufacturer's recommended procedure. The resulting blots were hybridized in a phosphate buffer (0.5 M Na phosphate, pH 7.2, 7% SDS, 1% BSA [Sigma A-4378], and 1 mM EDTA) at 66°C, with gene probes labeled with random primed end labeling (Paul et al., 1987; Paul and Ferl, 1993).

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