



Mini-review

Higher-order chromatin structure: looping long molecules

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Abstract

Chromatin structure plays a variety of roles in eukaryotes, ranging from the structural organization of the genome to the facilitation of transcription factors and remodeling of individual gene promoters. Higher-order chromatin structure typically refers to those structural features of the genome that serve to facilitate large-scale condensation and packaging. It is becoming increasingly clear, however, that large-scale features that create loop domains play an important role in the management and functional organization of the genome as well. Recently, plant models have made significant contributions to our understanding of higher-order chromatin structures in eukaryotes.

Introduction

The degree of DNA condensation that is necessary to package the genomes of higher eukaryotes into the nucleus staggers the imagination. Both man and maize have genome sizes in the order of 10^9 bp of DNA – about 1 meter's worth! Even cutting it up into chromosomal sections, it is still difficult to imagine the topological problems faced by packing such lengths of material into a nucleus that is only 5–10 μm across. A 1:100 000-scale model can be constructed with monofilament fishing line and a large beach ball. Six-pound-test monofilament line is 0.2 mm thick, 100 000 \times thicker than DNA, thus it would take some 100 km of this line to represent a human or maize-size genome. To complete the model, 100 1-km spools of monofilament must be unwound (no easy task in itself) and stuffed into a large beach ball (0.5–1 m across). While this feat may be possible, the ability to re-establish an ordered structure without the introduction of knots and breaks is unlikely. Consider not only the problem of fitting this material into a confined space, but also accessing many different sections for processes such as transcription. Finally, imagine replicating the entire mass, then separating duplicate filaments without a single knot, break or tangle. Of

course, a maize cell has no trouble doing this, because the DNA in a maize cell exists not as an unordered ball of monofilament, but instead exists as an ordered array of chromatin.

Chromatin does three fundamental things for the genome. First, the association of histones and other positively charged proteins with the highly negatively charged DNA renders the DNA molecule more flexible, thereby enabling the bending and coiling required for packaging and regulatory processes. Second, chromatin implements the structural organization and packaging of the genome. Third, matrix proteins create spatially fixed points in the genome, resulting in localized topologies that influence genome regulation and processing. Full attention to the functions associated with each type of chromatin structure is beyond the scope of this article, but there are many excellent reviews on this subject (e.g. Pruss *et al.*, 1995; Franklin and Cande, 1999).

The hierarchies of chromatin structures that confer increasing degrees of condensation and organization on the genome are referred to as 'orders'. First-order chromatin describes the 11 nm nucleosome array, and second order refers to the 30 nm fiber that is created by coiling the nucleosome array into a solenoid structure. Virtually every other feature of chromatin that

participates in further condensation is collected under the heading 'higher order'. Higher-order chromatin describes the anchored coils, loops and folds that further condense the length of the 30 nm fiber, and create organized domains and structures within the genome. The purpose of this mini-review is to focus on the loop features of higher-order chromatin structures in plants, and to place these features in a meaningful context for organization, management and regulation of plant genomes.

The genome is a collection of loops

The idea that chromatin is organized into loop domains is actually as old as the original observation of loops in amphibian lampbrush chromosomes in the late 1800s (Flemming, 1882). Almost 100 years later, Paulson and Laemmli (1977) gently removed histone proteins from metaphase chromosomes and saw that after extraction DNA remained attached to an insoluble proteinaceous structure. The micrographs showed the DNA spreading out as large loops attached at their bases to a chromosome-shaped scaffold. When interphase nuclei were similarly depleted of histones, they also exhibited an insoluble protein matrix surrounded by a halo of DNA loops. The image of loops of DNA issuing from a defined scaffold or matrix of protein was a compelling one. It made sense that the genome would have to be organized as such to accommodate the high degree of condensation required to package chromatin fibers in an ordered fashion. The morphological measurement of the loops from histone-depleted chromosomes ranged from 30 to 100 kb. This observation was in keeping with the theoretical expectations of how the chromatin fiber might be condensed to create a structure the size and shape of a metaphase chromosome, but did these loops reflect native structures, and those of the less highly condensed chromosomes of interphase? Microscopy experiments with chromosomes treated in low-ionic-strength buffer to produce a relaxed, but otherwise unmodified state, indicated that native chromosomes were indeed organized with a radial organization of loops. From these observations the 'radial loop' theory of chromosome structure was born (Marsden and Laemmli, 1979; see Figure 1). However, is the genome similarly organized during interphase? Is the same organization of loops maintained throughout the cell cycle? Do specialized, sequence-specific matrix-associated proteins anchor the basements of the loops? So far, it seems that

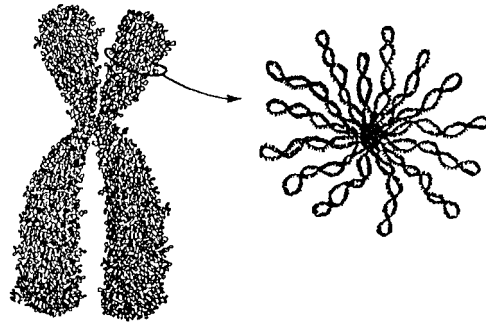


Figure 1. Radial loops in metaphase chromosomes. In the radial loop model of metaphase chromosome organization, domains of 30 kb to 100 kb emanate from a central protein scaffold that spirals through the axis of each chromosome arm.

the answer to all these questions is a carefully qualified 'yes'. However, these questions have not been answered sequentially or independently but, rather, as an understanding develops on one front, aspects of another fall into place, and previously unrelated features connect by extrapolation from specialized examples.

In the radial loop model, large chromatin fiber loops are arranged around the axis of the central scaffold of protein that runs the length of the chromosome. The regions of DNA that form the attachment to the scaffold are referred to as scaffold attachment regions (SARs). The nature of the scaffold is important as it, too, must be a dynamic structure that is capable of various states of condensation. To accomplish this, either the chromosome scaffold is disassembled and reassembled as the degree of condensation through the cell cycle dictates, or the scaffold structure must be flexible enough to accommodate extremes of compaction. Analyses of the proteins of the scaffold reveal that at least the potential for the latter scenario exists. Many of the scaffold proteins are required for proper condensation of chromosomes, and several appear to have protein motor motifs that could function in retracting and expanding the scaffold structure itself (Martelli *et al.*, 1996). Another major component of the scaffold is topoisomerase II (topo II) (Adachi *et al.*, 1991), an enzyme that resolves topological constraints in DNA and is required for condensing and decondensing supercoiled loops. Topo II preferentially binds to an AT-rich motif, thus, the axis of a chromosome should abound in AT motifs. Metaphase chromosomes, stained with the highly AT-specific dye daunomycin and, visualized with confocal microscopy, show that AT sequences line up along the chromosome axis, following a helical track from cen-

tromere to each telomere. Differentially stained chromatin fibers emanate from the AT-rich axis, which was dubbed the 'AT queue' (Saitoh and Laemmli, 1994). These observations suggest that the condensation of the genome into chromosomes proceeds by a gathering and contraction of chromatin loops that are anchored at AT-rich sequences, and that topo II probably plays a role in the condensation and supercoiling process. Do the proteins of the AT queue participate in the molecular organization of the chromosome at interphase as well?

Early studies also showed evidence of a proteinaceous interphase scaffold, or nuclear matrix, in histone-depleted nuclei (Mirkovitch *et al.*, 1984). As with chromosome scaffold proteins, topo II dominates the composition of the nuclear matrix, implying that a similarly organized arrangement of loop attachments occurs at the nuclear matrix during interphase.

Initially, the investigations of genome attachment sites to the nuclear matrix observed from interphase nuclei were biochemical in nature, being conducted predominantly with isolated nuclear matrices or in histone-depleted nuclei. These DNA regions were first defined operationally, either by determining the ability of a sequence to bind to isolated nuclear matrices *in vitro*, or by characterizing the sequences that remain attached to the nuclear matrix in isolated, histone-depleted nuclei (Mirkovitch *et al.*, 1984). Regions of the genome that remain associated with the interphase nuclear matrix are referred to as matrix attachment regions (MARs). MARs so identified among a number of plant and animal species were AT-rich in nature and contain topo II recognition sites. These characteristics are consonant with those seen for the basements of the metaphase chromosome loops. A puzzling inconsistency, however, arises from a comparison of apparent and deduced loop size. Microscopic observations suggest that the size of the loops from metaphase chromosomes are 30 to 100 kb, thus the distance between attachment regions would be assumed to be between 30 and 100 kb, as well. However, the distance between the operationally defined MARs of the interphase genome typically ranges between only 3 and 30 kb (Davie, 1995). Although there have been debates as to whether either of these observations are artifactual in nature, current perspectives see both observations as valid, and both can be embraced by reasonable models of genome organization.

Defining loops

A loop can be defined in two ways: by the points that anchor it and by the length of material that comprises it. Cytological observations aside, the presence of loops in the genome can be deduced by analyzing nuclei in ways that reveal either the anchor points of the loops, or the material freed from those points of attachment. It is clear, however, that associations with various nuclear structures must play a variety of roles in the functional organization of the genome. So it is not inconsistent to think that variety exists in the nature of anchor points, and therefore, in their susceptibility to reagents and techniques used to detect them.

Currently, almost any type of matrix-associated sequence in the genome is referred to as a MAR. All MARs do share common features, which appear to hold true among many genomes. MARs are inclined to be AT-rich and have a few conserved motifs (such as topo II sites) (Cockerill and Garrard, 1986; Laemmli *et al.*, 1992; Davie, 1995), but there is no clear consensus sequence for MARs. Indeed, the sequence variety itself in these 200–1000 bp AT-rich stretches may allow for a diversity of sequence motifs that contribute to specialization of function. Currently, only a few conserved motifs have been assigned any sort of role within the MAR (Boulikas, 1995). Another conserved characteristic of MARs appears to be segments of altered DNA topologies in the region. This conserved local topology creates sections of non-B form DNA that are hypersensitive to a variety of reagents, facilitating a host of *in vivo* diagnostic tools (Bode *et al.*, 1996; Razin, 1996).

Anchoring to the matrix can define functional units of the genome

Anchoring a section of the genome to create a topologically independent domain enables one section of the genome to be insulated from the adjoining section. Thus it is not surprising that MARs are very often found to flank genes, and gene clusters, and may serve to physically mark the boundary of certain active chromatin domains (Eissenberg and Elgin, 1991; Geyer, 1997). MARs also appear to be important for the proper regulation of genes within the topological domains that they define (reviewed in Holmes-Davis and Comai, 1998). DNA topology itself influences gene expression in eukaryotes (e.g. Owen-Hughes *et al.*, 1992; Dunaway and Ostrander, 1993; Liang and Gar-

rard, 1997), and in animals there are several examples of genes in which regulatory regions correlate with MARs (e.g. Bodnar and Bradley, 1996) and with the topology of MARs (Bode *et al.*, 1996). In plants, MARs with altered topology exist in the 5'-flanking MAR of maize *Adh1* (Paul and Ferl, 1993), and a tobacco chitinase gene (Fukuda, 1999). Analyses with isolated MAR DNA-binding proteins from pea show that the structurally narrow minor groove of the AT-rich target sequence is important for protein binding (Hatton and Gray, 1999).

The organization of genes into individual, relatively small MAR-defined domains of 3–10 kb seems to be a common pattern in plant genomes. The four genes in the 17.1 kb of the soybean lectin locus are segregated into separate domains (Breyne *et al.*, 1992), as are the tomato heat-shock cognate 80 gene (Chinn and Comai, 1996) and the β -phaseolin gene (van der Geest and Hall, 1996). In the 16 kb plastocyanin region of the *Arabidopsis* genome, three MARs partition the area into smaller, separate domains containing individual genes (van Drunen *et al.*, 1997). MARs also appear to partition chromosomal segments in several plant genomes into classes of sequence, as well as genes. MARs have been strongly correlated with the junctions of repetitive and low-copy-number DNA sequence blocks, apparently segregating highly repetitive DNAs into the regions between the MARs (Avramova *et al.*, 1998). The *Adh1* gene in maize is found positioned in such a continuum as a separate MAR-defined domain.

MARs have also attracted considerable attention in plant gene regulation because evidence points to their involvement in influencing transgene expression. The idea here is that a MAR-flanked transgene creates its own topological chromatin domain upon insertion, insulating it from the influences of the surrounding genome. Recent studies (Ulker *et al.*, 1999; Vain *et al.*, 1999) and reviews (Spiker and Thompson, 1996) indicate, however, that the establishment of chromatin domains in MAR-flanked genes and transgenes remains an inferred, rather than demonstrated, conclusion.

DNA freed from the matrix can define organizational units of the genome

Although the radial loop model of genome organization is almost universally accepted, controversy remains as to the nature of the loop basements, i.e. the attachment sites of the organized loops of individual chromosomes especially during interphase. Dauno-

mycin staining demonstrates that the loop basement sequences are AT-rich, and the topology of the loops infers an affinity for topo II, both hallmarks of the MARs discussed above. The distinction, however, is in using the presence of biochemically identified MARs to infer the presence of a loop structure bounded by MARs as opposed to identifying loops visually and inferring that attachments delimit such structures. The presumed loops are defined solely on the basis of the linear distribution of sequences that can bind to the nuclear matrix. Thus, if a gene is flanked by two MAR sequences, 4 kb apart, it is inferred that the region is organized in the genome as a 4 kb loop. By these criteria the genomes of eukaryotes would be partitioned into domains 3–30 kb in length. However, the morphological measurement of the loops from the histone-depleted chromosome of 30 to 100 kb suggests that another layer of organization may be superimposed on the 3–30 kb domains inferred to exist between MARs.

Investigation into the molecular nature of structures creating the cytological loops was pioneered by an approach that maintained native nuclear structures while employing diagnostic procedures that identified regions of the genome associated with the nuclear matrix (Jackson *et al.*, 1990). Permeabilized cells imbedded in agarose beads in a 'physiological' buffer were treated with a battery of restriction enzymes, and the released DNA fragments were removed by electrophoresis. The fragments remaining attached to the matrix were called loop attachment sequences (LASs). Interestingly, the average size of fragments freed from the matrix was 86 kb, more in keeping with the expected loop size based on cytological observations. Furthermore, the size of the presumed loops remained constant throughout the cell cycle, suggesting that some aspect of the cytological loops observed in the metaphase chromosomes are maintained throughout the cell cycle.

Other approaches have focused on maintaining native features of the genome in the definition of chromatin domain loops and have taken advantage of structural features of native matrix attachment regions such as topo II sites and the inherent DNase I hypersensitivity of non-B DNA (reviewed in Razin, 1996). Topo II sites are vulnerable to the actions of cytotoxic drugs such as VM26, which can be applied *in vivo*. This type of topo II poison creates a cleavable complex at the topo II/DNA interface. Thus, any cuts in the genome resulting from VM26 treatments indicate topo II binding sites (Gromova *et al.*, 1995a,b). Judicious

use of DNase I in limited digestions can create a set of cleavage sites that overlap those seen by the actions of VM26. The size of fragments that are created by these topo II and DNase I cleavage reactions are in the range of 30 to 100 kb, again reflecting the cytological expectations for structural loops in the genome. Attachment regions that serve to anchor sections of the genome into these organizational loops are referred to as loop basement attachment regions (LBARs), to distinguish them from among the general category of MARs. However, the presence of LBARs at the ends of these released domains remains an inferred, rather than demonstrated, conclusion.

In maize and *Arabidopsis*, the organization of the loop domains as defined by loop basement attachment regions has been examined in detail. The genomes of these plants can be cleaved at LBARs to create a distribution of fragments that is thought to reflect units of structural loops. (The genomes can be selectively cleaved by limited DNase I digestions and by the creation of cleavable complexes at the loop basements with topoisomerase II poisons such as VM26.) Although the median size of these LBAR-defined structural loops is about 45 kb in maize, the distribution ranged from about 20 kb to a little more than 100 kb (Paul and Ferl, 1998). These observations from maize correlate well with what is known about the structural organization of animal genomes, but there is an additional observation gained from these experiments that may influence models for the structural organization of all higher eukaryotic genomes. Probing the fragments liberated with DNase I or topo II-mediated cleavage with gene-specific sequences revealed that individual genes can consistently occupy a discrete loop or domain. For instance, the maize *Adh1* gene hybridizes to a distinct 90 kb band in a distribution of 20–100 kb loop fragments. This result suggests that a particular gene consistently occupies an LBAR-defined loop, which implies that the distribution of LBARs is not random. If the distribution of LBARs was random in the genome, hybridization with specific genes would be uniformly distributed among the loop-sized fragments.

MARs and LBARs in concert: putting it all together

Challenges remain in understanding the higher-order chromatin structures such as the structural relationship of MARs and LBARs. There are likely to be

many types or classes of matrix associations that organize the eukaryotic genome. Two obvious categories include matrix associations that are the result of a transient activity of the genome (such as those that contribute to creating transcriptionally competent chromatin, the classic MAR; Holmes-Davis and Comai, 1998), and those that might represent more permanent associations of DNA to remnants of the scaffold that partition the genome into structural loops (Razin and Gromova, 1995; Razin, 1996). Research has shown that not all characterized MARs participate equally in the macro-organization of the genome. Nor do all MARs respond identically to the reagents and protocols used for chromatin analyses. The idea that chromatin is composed of transient and permanent chromatin structures confers more flexibility in the way we define higher-order structures. Thus, discrepancies in MAR biochemical signatures may simply reflect distinctions in the role of specific MARs in the genome.

Thus far, exogenously defined MARs have yet to be connected with the site of a large-scale organization loop basement, and the matrix-binding characteristics of LBARs have not yet been demonstrated. In an example from maize, hybridization analyses with fragments liberated by *in vivo* cleavage at presumed LBARs demonstrated that an LBAR was not coincident with the previously defined MAR of the 5'-flanking region of the maize *Adh1* gene (Avramova and Bennetzen, 1993; Paul and Ferl, 1993). However, this does not imply that the either MARs or LBARs are artifacts of *in vitro* analyses, but, rather, this example may support the idea that different types of matrix associations differentially contribute to the organization of the genome. The primary role of MAR sites of a more transient nature may be in creating shorter-term topologies favorable for transcription. In maize *Adh1*, a MAR defines the 5' edge of the DNase I hypersensitive region gene promoter, implying a role in maintaining that topology (Paul *et al.*, 1987). This role is supported by the presence of a recurring sequence motif embedded in the MAR that changes its topology with the degree of torsional stress in the region (Paul and Ferl, 1993). This motif is very similar to the SATB1 recognition sequence, a site that plays a role in the organization of the human genome (Bode *et al.*, 1992). LBARs, however, may be a very different sort of MAR, one that is more permanent in nature and defines the anchor point for the classic chromatin loop.

A working model of higher-order genome organization for typical eukaryotes such as man or maize is

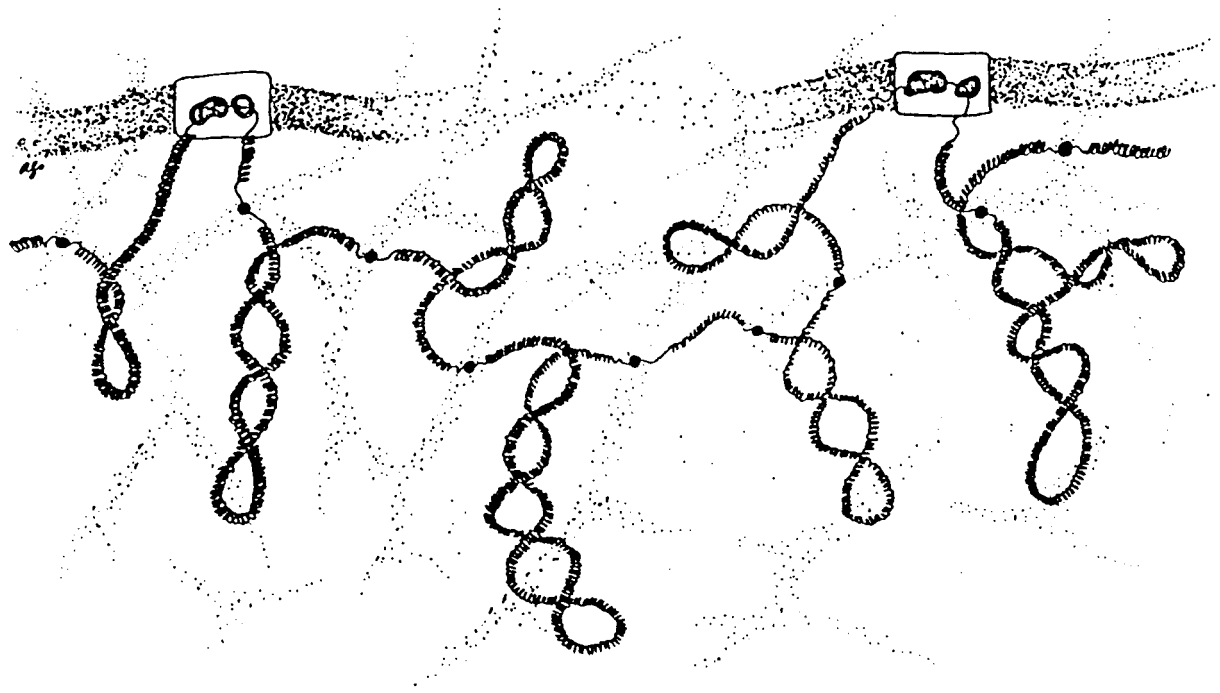


Figure 2. Organization of interphase loops and domains. In this model of interphase chromosome organization, the domains of the metaphase radial loop remain attached to dispersed remnants of the metaphase scaffold. The points of attachment to these scaffold remnants are the loop basement attachment regions, or LBARs, which are represented by boxes. The LBARs are sensitive to topo II poisons and DNase I digestion, such that the loop domains between LBARs can be freed. The sizes of the freed loop domains matches the sizes of domains estimated from metaphase loop measurements at 30 to 100 kb, so it is likely that LBAR attachment reflects the organization of the metaphase chromosome and carries that organization into interphase. Additional associations of the genome are formed with the nuclear matrix such that subdomains are created within the structural loops. These subdomains are mediated by matrix attachment regions, or MARs, which are represented by solid circles. MARs create localized topologies that further organize the loop into domains that are important for gene regulation.

shown in Figure 2. Any such model needs to incorporate several observations. First, the loops attached to the metaphase chromosome scaffold range in length from 30 to 100 kb. Second, biochemically defined MARs are typically found 5–30 kb apart in the interphase genome. Third, the loops freed by cleavage at LBARs in the interphase chromatin range in length from 20 to 100 kb. Fourth, genes occupy discrete loops within higher-order structures. Because of the similarity between the size of the loops attached to the metaphase chromosome scaffold and the size of the fragments liberated by cleavage at interphase LBARs, it is possible that LBARs do, in fact, represent the AT queue seen in the metaphase chromosome scaffold. This connection implies that the chromosome scaffold is maintained throughout the cell cycle in some capacity, undergoing morphological changes in concert with the condensations and expansions of the genome. The nuclear matrix material may be a distinct structure or it may be an extended feature of the chromo-

some scaffold, although the former possibility seems most likely. The biochemically characterized MARs are likely to reflect interactions of the nuclear matrix with subsections of the chromosomal loops to create smaller topological domains. These smaller domains participate more directly with the transcriptional organization and regulation of the genome, and are often coupled to a variety of MAR-associated proteins that confer specialized features to these attachments, such as the topology remodeling SATB1 (Bode *et al.*, 1992; de Belle *et al.*, 1998) and the filament-like MP1 (Meier *et al.*, 1996; Gindullis and Meier, 1999).

Finally, the observation that genes appear to occupy discrete loops within the genome suggests a sophistication of higher-order packaging that was heretofore unknown. Higher-ordered structures were once assumed to be merely features required to meet the packaging needs of large genomes. However, the degree of directed organization implied by the placement of specific genes on discrete loops suggests that

genome packaging is not random, and that even the higher-order chromatin structures contribute to the functional organization of the eukaryotic genome.

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