Chromosomal Loop Organization in Eukaryotic Genomes

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1 Remaining Questions in Gene Regulation Relate to Nuclear Organization

Considerable progress has been made in understanding the mechanism of gene regulation in eukaryotic cells. Protein–DNA and protein–protein interactions are believed to bridge specific promoter and enhancer elements, thereby creating a favorable environment for RNA polymerase to initiate transcription (Ptashne 1988). Over the next few years it is predictable that perhaps hundreds of genes which encode regulatory proteins will be cloned, sequenced, and functionally characterized. While this information will be fundamental, these studies do not address the next important problem, namely how nuclear organization might participate in gene expression.

It has been known for many years that gene expression is influenced by chromosomal position. Examples include position effect variegation in *Drosophila* (Spofford 1976), X-chromosome inactivation (Lyon 1974), and variation in gene expression depending on integration site (Spradling and Rubin 1983). The transcriptional activation of the c-myc gene after translocation into the immunoglobulin gene locus (Kakkis et al. 1986) or transvection in *Drosophila,* which can result in the "curing" of a *cis*-acting mutation by synapses with the allelic partner (Zacher et al. 1985), are striking examples of position effects. In the broader sense, active chromatin domains appear to be compartmentalized in the nucleus (Hutchison and Weintraub 1985), and DNA sequences generally maintain their position in the interphase nucleus from one cell cycle to the next (Cremer et al. 1982). Furthermore, specific gene transcripts appear to be vectorally transported from their site of synthesis to a single or small subset of nuclear pores (Lawrence et al. 1989), suggesting that genes might be specifically "gated" as proposed earlier (Blobel 1985). Therefore, gene organization in the nucleus appears to be functionally ordered, but the principles that underlie this organization remain largely to be elucidated.

2 Chromosomal Loops: Fundamental Elements of Nuclear Organization

Just as in prokaryotic chromosomes (Worcel and Burgi 1972), DNA within eukaryotic interphase nuclei and mitotic chromosomes is organized into topo-

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logically constrained looped domains averaging 50–100 kb in length (Benyajati and Worcel 1976; Cook and Brazell 1976; Paulson and Laemmli 1977; Vogelstein et al. 1980; Pettijohn, this volume p 152). It seems likely that interphase chromosomal loop organization may play crucial roles in localizing pertinent *cis*-acting sequences to their correct nuclear environment. Thus, although the effect of chromosome position on gene expression can be explained by the presence of endogenous enhancers or silencers near the sites of integration of introduced genes, the mechanisms that govern precision of enhancer and silencer action within their own loci need also to be considered. Since these *cis*-acting sequences work over long distances, presumably by forming "mini-loops", nuclear order must play an important role to ensure, for example, that an enhancer from one genetic locus does not accidentally activate the wrong promoter. Thus, it is believed that the organization of the eukaryotic genome into chromosomal loops facilitates the interactions of linked *cis*-acting sequences with trans-acting factors, within a loop domain, but not between loop domains.

3 Identification of Chromosomal Loop Attachment Sites

Early studies on nuclear structure provided evidence for a proteinaceous framework that underlay histone and DNA-depleted nuclei and set the precedent for nomenclature by coining the term "nuclear matrix" (Berezney and Coffey 1974). Analysis of the association of specific DNA sequences with the nuclear matrix was first performed by Vogelstein and co-workers (reviewed in Zehnbauer and Vogelstein 1985). Since the techniques used in these studies employed high salt extraction which can sometimes lead to artifactual coprecipitation of proteins (e.g., Roberge et al. 1988), and because attachment often was found to encompass very long stretches of DNA, alternative techniques were sought to identify the "short" attachment site sequences.

The first demonstration of specific defined sites of attachment was by Mirkovitch et al. (1984) in the *Drosophila* histone and hsp70 genes. These investigators developed the lithium diiodosalicylate (LIS) nuclear-halo mapping procedure, a method employing a mild detergent instead of high salt, thus eliminating the artifacts associated with high salt-induced precipitation. This procedure permitted the localization of the endogenous sequences that remain attached to the matrix after histone depletion. However, since it is now known that exogenous sequences can bind under these conditions, uncertainty arises as to which sequences are really attached in vivo (see 1.8.0). For this reason, these sites should be considered as potential matrix-binding sequences. An in vitro assay to localize "matrix association regions" (*MARs*) within exogenously added cloned genes permitted the first demonstration that the attached sequences in the LIS nuclear-halo assay actually were *cis*-acting and could mediate binding in the absence of flanking elements (Cockerill and Garrard 1986a). Using the in vitro technique, it was possible to demonstrate that a single mouse *MAR* sequence could recognize multiple (> 10,000) binding sites per matrix and that *MARs* from *Drosophila* genes could compete for these same binding sites in the mouse nuclear matrix (Cockerill and Garrard 1986a). Other studies showed that a mouse *MAR* sequence would also specifically bind to the yeast nuclear matrix (Cockerill and Garrard 1986b). These results led us to propose that both *MAR* sequences and their matrix binding sites have been conserved during evolution (Cockerill and Garrard 1986a, b), a supposition which has now been supported by other laboratories (Amati and Gasser 1988; Izaurralde et al. 1988). Furthermore, both the nuclear-halo and in vitro assays appear to yield similar results for mapping attachment site sequences (Cockerill and Garrard 1986a; Izaurralde et al. 1988; Phi-Van and Stratling 1988), although minor differences have sometimes been noted (Cockerill et al. 1987; Dijkwel and Hamlin 1988).

Gasser and Laemmli (1986a) have introduced the term "scaffold attached regions" (*SARs*), a strictly analogous term for *MAR*. These two nomenclatures sometimes tend to cause confusion in the literature even though they appear to

Table 1. Examples of *MARs* and *SARs*

Genetic origin	Position(s)	Reference
Human		
β-Globin locus	Intronic and flanking	Jarman and Higgs (1988)
HPRT	Intronic	Sykes et al. (1988)
β-IFN	5′ and 3′ flanks	Bode and Maass (1988)
Chr. 21 (LINE)	5′ and 3′ flanks	Sperry et al. (1989)
Factor IX	5′ and intronic	Beggs and Migeon (1989)
OTC	Exonic	Beggs and Migeon (1989)
Mouse		
κIgL	Intronic (Enh)	Cockerill and Garrard (1986a)
μIgH	Intronic (Enh)	Cockerill et al. (1987)
γ_3, γ_1, γ_{2b} IgH	5′ to S regions	Cockerill (unpub.)
β-Globin locus	5′ flank	Greenstein (1988)
Rabbit		
κ_1 IgL	Intronic (Enh)	Sperry et al. (1989)
Chicken		
Lysozyme	5′ and 3′ flanks	Phi-Van and Stratling (1988)
Hamster		
DHFR	Intronic	Kas and Chasin (1987)
DHFR	5′ and 3′ flanks	Dijkwel and Hamlin (1988)
Drosophila		
Histone gene repeat	5′ to H1	Mirkovitch et al. (1984)
hsp70 (87A7, C1)	5′ flanks	Mirkovitch et al. (1984)
Sgs-4	5′ and 3′ flanks	Gasser and Laemmli (1986a)
ftz	5′ and 3′ flanks	Gasser and Laemmli (1986a)
AdH	5′ and 3′ flanks	Gasser and Laemmli (1986a)
320 Kb (rosy locus)	5′ and 3′ flanks (4 sites)	Mirkovitch et al. (1986)
Yeast		
TRP1	*ARS1*	Amati and Gasser (1988)
Chr 3	*CENIII*	Amati and Gasser (1988)
Virus		
SV40	T-ag-Exon	Pommier et al. (1990)
Polyoma	T-ag-Exon	Fluck (unpub.)

refer to the same sequences. Since the term "scaffold" had been introduced earlier by Paulson and Laemmli (1977) to describe the mitotic chromosome backbone, and Berezney and Coffey (1974) had already coined the term "matrix" for the interphase nuclear framework, we believe that *MAR* should be used to describe the potential attachment sites for interphase chromosomes and the term *SAR* for the mitotic counterparts. So far, however, these terms have been used interchangeably.

MARs have been identified in many eukaryotic biological systems, including human, mouse, hamster, *Drosophila,* yeast, and viruses (Table 1).

4 Functions of Chromosomal Loop Attachment Sites

4.1 Substrates for Topoisomerase II

The functions of *MARs* are only beginning to be understood. Several *MARs* have been shown to specifically bind topoisomerase II (topo II) (Sander et al. 1987; Sperry et al. 1989). *MARs* with high affinity for topo II are probably sequestered by this protein at the bases of the DNA loops in metaphase chromosomes, since this enzyme is a major component of the mitotic chromosomal scaffold (Earnshaw and Heck 1985; Gasser et al. 1986; Boy de la Tour and Laemmli 1988). Besides structural roles in mitotic chromosomal loop organization, these sequences may serve as substrates for topo II both in the decatenation of intertwined replicated duplexes (Holm et al. 1985) and in the relaxation of torsional stress introduced by transcription and replication (Brill and Sternglanz 1988; Wu et al. 1988).

4.2 Facilitators of Transcription

MARs sometimes are located near enhancer elements, in immunoglobulin genes (Cockerill and Garrard 1986a; Cockerill et al. 1987), in *Drosophila* genes (Gasser and Laemmli 1986a), and in the human β-globin gene locus (Jarman and Higgs 1988). These enhancer-linked *MARs* are occasionally found within introns and may functionally differ from *MARs* located at the boundaries of chromatin domains. The *MAR,* but not the enhancer, has been deleted from the rabbit $\varkappa 2$ immunoglobulin gene (Sperry et al. 1989). Interestingly, expression of this gene is very low and often undetectable (Emorine and Max 1983). Furthermore, when a segment of the *MAR* becomes spontaneously deleted from the rabbit $\varkappa_1$ immunoglobulin gene, or when the *MAR* is removed from the mouse $\varkappa$ immunoglobulin gene by reverse genetics, expression is reduced about fourfold (Akimenko et al. 1986; Blasquez et al. 1989a; Xu et al. 1989). Therefore, it seems clear that the *MARs,* whose position next to the enhancer within the $\varkappa$ immunoglobulin genes has been conserved during evolution, serve a positive role in transcription.

At least three experimentally testable models can be formulated on the mechanism by which enhancer-linked *MARs* might work to facilitate transcription.

Model I: The *MAR* is a subnuclear targeting sequence that needs to be near *either* an enhancer or a promoter. The promoter–enhancer complex would be directed by the *MAR* to a nuclear location rich in the transcriptional machinery.

Model II: The *MAR* needs to be downstream of the promoter in order to relieve positive supercoiling, induced by transcription, via topoisomerase II swiveling (Wu et al. 1988).

Model III: To facilitate enhancer activation, the *MAR* must be adjacent. These two sequences, however, need not be in any specific configuration with respect to the promoter.

4.3 Boundaries Between Chromatin Domains

Properties of active chromatin often extend well beyond the 5′ and 3′ boundaries of transcription units and *MARs* may serve to functionally punctuate such chromatin domains (Gasser and Laemmli 1987). *MARs* have been identified at the ends of the 19 kb chicken lysozyme DNase-I-sensitive domain (Phi-Van and Stratling 1988). The *Drosophila fushi tarazu* gene appears to be more sensitive to chromosomal position effects when lacking terminal *MARs* (discussed in Gasser and Laemmli 1986a). A β-globin mini-locus can be created that exhibits expression that is roughly proportional to gene copy number and independent of chromosomal integration site (Grosveld et al. 1987); this gene construct appears to have *MARs* at each end (Jarman and Higgs 1988). Similarly, when a reporter gene is flanked by a 5′*MAR* of the chicken lysozyme gene, expression is stimulated and sensitivity to chromosomal position of integration is diminished (Stief et al. 1989). Thus, *MARs* may serve as boundaries between chromatin domains.

4.4 Replication Origins, Terminators, and Centromeres

Amati and Gasser (1988) have shown that *ARS* and *CEN* elements are *SARs* in *Saccharomyces cerevisiae;* these sequences appear to recognize both common and different components of the binding sites found in nuclear scaffolds. In addition, a *MAR* localized in the human HPRT gene serves as an *ARS* in yeast (Sykes et al. 1988), and sequences which have been suggested to be putative replication origins for the dihydrofolate reductase gene may be near *MARs* (Dijkwel and Hamlin 1988). However, an analysis of the direction of replication fork movement within this locus suggests that *MARs* comap with termination regions (Handeli et al. 1989). Thus, evidence exists that a subfraction of *MARs* may be centromeres, replication origins and terminators.

5 Dysfunction of Chromosomal Loop Attachment Sites: Illegitimate Recombination

Illegitimate recombination breakpoints, although obviously rare in frequency, do not occur totally at random positions in the genome. Since those *MARs* which possess high affinity for topo II are likely to be a major class of sequences that interact with the enzyme in living cells, it is logical to predict that these elements may also be targets for illegitimate recombination, particularly in view of the elegant studies performed in model in vitro systems (Bae et al. 1988). In fact, we have localized *MARs* at sites of chromosomal DNA insertion, deletion, and translocation. Sequence analysis of the reciprocal products of the chromosomal translocation that occurred in the mouse plasmacytoma PC7183 reveals that only 14 bp was deleted from the *MAR* of the immunoglobulin κ gene and we have found that this precise region has four strong topo II cleavage sites. Furthermore, one cleavage site corresponds exactly to the sequence at the 5′-junction of the recombination event (Sperry et al. 1989). A *MAR* has been deleted from one of the two rabbit κ immunoglobulin genes, and *MARs* reside next to a LINE within the recombination junction of a human ring chromosome 21 (Sperry et al. 1989). These results, taken together with other accounts of non-homologous recombination in the literature (Table 2), lead to the proposal that a dysfunction of *MARs* is illegitimate recombination. Evidently, the association of topo II with *MARs* may impose a heavy genetic load upon these sequences, since under adverse conditions the enzyme apparently creates recombinogenic double-stranded DNA breaks.

Table 2. *MARs* at sites of illegitimate recombination

Example	Genetic event	Reference
Human		
Chr. 21 LINE	Insertion	Sperry et al. (1989)
Chr. 21	Ring formation	Sperry et al. (1989)
β-Globin Gene	Deletions	Jarman and Higgs (1988); Anand et al. (1988)
Mouse		
κ IgL	Translocation	Sperry et al. (1989)
Rabbit		
κ IgL	Deletions	Sperry et al. (1989)
Hamster		
Dihydrofolate reductase	Amplification	Dijkwel and Hamlin (1988)
Adenylate deaminase[a]	Amplification	Hyrien et al. (1987)

[a] Presumptive MAR.

6 Sequences at Chromosomal Loop Attachment Sites

MARs are at least 250 bp long, AT-rich ($\geq$ 65%), often contain the topo II consensus sequence of Sander and Hsieh (1985) and other AT-rich sequence motifs (see below), sometimes reside near *cis*-acting regulatory sequences (see above), and are evolutionarily conserved (Cockerill and Garrard 1986a, b; Cockerill et al. 1987; Gasser and Laemmli 1986a, b; Amati and Gasser 1988; Izaurralde et al. 1988; Mirkovitch et al. 1986, 1988). However, the sequences themselves are not sufficiently similar to cross-hybridize among themselves. Besides the topo II consensus, *MARs* often contain other multiple DNA sequence motifs, including A-boxes and T-boxes (Gasser and Laemmli 1986a), and the ATATTT and ATATTTTT motifs (Cockerill and Garrard 1986a). Other experiments suggest that the KpnI family of repetitive sequences might constitute a subclass of *MARs* (Chimera and Musich 1985; Beggs and Migeon 1989). The *Drosophila* histone gene *MAR* contains at least two barriers to exonuclease III (Gasser and Laemmli 1986b). The mouse κ gene *MAR* contains multiple protein-binding sites, including 11 barriers to exonuclease III when specifically bound to the matrix (Blasquez et al. 1989b), and numerous overlapping topo II binding and cleavage sites (Sperry et al. 1989). *MARs* may require a minimum length to function if they must wrap around multiprotein complexes, as is the case for the prokaryotic DNA gyrase, which protects 205 bp in DNase I footprinting experiments when bound to *REP* sequences, the likely prokaryotic counterpart of *MARs* (Yang and Ames 1988). Indeed, *MARs* appear to contain regions of bent DNA (Blasquez et al. 1989c; Homberger 1989). Therefore, we currently view a *MAR* as a relatively long element (> 250 bp) containing several classes of sequences that are binding sites for different proteins. Since functional assays for these sequences are only now becoming available, and the proteins that interact with these sequences largely remain to be identified, the precise DNA sequences that constitute a functional *MAR* remain to be determined. In summary, although it is not at present possible to predict a *MAR* from the nucleotide sequence of a given DNA segment, *MARs* have many characteristic features in common (Fig. 1).

Although the initial *MAR* we identified possessed several of the topo II consensus sequences of Sander and Hsieh (1985) (Cockerill and Garrard 1986a), in vitro analysis has revealed that the true sites of enzymatic fragmentation do not correspond to the consensus prediction (Sperry et al. 1989), nor do such sites correspond to another topo II consensus (Spitzner and Muller 1988). It is to be emphasized that only limited lengths of sequences have been studied in an effort to derive a consensus sequence and in these cases no attention has been placed

THE *MAR* SEQUENCE FAMILY

- (A+T)-rich, ≥ 250 bp
- TOPOISOMERASE II CONSENSUS SEQUENCES
- EVOLUTIONARILY CONSERVED
- POSITIONED BETWEEN GENES, SOMETIMES WITHIN INTRONS AND NEAR ENHANCERS
- RECOGNIZE ABUNDANT NUCLEAR BINDING SITES (≥10,000 PER NUCLEUS)

Fig. 1. Common features of *MARs* and *SARs*

on starting first with sequences which exhibit high affinity to *bind* the enzyme. Simply randomly selecting DNA sequences for in vitro cleavage site mapping has probably led to an incomplete picture for a bona fide topo II consensus cleavage sequence hierarchy, if one indeed exists. In summary, it seems clear that it is not always possible to predict a *MAR* from available topo II consensus sequences, nor is it possible to predict the actual sites of cleavage from such sequences.

7 Proteins at Chromosomal Loop Attachment Sites

Identification of the proteins that specifically bind to *MARs* remains incomplete. Although *MARs* possess topo II consensus sequences and often specifically bind this protein (see above), nuclei of non-dividing cells still possess organized chromosomal loops and abundant *MAR* sequence binding sites (Cockerill and Garrard 1986a; Cockerill et al. 1987; Phi-Van and Stratling 1988), even though they apparently lack topo II (Heck and Earnshaw 1986; Heck et al. 1988; Fairman and Brutlag 1988). Furthermore, not all *MARs* strongly bind topo II, whereas certain sequences that are *not MARs* exhibit marked binding specificity (Sperry et al. 1989). Thus, the ability to specifically bind topo II is neither a necessary nor a sufficient condition to specify a *MAR*. Clearly, other proteins must participate in chromosomal loop organization at these sequences.

Proteins other than topo II that mediate or are closely linked to *MAR* binding possibly may include the nuclear lamins, and/or SC2, a mitotic scaffold protein (Lewis and Laemmli 1982). In addition, tightly bound proteins, possibly covalently linked, have been reported associated with isolated DNA and these may participate in nuclear attachment (Avramova and Tsanev 1987; Cress and Kurath 1988). However, it is difficult to rule out artifactual induction of covalent bound formation in these cases (e.g., Blanco and Gottesfeld 1988). Clearly, in vivo crosslinking conditions will be necessary to prove unequivocal physical contact between *MARs* and the protein of interest for which a monospecific antibody probe is available.

8 Frequency, Regulation, and Nuclear Location of Chromosomal Loop Attachment Sites

The periodicity of *MARs* along eukaryotic DNA is variable, ranging from one every 5 kb for the *Drosophila* histone gene repeat (Mirkovitch et al. 1984) to none detectable in a 140 kb contiguous stretch of the human α-globin gene complex (Jarmin and Higgs 1988) (see also Table 1). Based on a limited number of studies, the average frequency so far seems to be roughly once every 30 kb in eukaryotic DNA.

An interesting question is whether attachment of *MARs* is developmentally regulated. Attachment patterns have been studied for tissue-specific or X-linked genes which are differentially expressed. In all these cases, attachment seems to be constitutive and not correlated with repression or transcription (Cockerill and

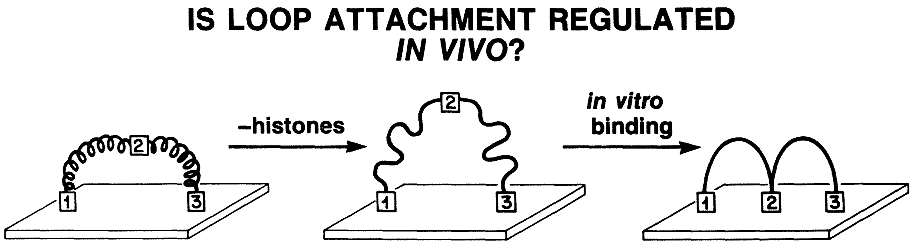

Fig. 2. Schematic view of a potential complication of the nuclear-halo technique in evaluating in vivo association of *MARs*. Three *MAR* sequences are depicted by the *numbered boxes*. *MAR*2 is not normally associated in vivo, but after removal of the histones with LIS the exposed sequence can associate with the scaffold since it is known that not all binding sites are occupied in such preparations

Garrard 1986a; Jarmin and Higgs 1988; Phil-Van and Stratling 1988; Greenstein 1988; Beggs and Migeon 1989). However, it is important to point out that the LIS nuclear-halo mapping procedure does not preclude the artifactual binding of a *MAR* which was not originally attached via the scheme shown in Fig. 2 (Gasser and Laemmli 1986a; Izaurralde et al. 1988). Identification of *MARs* by existing assays only gives information for potential in vivo contacts. Furthermore, Imler et al. (1987) have shown that segments within the *MARs* which flank the mouse immunoglobulin gene heavy chain enhancer act as negative regulators in fibroblasts, leading to the provocative possibility that modulation of attachment could regulate enhancer function (Scheuermann and Chen 1989). This idea is also consistent with the recent observations of Gasser and co-workers, which implicate loop formation as a mechanism for silencer action in the yeast mating type locus (Hofmann et al. 1989). Clearly, an in vivo crosslinking technique will be necessary to determine which of the potential attachment sites are actually used in living cells.

Another interesting question is whether or not *MARs* are closely associated with each other at the entry and exit points of a given chromosomal loop from the matrix, as appears to be the case for the *SARs* associated with the mitotic chromosomal scaffold (Paulson and Laemmli 1977). In the case of a Balbiani ring transcription loop in the polytene chromosomes of *Chironomus,* however, an electron microscopic study has shown that the loop anchoring sites are *spatially separated* from each other (Ericsson et al. 1989). Perhaps the use of DNA crosslinking reagents in the future may provide an answer to this question for genes in the diploid interphase nucleus.

At least 10,000 *MAR* sequence binding sites exist in matrices or scaffolds prepared from mammalian sources (Cockerill and Garrard 1986a; Izaurralde et al. 1988). Whether these sites reside at the nuclear periphery or internally is not known. Clearly, these sites must undergo considerable reorganization during the cell cycle, leading a series of complex supramolecular assembly and disassembly processes which will be difficult to dissect without a tractable genetic system (Fig. 3). A nuclear skeleton within the interphase nucleus composed of intermediate filaments has been reported (Capco and Penman 1983; Jackson

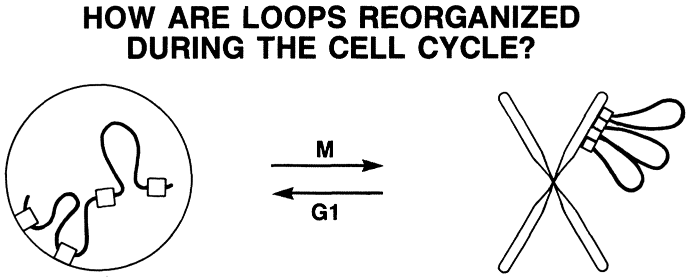

Fig. 3. Schematic view of the nuclear location and reorganization of chromosomal loops during the cell cycle. Loops in the interphase nucleus may be attached through *MARs* (*boxes*) being associated with the nuclear periphery or with internal structures. During mitosis these attachments sites must be reorganized into the mitotic chromosomal scaffold, in which the points of entry and exit of the DNA loops from this structure appear immediately next to each other. Whether the same, a subset, or a largely different set of attachment sites exist between the interphase and mitotic states remains to be investigated

and Cook 1988). Although nuclear matrices (Berezney and Coffey 1974) and scaffolds (Mikovitch et al. 1984) are operationally defined entities, the possibility of an underlying internal structure in the nucleus is exciting but remains controversial. Clearly, this framework could serve to organize chromosomal loops in association with other proteins, and create trafficking pathways for export of nuclear RNA and import of cytoplasmic constituents. Furthermore, the scaffold of mitotic chromosomes possesses a helical arrangement of topo II molecules (Boy de la Tour and Laemmli 1988). It seems clear that we are only beginning to appreciate the principles of nuclear order. Although this is a technically difficult area for study, it is clearly important and deserves considerable future attention.

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