Journal of Cellular Biochemistry 102:1067–1075 (2007)

PROSPECTS

Nuclear Architecture: Is It Important for Genome Function and Can We Prove It?

Julio Mateos-Langerak,[1] Sandra Goetze,[1] Heinrich Leonhardt,[2] Thomas Cremer,[2] Roel van Driel,[1] and Christian Lanctôt[2]*

[1]Swammerdam Institute for Life Sciences, University of Amsterdam, Amsterdam, The Netherlands
[2]Department Biologie II, Ludwig-Maximilians Universität, Martinsried, Germany

Abstract Gene regulation in higher eukaryotes has been shown to involve regulatory sites, such as promoters and enhancers which act at the level of individual genes, and mechanisms which control the functional state of gene clusters. A fundamental question is whether additional levels of genome control exist. Nuclear organization and large-scale chromatin structure may constitute such a level and play an important role in the cell-type specific orchestration of the expression of thousands of genes in eukaryotic cells. Numerous observations indicate a tight correlation between genome activity and nuclear and large-scale chromatin structure. However, causal relationships are rare. Here we explore how these might be uncovered. J. Cell. Biochem. 102: 1067–1075, 2007. © 2007 Wiley-Liss, Inc.

Key words: nuclear organization; chromatin; gene expression; high-throughput screening; gene positioning; macromolecular crowding; magnetic tweezers

NUCLEAR ORGANIZATION AND GENOME FUNCTION: AN UNRESOLVED QUESTION

It is well established that gene expression in higher eukaryotes is controlled at least at two levels [van Driel et al., 2003]. One involves binding of transcription factors to promoter and enhancer sequences and local remodeling of chromatin structure. The other is at the level of gene clusters that undergo functional transitions which affect all genes in the locus. A good example of the latter is the β-globin loci in mouse and human, which switch during hematopoietic differentiation from a silenced chromatin state to one that is permissive for gene expression [Fu et al., 2002]. Other examples are the MHC locus and the HoxB and HoxD gene clusters in mouse, which upon activation undergo major structural changes that can be visualized by light microscopy [Volpi et al., 2000; Chambeyron and Bickmore, 2004; Chambeyron et al., 2005; Morey et al., 2007]. These structural changes are most likely coupled to major epigenetic modifications at the loci. Whether additional levels of regulation exist, in particular at the level of nuclear organization, is an unresolved problem. If so, there would be broad implications for our understanding of the orchestration of gene expression across the mammalian genome [van Driel et al., 2003; Kosak and Groudine, 2004; Fraser and Bickmore, 2007; Lanctot et al., 2007; Misteli, 2007]. This article provides a brief review of our present knowledge on nuclear organization, and hints at

Grant sponsor: Sixth Framework Program of the European Community; Grant number: LSHG-CT-2003-503441; Grant sponsor: Instituts de Recherche en Santé du Canada and the Humboldt Stiftung. Deutsche Forschungsgemeinschaft and the Nationales Genom Forschungsnetz.

Julio Mateos-Langerak's present address is Institute of Human Genetics, Centre National de la Recherche Scientifique, Montpellier, France.

Sandra Goetze's present address is Institute of Molecular Biology, University of Zurich, Winterthurerstrasse 190, 8057 Zurich, Switzerland.

Christian Lanctôt's present address is Institute for Research in Immunology and Cancer (IRIC), Université de Montréal, Montreal, Quebec, Canada.

*Correspondence to: Christian Lanctôt, Institute for Research in Immunology and Cancer (IRIC), Université de Montréal, P.O. Box 6128, Station Centre-ville, Montreal, Quebec, Canada H3C 3J7.
E-mail: christian.lanctot@umontreal.ca

Received 3 July 2007; Accepted 9 July 2007

DOI 10.1002/jcb.21521

what we believe to be the experiments that need to be carried out in order to resolve a fundamental dilemma in the field. Does the cell control genome function, for example, gene expression, to some extent via changes in the spatial organization of the nucleus? Or, alternatively, are the observed changes in nuclear structure the result, rather than the cause, of changes in gene expression?

NUCLEAR ORGANIZATION IN ANIMALS: STATE OF THE ART

Present knowledge on higher order chromatin arrangements in animals is based mainly on the analysis of peripheral lymphocytes and a very limited number of cultured cell types such as fibroblasts. Little is known about possible differences in the nuclear organization of different cell types within their intact tissue environment. Furthermore, data comparing cell types generated during ontogeny are urgently needed. Therefore, we lack compelling evidence at the present to support or refute the hypothesis that "discrete 3D structures (each characteristic of a given differentiated state) develop from an omnipotent 3D structure of the zygotic genome" [Blobel, 1985]. Available evidence shows that the structural aspect of the nucleus is closely related to the differentiation state of the cell and that it changes in the diseased state [Zink et al., 2004; Gilbert et al., 2005]. Profound changes in nuclear organization were also observed during early stages of development and during postmitotic terminal differentiation of certain tissue cell types [Solovei et al., 2004; Merico et al., 2007].

Results of studies performed at the light microscopy level indicate that each chromosome occupies its own chromosome territory (CT) inside the nucleus (for review see [Cremer and Cremer, 2001, 2006a,b; Cremer et al., 2006]). The extent of intermingling between chromatin fibers of neighboring CTs is still an unresolved matter [Albiez et al., 2006; Branco and Pombo, 2006]. A recurrent theme in the field of nuclear organization is the differential distribution of gene-dense and gene-poor chromatin. In the rather spherical human lymphocyte nuclei, territories of gene-dense and highly expressed chromosomes are typically located more towards the center of the nucleus whereas gene-poor and weakly expressed ones are often associated with the nuclear envelope [Croft et al., 1999; Cremer et al., 2001]. A similar arrangement of early and late replicating chromatin was found in the nuclei of evolutionary distant species, for example, chicken [Habermann et al., 2001] and *Hydra vulgaris* [Alexandrova et al., 2003], and in the micronuclei of the spirotrichous ciliate *Stylonychia lemnae* [Postberg et al., 2005]. While a radial distribution of CTs was also noted in the ellipsoid human fibroblast nuclei, 3D fluorescent in situ hybridization (FISH) results revealed a chromosome size dependent pattern in this cell type: CTs of the largest chromosomes were typically observed close to the nuclear equator whereas CTs of small chromosomes were located remote from it [Bolzer et al., 2005; Neusser et al., 2007]. Interestingly, the radial distribution of gene-dense and gene-poor CTs was found to be recapitulated at the subchromosomal level, in that gene dense and highly expressed loci were also observed more towards the nuclear center and gene-poor and weakly expressed loci were located more towards the nuclear envelope [Goetze et al., 2007]. Several reports have provided correlative evidence between the 3D structure of individual CTs and parameters such as DNA content, gene density, transcriptional activity and replication timing of chromosomal subdomains.

Important aspects of large-scale[1] chromatin organization have been revealed by electron microscopy, showing that a considerable fraction of the interphase chromatin fiber is folded in relatively compact subchromosomal domains, in such a way that silent chromatin lies inside the domains whereas transcriptionally active chromatin lies at or near the surface of the domains, that is, the perichromatin area [Fakan, 1994; Cmarko et al., 1999]. Interestingly, Polycomb group (PcG) proteins, often associated with epigenetic silencing of genes, also accumulate in the perichromatin area, suggesting that both active and silenced transcription units are present in this region [Cmarko et al., 2003]. These and many other studies demonstrate a strong correlation between genome function, in particular transcription, and large-scale chromatin organization in the interphase nucleus. A major

[1]"Large-scale" structure is operationally defined as structural aspects that can be visualized by classical light microscopy.

challenge is now to design experiments to determine whether this correlation is a causal one. For instance, what would happen if the organization of chromosomes in territories or the radial distribution of highly active and less active chromatin in the nucleus would be disturbed? Would this result in changes in gene expression patterns? If the answer is affirmative, it might lead to an important and unexplored level of control of genome function. Alternatively, it may be that these aspects of nuclear organization are the result of intrinsic properties of chromatin which do not play an active role in gene regulation. Here we address this and related questions by pointing to experimental approaches that could be used to settle this issue.

NUCLEAR COMPONENTS INVOLVED IN LARGE-SCALE NUCLEAR ORGANIZATION

How can we explore the molecular mechanisms that control large-scale chromatin organization in the interphase nucleus? One approach is to identify the components that are involved, manipulate their activity and monitor the effect on nuclear organization and genome function. Recently, a small number of proteins that may play a role in maintaining nuclear architecture have been identified. We begin by discussing the properties of some of these proteins.

Special AT-binding protein 1 (SATB1) is a protein that binds to S/MAR sequences [Liebich et al., 2002] at the base of putative chromatin loops [Cai et al., 2006; Kumar et al., 2007]. In the nuclei of thymocytes, where SATB1 is most abundant, the protein forms a cage-like network that is thought to mirror the cell type specific spatial arrangement of the chromatin fiber and to be essential for proper gene expression [Cai et al., 2006]. Consistent with this idea, targeted mutagenesis or knock-down of the SATB1 gene leads to global dysregulation of gene expression and concomitant changes in the looping patterns of three loci that were investigated at the biochemical level [Yasui et al., 2002; Cai et al., 2006; Kumar et al., 2007]. CTCF is another nuclear protein that is involved in establishing long-range *cis* and *trans* chromatin interactions [Kurukuti et al., 2006]. Based on results of biochemical analyses and 3D FISH, Ling et al. [2006] suggested that CTCF mediates the physical interaction between the maternal H19/Igf2 allele and the paternal Wsb1/Nf1 allele. Knock-down experiments demonstrated that this is indeed the case and again revealed a causal link between gene expression and large-scale chromatin structure. Interestingly, CTCF binds to genomic sequences called boundary elements, or insulators, that might be involved in compartmentalization and clustering of the genome during interphase [Gaszner and Felsenfeld, 2006; Valenzuela and Kamakaka, 2006]. Evidently, SATB1, CTCF and related proteins as well as the genomic S/MAR and insulator elements are candidate components of large-scale chromatin organization in the interphase nucleus.

Another component that has been shown to affect large-scale chromatin structure is the nuclear lamina. Mutations in genes coding for structural proteins of the nuclear lamina or for proteins involved in their posttranslational processing give rise to a dramatic change in nuclear shape and reduce the amount of peripheral heterochromatin. Some of these mutations are associated with the Hutchinson–Gilford Progeria syndrome, which is characterized by premature aging of the affected individuals [Gruenbaum et al., 2005; Hennekam, 2006]. Despite intense investigation of the nuclear lamina, no systematic studies have been carried out that analyze the effects of changing the composition of this structure on nuclear organization and genome-wide transcriptional activity.

PcG proteins are also thought to play a role in large-scale nuclear organization. PcG proteins bind to Polycomb response elements (PREs), thereby maintaining the silenced state of nearby genes. In *Drosophila melanogaster* PREs that are located at very distant loci in the genome, even on different chromosomes, cluster in the interphase nucleus when the genes controlled by these PREs are silenced [Grimaud et al., 2006; Vazquez et al., 2006].

Finally, methylcytosine binding proteins (MeCPs and MBDs) represent an intriguing link between nuclear genome architecture, differentiation and disease. The founding member of this protein family is MeCP2, which specifically binds methylated DNA and is upregulated during differentiation, for example, myogenesis and neurogenesis. This differentiation-dependent upregulation is accompanied by large-scale genome reorganization, in particular by progressive clustering of centromeric and pericentromeric heterochromatin. Overexpression of MeCP2

caused clustering of heterochromatin in undifferentiated myoblasts, indicating a causal relationship between MeCP2 expression level and genome organization [Brero et al., 2005]. Mutations affecting MeCP2 function are linked to Rett syndrome, a frequent neurodevelopmental disease [Amir et al., 1999].

HIGH-THROUGHPUT APPROACHES TO IDENTIFY NEW COMPONENTS INVOLVED IN NUCLEAR ORGANIZATION

How can novel gene products that are involved in establishing and maintaining nuclear organization be identified? A powerful approach is to combine high-throughput light microscopy and RNAi technology [Pepperkok and Ellenberg, 2006]. Specific aspects of large-scale chromatin structure could be monitored quantitatively in cultured cells by expressing a fluorescently tagged core histone protein [Kanda et al., 1998]. However, considerable cell-to-cell variation in the spatial distribution of chromatin creates a major hurdle in the analysis of such image data sets [Goetze et al., 2007]. Therefore, an initial study should carefully determine what structural parameters to use in a quantitative analysis. If this problem can be solved, an automated and high-throughput analysis could be carried out to identify candidate proteins that are important in large-scale nuclear organization. By exploiting the possibilities of multicolor imaging, the initial study could be expanded to screen simultaneously for changes in the large-scale structure of chromatin and in the distribution of other nuclear structures, such as splicing factor domains ("speckles") [Lamond and Spector, 2003] and nucleoli. If gene products can be identified that, when knocked down, result in changes in nuclear organization, the next step would be to elucidate their role in gene regulation. These gene products would represent ideal candidates to include in a more detailed analysis of the molecular mechanisms underlying the relationship between genome function and large-scale chromatin structure in the nucleus. The approach that we propose here has limitations since it cannot be used to assay nuclear activities that can be carried out by sets of different proteins. For instance, the looping of chromatin is likely to be the result of the action of a variety of proteins that can complement each other, making it uncertain that knocking down a single gene product will have an effect that is measurable at the light microscopy level.

IN SITU MANIPULATION OF NUCLEAR ORGANIZATION BY GENETIC ENGINEERING

The position of a gene in the nucleus has been shown to correlate with its transcriptional activity. Proximity to the nuclear envelope or pericentromeric heterochromatin is generally associated with gene silencing [Fisher and Merkenschlager, 2002; Williams et al., 2006; Goetze et al., 2007; Landeira and Navarro, 2007]. In cycling cells, the position of a gene is established during chromosome decondensation at the end of telophase and in early G1 [Walter et al., 2003; Thomson et al., 2004]. After that, chromatin mobility becomes constrained to a small region of the nucleus and the position of genes at the large-scale chromatin level is more or less fixed. Interestingly however, a gene can be relocated upon transcriptional activation, as was shown for a transgene that moved towards the nuclear interior after binding a strong transcriptional activator [Chuang et al., 2006]. Furthermore, although chromatin mobility tends to be more confined in differentiated cells [Thakar and Csink, 2005; Thakar et al., 2006], examples of dramatic changes in the nuclear architecture of postmitotic cells have been reported [Solovei et al., 2004]. These and other observations underscore the correlation between the activity of a gene and its position and local environment inside the interphase nucleus. However, they do not tell whether repositioning is causing a change in activity or is the result of activation. We suggest that changing the position of a locus in the cell nucleus and monitoring the effect on its transcription and replication might be one way to determine whether causal relationships exist between nuclear organization and gene activity.

In fact, such a causal relationship has already been shown in the yeast *S. cerevisiae* by artificially tethering a de-repressed mutant of the HM mating type locus to the nuclear membrane. This was done by engineering GAL4 binding sites in the HM locus and expressing the GAL4 DNA-binding domain fused to an integral nuclear membrane protein [Andrulis et al., 1998]. Targeting of the locus at the nuclear periphery was associated with transcriptional silencing,

indicating that in this case repositioning leads to changes in transcriptional activity. A similar experimental approach is feasible in higher eukaryotes, including in mammalian cells, using the lacO-lacR (lac operator/lac repressor) technology developed by the Belmont [2001] group. This technique is based on the high affinity of the lacR protein for the lacO sequence, and has been used extensively to analyze the nuclear position of transgenes during the cell cycle in various cells [Marshall et al., 1997; Vazquez et al., 2001; Chubb et al., 2002]. Insertion of an array of lacO sites into a highly active region of the genome, as defined for instance by the human transcriptome map [Caron et al., 2001; Versteeg et al., 2003], and expression of a construct encoding a nuclear lamina protein fused to the lacR protein may result in tethering of the locus to the nuclear envelope. Moreover, the recent discovery that small antigen-binding fragments derived from antibodies of camelidae bind their antigens in living mammalian cells offers new possibilities to analyze and manipulate nuclear architecture [Rothbauer et al., 2006]. The effect of nuclear repositioning on gene expression close to and further away from the lacO array will be very informative about any relationship between nuclear localization and gene activity. Such studies should be combined with structural analysis using FISH, not only to confirm the repositioning of tagged chromatin in the nucleus, but also to analyze changes in the nuclear position and structure of the corresponding chromosome. An important question is whether the whole chromosome is repositioned, or whether tethering only results in the looping out of that part of the chromosome that carries the lacO array. It will be essential to determine to what extent repositioning leads to changes in gene expression and epigenetic state: are only the genes close to the envelope binding site affected or are loci further away on the linear genome also affected, and, if so, where are these distant loci physically localized: close to the envelope or more inside the nucleus? Although the lacO-lacR technology can in principle be used to target loci to any nuclear compartment, the nuclear lamina is probably the most tractable nuclear compartment for this type of experiments because a number of proteins are known to be exclusively localized to the lamina and not to occur in a soluble form which would compete with the lamina-associated binding [Gruenbaum et al., 2005]. Moreover, the lamina assembles onto the chromatin after mitosis [Leung et al., 2004], which means that binding of the locus to the lamina does not depend on diffusion in the highly concentrated environment of the interphase nucleus.

ALTERNATIVE WAYS TO MANIPULATE NUCLEAR ORGANIZATION

An alternative but unexplored way to change nuclear organization is to exert forces on chromatin in the intact cell. A promising technique relies on the use of magnetic tweezers in combination with magnetic nanosized beads [de Vries et al., 2005]. So far, this technology has been used in the nucleus only for relatively large beads (about 1 μm in diameter) that are microinjected into the nuclei of living cells [de Vries et al., 2007]. Under the experimental conditions tested, the nucleus behaved as an elastic medium through which the bead could not be displaced. Smaller magnetic particles might move over larger distances inside the nucleus. Nanobeads coated with, for instance, an antibody against core histones would firmly bind chromatin at the site of microinjection, which may then be repositioned at will using magnetic tweezers.

Another way to change nuclear organization is to manipulate macromolecular crowding [Hancock, 2004a,b]. Macromolecular crowding can increase the strength of molecular interactions in environments that have a high concentration of macromolecules (in the 100 mg/ml range inside of cells) by two orders of magnitude or more and can be induced by any type of bio-macromolecule, such as proteins, and by large exogenous molecules, such as polyethylene glycol [Ellis, 2001; Minton, 2006]. Lowering the macromolecular concentration in cells results in the reversible dissociation of nucleoli and PML bodies [Hancock, 2004b]. This can be achieved in living cells by allowing nuclei to expand in medium of low monovalent cation concentration. The effect on large-scale chromatin structure and genome function, in particular transcription, has not yet been explored under these conditions. It is conceivable that reorganization of chromatin structure will be easier in a less crowded environment, because many chromatin interactions are weakened. It is also conceivable that the architecture of the normally compact chromosome territories could change considerably at lower macromolecule

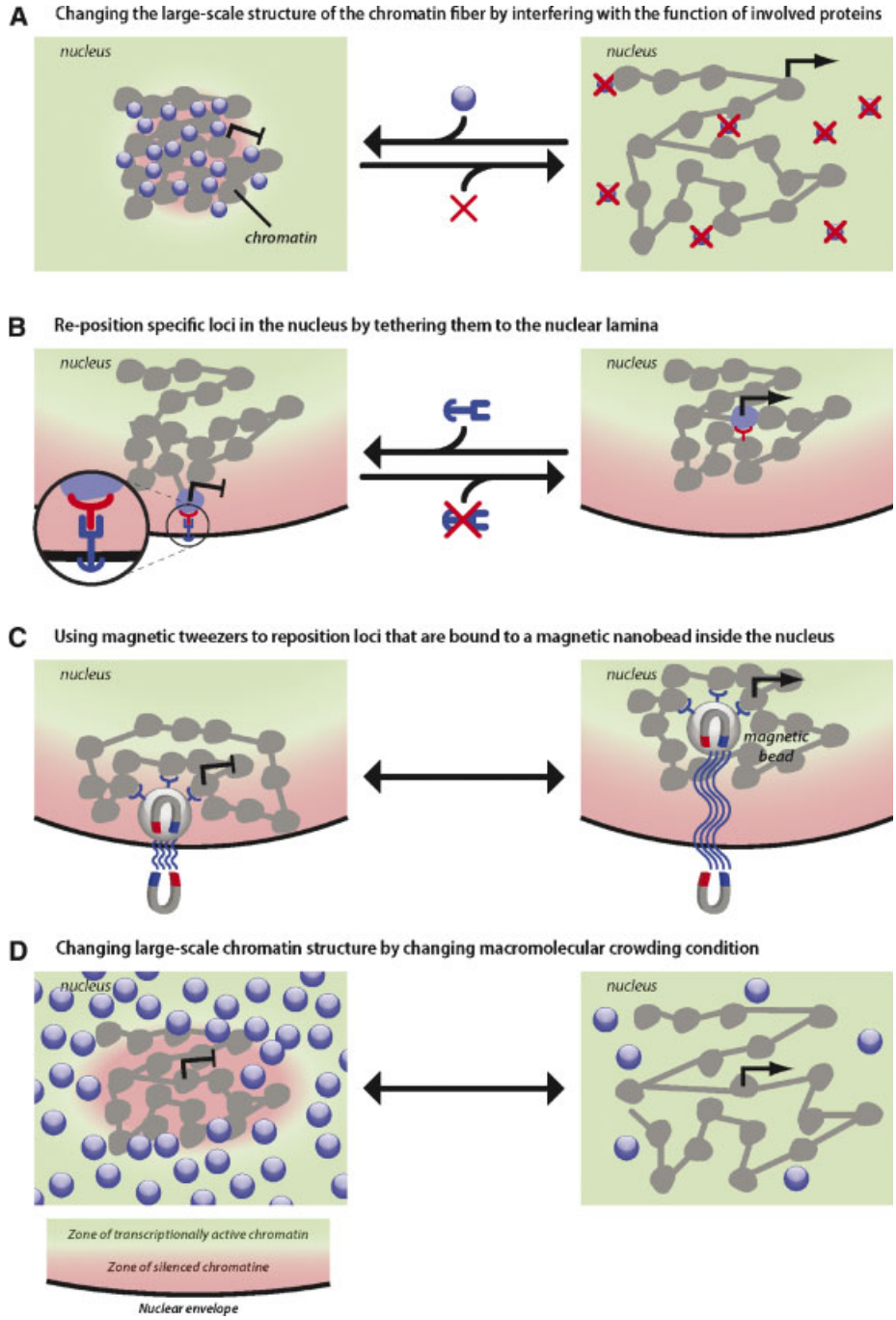

Fig. 1. Experimental approaches to manipulate nuclear architecture. In all panels, chromatin is depicted as a network of 1 Mb domains. Silencing and activating environments are colored pink and green, respectively. Blunted arrows (panels on the **left**) indicate transcriptional repression whereas forward arrows (panels on the **right**) indicate transcriptional activity. **A**: Adding (e.g., via overexpression, **left panel**) or removing (e.g., via knockdown, **right panel**) proteins involved in the large-scale organization of chromatin result in changes in gene expression. **B**: Changing the positioning of a gene from the nuclear periphery (**left panel**) to the nuclear interior (**right panel**) triggers its transcription. In the silenced state (left panel), the gene is anchored via a lac repressor moiety (red) that binds neighboring sequences and that is fused to a lamin-binding moiety (blue). **C**: Magnetic attraction (**left**) or repulsion (**right**) can be used to alter the positioning of a nanobead within the nucleus. If the nanobead is coated with, for instance, histone-binding moieties (blue), then movement of the nanobead should be accompanied with displacement of the bound chromatin, in this case from the nuclear periphery to the nuclear interior. This chromatin movement could trigger transcriptional activation, as in B. **D**: Increasing (**left**) or lowering (**right**) macromolecular crowding may result in changes in gene expression. This can be achieved by changing the ionic concentration in the extracellular medium. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

concentration; intermingling between chromosomes may increase. A method to obtain the opposite effect has recently been reported. Indeed, the Cremer group was able to reversibly compact chromatin in living cells by transferring cultured cells to medium of high osmolarity [Albiez et al., 2006]. It is likely, although not proven, that this procedure results in an increase in the intranuclear concentration of macromolecules, thereby increasing crowding and thus compaction of chromatin. Importantly, the effect of increased crowding on biological structure is reversible; biological functions are restored after a return to normal conditions. Manipulating intracellular macromolecular crowding conditions appears to be a promising route towards changing large-scale chromatin structure in vivo, thereby allowing causal relationships between nuclear organization and genome function to be identified.

CONCLUSION

In this article we described a number of different approaches to resolve the fundamental question of whether cells actively control the spatial organization of the cell nucleus, and in particular large-scale chromatin structure, to regulate genome function. We outlined several fundamentally different experimental routes to explore the relationships between nuclear organization and gene expression. These are depicted on Figure 1. If relationships of a causal nature exist, then a novel and exciting new field of gene control mechanisms will have been opened.

ACKNOWLEDGMENTS

We thank I. Solovei, Ludwig-Maximilians University Munich for skillful help with Figure 1. This work was supported by the Sixth Framework Program of the European Community as part of the 3DGENOME program (Contract number: LSHG-CT-2003-503441). C.L. was supported by fellowships from the Instituts de Recherche en Santé du Canada and the Humboldt Stiftung. Work by T.C. has been funded by the Deutsche Forschungsgemeinschaft and the Nationales Genom Forschungsnetz (NGFN).

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