Experimental evidence for the influence of molecular crowding on nuclear architecture

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Summary

Many compounds in the cell nucleus are structurally organized. To assess the influence of structural organization on nuclear function, we investigated the physical mechanisms of structure formation by using molecular crowding as a parameter for nuclear integrity. Molecular crowding promotes compaction of macromolecular compounds depending on their size and shape without the need for site-specific interactions. HeLa and MCF7 cells were incubated with hypertonic medium to increase crowding of their macromolecular content as a result of the osmotic loss of water. Supplementation of sucrose, sorbitol or NaCl to the growth medium shifted nuclear organization, observed by fluorescence and electron microscopy, towards compaction of chromatin and segregation of other nuclear compounds. With increasing hypertonic load and incubation time, this nuclear reorganization proceeded gradually, irrespective of the substances used, and reversibly relaxed to a regular phenotype upon re-incubation of cells in isotonic growth medium. Gradual and reversible re-organization are major features of controlled de-mixing by molecular crowding. Of fundamental importance for nuclear function, we discuss how macromolecular crowding could account for the stabilization of processes that involve large, macromolecular machines.

Key words: Chromatin compaction, Excluded volume effect, Microcompartmentalization

Introduction

Many compounds in the cell nucleus are non-randomly distributed (Schul et al., 1998; Dandr and Misteli, 2001; Misteli, 2005), indicating microcompartmentalization beyond the level of stoichiometric interaction of reaction partners. But by what mechanism is this structural organization established?

Different types of structural organization can be observed in the nucleus. With chromatin for example, interchromosomal neighborhoods compact territorially into domains, which can be visualized by the selective labeling of chromosome bands, arms and whole chromosomes (Manuelidis, 1985; Scharadin et al., 1985; Lichter et al., 1988; Leitch et al., 1991; Dietzel et al., 1998; Cremer et al., 2006). Functional importance has been assigned to this territorial organization of chromatin, on the assumption that genomic sequences inside domains are less accessible to regulatory factors compared with those at the surface (Zirbel et al., 1993; Cremer et al., 1993; Kurz et al., 1996; Scheuermann et al., 2004; Cremer et al., 2006). Apart from this 'processive' compaction of chromatin, replication timing reveals another type of pattern: Isochronously replicating domains stably persist through cell divisions (Jackson and Pombo, 1998; Zink et al., 1999; Zink, 2006). A third type of chromatin organization is reflected by the concentration of more densely packed chromatin in peri-nuclear and peri-nucleolar regions. Apart form the organization of chromatin, many nuclear proteins and ribonucleoprotein particles are enriched in domains of characteristic size, shape and composition. The structure of some of these domains, notably nucleoli, replication factories and transcription foci, relates to their dedicated activity. Thus, the typical organization of nucleoli into fibrillar centers, fibrillar components and granular components expresses distinct processes in the formation of ribosomal subunits (Hernandez-Verdun, 2006). The structural integrity of other entities, such as speckles, Cajal bodies and PML bodies, appears less determined by productive activity. Speckles, for example, still persist upon polymerase II inhibition when splicing ceases (Huang et al., 1994).

Structural organization of the cell nucleus involves different levels of pattern formation. Modifications such as SUMOylation, which is required for PML to accumulate in PML bodies (Maul et al., 2000; Shen et al., 2006), and phosphorylation, which releases components from speckles (Misteli et al., 1998), are switches at the level of information transfer. However, molecules cannot make decisions such as where to go if phosphorylated and where else to go if not. Therefore, at a physical level, their localized accumulation reflects an increased length of stay. Both the site-specific interaction with a lattice such as the nuclear matrix, and the phase separation by non-specific interactions are possible mechanisms by which nuclear compounds might accumulate in local microcompartments.

The existence of a nuclear matrix had been proposed for a long time [for a historical background see Pederson (Pederson, 2000)]. However, a global matrix can barely account for all the spatial and temporal flexibility of nuclear organization (Hancock, 2000; Pederson, 2000). Nevertheless, skeletal elements do exist in the nucleus: the lamins, which are intermediate filament proteins of the nuclear lamina and structurally link peripheral chromatin via the nuclear envelope to the cytoskeleton (Grüenbaum et al., 2005). As a result of...
Hypertonic incubation causes compaction of chromatin in living cells: MCF7 cells were incubated for 20 minutes with growth medium enriched with varying concentrations sucrose as indicated. The cells were fixed and stained with ToPro3 to observe the chromatin distribution by confocal laser scanning microscopy. The confocal sections demonstrate the progress in chromatin compaction with increasing load of sucrose. Neither chromosome bands nor individual chromosomes became discernible. Bar, 10 μm.

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Results

The influence of water stress on the nuclear architecture of MCF7 and HeLa cells was investigated by adding sucrose, sorbitol or NaCl to the growth medium. Re-arrangements of chromatin and the SC35 domains were assessed by fluorescence light microscopy. Electron microscopy was employed to reveal re-organization of the nucleus at the ultrastructural level.

Hypertonic treatment provokes reversible chromatin compaction in living cells

The effect of hypertonic treatment most conspicuous by light microscopy is compaction of chromatin (Fig. 1). Using sucrose as the hypertonic agent, 40 mM supplied to the growth medium (DMEM) had no visible effect. However, after a 20-minute incubation in 80 mM sucrose medium, chromatin distribution shifted to a coarser pattern. In 160 mM sucrose medium, incubation in 80 mM sucrose medium, chromatin distribution appeared consistently reduced. Individual chromosomes or banding patterns reminiscent to metaphase-like chromatin compacted into portions, which became even more distinct with higher sucrose concentrations, up to the experimental limit of 640 mM sucrose. Individual chromosomes or banding patterns reminiscent to metaphase-condensation were not observed.

MCF7 and HeLa cells survived and also divided in medium containing 160 mM sucrose. After overnight incubation, the chromatin compaction appeared consistently reduced. Furthermore, after 3 weeks of culture in the hypertonic medium, chromatin distribution resembled that of normally grown cells (Fig. 2). By contrast, cells could not adapt to 320 mM sucrose and died overnight.

At 320 mM sucrose, a further process affected chromatin organization: the peripheral chromatin separated from the nuclear lamina, giving rise to a new nuclear compartment, the
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‘peripheral layer’, which was visibly free of chromatin. This process was observed in MCF7 as well as in HeLa cells (Fig. 3A). Formation of the peripheral layer involved complex structure formation, amongst others the establishment of SC35-positive foci along the outer border of peripheral chromatin (Fig. 3B). Remarkably, this process was not specific for sucrose. Sorbitol and NaCl had the same effects when applied at similar osmotic loads. By contrast, dextran of 10 kDa and 41 kDa in size, applied at a weight/volume concentration of 10%, which is equivalent to 320 mM sucrose, did not visibly influence chromatin organization (Fig. 3B).

The behavior of chromatin upon incubation with hypertonic media versus incubation with dextran changed upon digitonin permeabilization of cells (Fig. 4). Although permeabilization of the cell membrane eliminated compaction by NaCl and sucrose (sucrose not shown), chromatin compaction was then inducible by dextran. In particular, dextran still promoted chromatin compaction in a sequence following initial compaction by hypertonic media and de-compaction by digitonin.

HeLa cells stably transfected with H2B-GFP allowed the live observation of the compaction process (Fig. 5). The initial response to 320 mM sucrose administration was quick with most of the compaction process accomplished within 60 seconds (Fig. 5A). However, compaction proceeded further over 20 minutes with densitometry of the image series showing a continuous approach towards a final compaction level (Fig. 5C). As chromatin compaction proceeds, low-density forms still persist, such that the width of the distribution increases in parallel with its mean value. No distinct intermediate or terminal state of chromatin compaction was detected.

MCF7 and HeLa cells proliferated in hypertonic media up to about 490 mOsm. The higher loads of 320 mM sucrose, 320 mM sorbitol or 160 mM NaCl, however, were lethal: cells detached and died overnight. Nevertheless, even the peripheral layer was reversible and cells recovered to regular phenotype and productive growth at the normal rate, when returned to normal growth medium within 1 hour of hypertonic treatment. Throughout this compaction–de-compaction cycle, the initial
heterogeneity of chromatin distribution remained remarkably preserved (Fig. 5B). Note that instability in the hydration of the medium during observation did not allow us to extend the experiment for longer periods of time. Chromatin compaction started to resume after about 15 minutes because the medium was dried out by the CO₂ flush (not shown). However, histogram analysis of the documented part of the process also demonstrated a continuous shift, in this case towards the initial distribution (Fig. 5C).

Hypertonic treatment promotes the segregation of many nuclear compounds

Besides chromatin compaction, hypertonic treatment encompassed various other nuclear re-arrangements, visible by electron microscopy (Fig. 6), suggesting that hypertonic shock drives many nucleoplasmic compounds into differential segregation. At present, the significance of these segregation patterns is uncertain and descriptive denotations are chosen to underline their currently unknown composition.

Ultrastructural investigation of cells incubated for more than 15 minutes with either 320 mM or 160 mM sucrose medium before fixation revealed that their chromatin was compacted into distinctly delimited portions of homogeneous fine granularity (Fig. 6A,B). This was in stark contrast to control cells where the chromatin distribution was more dispersed in structures ranging from coarse granular to fine fibrillar textures (see Fig. 6C). Establishment of ‘dense bodies’ throughout the interchromatin space also appeared to be the same in both hypertonic conditions.

Upon incubation with 320 mM sucrose medium, a fine fibrillar material segregated in lacunas of distinct zoning. This segregation pattern was less distinct when cells were treated with the lower load of 160 mM sucrose: although local segregation of fine fibrillar material became evident, these areas appeared cloudy with fuzzy borders.

The peripheral layer, which appeared upon incubation with 320 mM sucrose medium, was of complex structural composition. Similarly to the interchromatin space of the same cells, the major part comprised a mass of fine fibrillar material, which filled the gap between peripheral chromatin and the nuclear envelope. Only spherical bodies of very low contrast, which were frequently found embedded in the peripheral layer, interrupted the otherwise homogeneous appearance. The interface with the retracted chromatin was decorated with small clusters of interchromatin granules. No intermediate signs of peripheral layer formation were found in cells treated with 160 mM sucrose. Adding dextran to the growth medium of living cells had no noticeable effect on nuclear architecture (Fig. 6C).

Discussion

The present study aims to demonstrate that macromolecular crowding influences nuclear architecture. The experimental approach of the study is based on the presumption that hypertonic shock enforces crowding in the nucleus owing to the osmotic extraction of water from the cells. We observe that nuclear organization gradually shifts towards compaction of chromatin and segregation of other nuclear compounds with increasing hypertonic load, and reversibly adopts a regular phenotype when cells are returned to normal, isotonic, growth medium.

Nuclear re-organization depends on the osmotic load of incubation

The quality and degree of nuclear re-organization reproducibly depended on the osmotic load of the incubation medium, irrespective of the osmotrope used. One distinct effect was the formation of the peripheral layer, i.e. the separation of peripheral chromatin from the nuclear rim and establishment of SC35-positive speckles at the new peripheral location. Added at similar osmotic loads, sucrose, sorbitol and NaCl all
caused this rather complex re-arrangement. In comparison, the osmotically ineffective dextran had no noticed influence on the nuclear architecture of intact cells.

Sucrose and sorbitol cannot freely permeate living cells (Jacobs, 1952). Therefore, they cannot act directly by their chemical nature. Their impact on nuclear organization rather relates to osmotic effects, notably loss of water from the cells. Sodium and chloride ions can cross the membrane barrier only across controlled channels. Thus, hypertonic saline also affects the water balance of the cell osmotically in so much as ion fluxes remain unchanged.

Cells potentially react to osmotic stress by compensating volume regulation, involving changes of ion flux across membrane channels and the production of so-called osmolytes (Lang et al., 1998). Therefore, our observations could relate to secondary effects of volume compensation rather than the increase in molecular crowding upon the initial loss of water. However, according to Tivey et al. (Tivey et al., 1985), HeLa cells do not enable regulatory cell volume increase during the first 2.5 hours of hypertonic incubation. Furthermore, Gilles et al. (Gilles et al., 1995) demonstrate for L929 cells, that hypertonic chromatin compaction no longer occurs after a long adaptation of the cells to hypertonic growth conditions. Hence, chromatin compaction is not a sign of compensatory volume regulation, rather volume regulation compensates for it. Accordingly, we observed reduced compaction after overnight incubation with 160 mM sucrose.

Fig. 6. Hypertonic shock causes a complex pattern of segregation of nuclear compounds. (A) Upon in-vivo incubation of MCF7 cells with 320 mM sucrose medium and subsequent preparation for electron microscopy, ultrathin sections show chromatin (ch) as distinct portions of homogeneous, dense granularity. Lacunas of fine fibrillar material (fm) comprise a major fraction of the interchromatin space. They frequently include body-like structures of very low contrast and spherical shape (sb). Between chromatin and the fine-fibrillar lacunas, granular and fibrillar parts were arranged in various ultrastructural units, among them interchromatin granule clusters (ig), and dense bodies (db). At the nuclear periphery, the peripheral chromatin retracted by several hundred nanometers giving rise to the peripheral layer (double-headed arrow), which predominantly comprises fibrillar material like that of the interchromatin space. Its interface with the retracted chromatin is defined by several structures, among them small clusters of interchromatin granules (ig). The nucleolus (no) is of compact shape and homogeneous granular composition. Association with perinucleolar chromatin appears reduced. (B) Treatment with 160 mM sucrose medium caused intermediate states of nuclear re-organization. Although chromatin (ch) compacted to portions of similar texture, and dense bodies (db) arose as with 320 mM sucrose load, fine-fibrillar material (fm) did not segregate and separate from other nucleoplasmic compounds as sharply, e.g. interchromatin granule clusters (ig). Furthermore, peripheral and perinucleolar chromatin still associated with the nuclear envelope and the nucleolus (no), respectively. (C) Incubation of cells with 10% dextran of 41 kDa size had no influence on nuclear ultrastructure. Like control preparations, chromatin (ch) remained dispersed and did not cluster into distinct portions. Fine-fibrillar material did not segregate from interchromatin granule clusters (ig) and chromatin. The nucleolus (no) is of normal, polymorphous appearance, differentially organized into fibrillar and granular parts, and associated with perinucleolar chromatin. Bars, 1 μm.
Crowding agents promote chromatin compaction
Chromatin compaction by hypertonic treatment of cells might be attributed to the rise of intercellular ion concentrations (Robbins et al., 1970; Delpire et al., 1985; Albiez et al., 2006), or of some other endogenous factor. Multivalent cations promote the condensation of DNA and chromatin (Bloomfield, 1998; Hansen, 2002). However, presuming that only water leaves the cell, no change in the ratio of counter ions to charged groups of chromatin could account for the observed shift in chromatin compaction. Nevertheless, permeabilization of the cells by treatment with digitonin abolished the potential of the hypertonic medium to promote chromatin compaction. Chromatin then appeared even less structured than in normal cells, suggesting that essential factors for chromatin compaction become depleted. However, chromatin compaction could still be induced by adding dextran to the medium of permeabilized cells. Thus, some parameter other than the concentration of ions and endogenous factors contributes to chromatin compaction.

Does molecular crowding affect nuclear architecture?
Taking into account the fact that the nucleoplasm is crowded owing to the abundance of macromolecules and macromolecular particles, reduction of the solvent (water) intensifies crowding. Volume exclusion strikes many compounds within a large scale of possible impact, depending on their size and shape. Hypertonic treatment not only promoted chromatin compaction. As demonstrated by electron microscopy, many other nuclear compounds segregated into distinct structural units. Accordingly, microcompartmentalization driven by the volume exclusion effect in crowded media accounts well for the observed complexity of nuclear re-organization.

As hypertonic environments extract water from the cells, the cellular volume decreases and the macromolecular content becomes more crowded. Although the regular phenotype of the nucleus represents the regular degree of crowding, enforced crowding shifts the system towards further segregation and compaction of some of its compounds. Upon permeabilization with digitonin, the cell equilibrates with the external medium, and crowding-dependent organization disappears concordantly. The macromolecular dextran has no obvious impact on the nuclear architecture of intact cells. Since dextran adds no relevant osmotic load to the medium it does not account for significant loss of water from the cells. However, macromolecular dextran acts as a ‘crowder’ itself. Its potential to promote chromatin compaction in permeabilized cells thus perfectly supports the model that molecular crowding influences nuclear architecture and that this is a cause of the nuclear re-organization upon hypertonic shock.

Hypertonic shock re-organizes the nucleus within the realms of its natural potential
Hypertonic treatment did not denature the cells. Even gross rearrangements, such as formation of the peripheral layer, reversibly relaxed within minutes of re-incubation in normal growth medium. Segregation patterns observable upon hypertonic treatment may thus represent the forced expression of regular, functional organization.

Time-lapse studies of chromatin compaction in HeLa H2B-GFP cells demonstrated the continuous approach of a final compaction level. Chromatin structure did not appear to switch among distinct condensation states. How does this relate to physiologically dense chromatin such as mitotic chromatin and heterochromatin?

As demonstrated by Robbins et al. (Robbins et al., 1970) and Pederson and Robbins (Pederson and Robbins, 1970), hypertonic shock does not create mitotic chromatin condensation. Concordantly, we did not observe individual chromosomes or mitotic banding. Furthermore, mitotic chromosome condensation is a complex process of congruent sister chromatid compaction and separation (Hirano, 2005; Nasmyth and Haering, 2005), which strictly depends on completion of replication. However, in our experiments, chromatin compacted throughout the entire population of the non-synchronous cell cultures.

The other type of physiologically condensed chromatin, heterochromatin, is not homogeneously defined. Biochemically different versions of heterochromatin exist, depending on the localized action of different chromatin architectural proteins (McBryant et al., 2006). Furthermore, biochemically defined heterochromatin does not necessarily match microscopically dense chromatin (Cmarko et al., 2003). Hypertonic compaction of chromatin, which we report here, was a global event, apparently involving all the chromatin of the nucleus. The process was reversible to an extent that the initial, heterogeneous pattern of chromatin distribution remained preserved within the experimental limits (see also Albiez et al., 2006), suggesting that endogenous markers of differential chromatin organization exist, which persist during hypertonic chromatin compaction. Murphy and Zimmermann (Murphy and Zimmermann, 1995) demonstrated that macromolecular crowding cooperatively supports DNA-binding factors in the compaction of lambda DNA. As DNA-binding factors add size to particular regions of DNA, they shift excluded volume-driven compaction to a weaker level of crowding than is required for unmodified chromatin. Thus, although compaction is forced when increasing the degree of crowding experimentally, the endogenous pattern will be restored upon re-incubation of cells at physiological conditions. Alternatively, crosslinking activities might stabilize the endogenous compaction state (Albiez et al., 2006). Crosslinkers need to be bivalent at least; however, ‘volume-exclusion modulators’ are functional at only a single binding site. Having the potential to proceed interchromosomally in a processive manner, crowding-induced compaction could account for the general observation that chromosomes are territorially organized (Lichter et al., 1991; Dietzel et al., 1998). By contrast, to promote this kind of territorial compaction by crosslinkers would require elaborate sensing capabilities to distinguish cis from trans interactions.

Molecular crowding potentially stabilizes nuclear processes
Our results suggest that macromolecular crowding is a parameter for the microcompartmentalization of the cell nucleus. Phase separation is one consequence of macromolecular crowding among others like the slow down of diffusion (Ellis, 2001), the obstruction of diffusion characteristics (Banks and Fradin, 2005), and the increase of thermodynamic activities (Minton et al., 1992). With respect to microcompartmentalization of the cell nucleus, three properties of the excluded volume effect appear of particular
interest: (1) Microcompartmentalization does not depend on crosslinking interactions; (2) Microcompartmentalization is not bound to a pre-established spatial network; (3) Compaction by volume exclusion is cooperative. Although molecular crowding promotes bundle formation upon directional ordering of elongated compounds, and may even cause the formation of networks, pattern formation is not focussed at pre-determined local nodes and along pre-determined orientations without the additional influence of modulating factors (Kulp and Herzfeld, 1995; Herzfeld, 2004). As absolute placement is an intrinsic property for interaction with a pre-formed lattice, a nuclear matrix would provide a higher level of order. However, crowding effects and matrix interaction are not mutually exclusive and can well support each other.

Cooperativity of compaction could be instrumental for processes like RNA polymerization and splicing, which use multi-molecular machinery. Assuming that subunits bind independently, the probability that an essential factor is missing increases with the number of subunits involved. Therefore, cooperativity becomes essential for the stable association of many factors into large complexes. In contrast to cooperativity of binding, association supported by volume exclusion intensifies as the complex grows in size (Ellis, 2001; Marenduzzo et al., 2006a). In this sense, molecular crowding could function as an environmental parameter to stabilize individual nuclear processes.

Materials and Methods

Cells

HeLa cells stably transfected with histone H2B-GFP (kindly provided by K. F. Sullivan, Scrpps, LaJolla, CA) and MCF7 cells were grown on glass coverslips to semi-confluence in DMEM supplemented with penicillin, streptomycin, glutamine (100 μg/ml each) and 10% heat-denatured FCS. This medium will be referred to as normal growth medium. Its total osmolality was measured as 307 mOsm. To apply water stress, cell cultures were incubated with different loads of sucrose, sorbitol or sodium chloride, added to the normal growth medium. Media were pre-warmed to 37°C before application; incubation of cells took place in controlled CO2 (4%) and Leica Microsystems, Germany) either of fixed cell preparations or living cells on glass slides with VectaShield (Vector, Burlingame, CA) for direct observation.

Electron microscopy

Cells grown on coverslips were fixed for 1 hour at room temperature in 4% formaldehyde, 1% glutaraldehyde (EM-grade, Sigma) and 1 mM MgCl2 in 100 mM sodium phosphate buffer at pH 6.8, rinsed in buffer, post-fixed in 1% buffered OsO4 for 30 minutes, dehydrated in aqueous ethanol of increasing concentration (30%, 50%, 70%, 100%), transferred to propylene oxide and embedded in epoxy resin (Epon 812, Sigma). The cover slips of polymerized blocks were removed by thermal stress under liquid nitrogen. Ultrathin sections were prepared at 70 nm nominal thickness, post-stained in aqueous lead citrate and uranyl acetate and observed in a Philips 410 transmission electron microscope at 80 kV.

Digitonin permeabilization

For mild permeabilization, cells were incubated with 40 μg/ml digitonin, added directly to the incubation medium from a 1000X stock-solution freshly prepared in DMSO (CalBiochem, Germany). Permeabilization took 5 minutes, as checked by uptake of Texas-Red-labeled, 2 kDa dextran (Invitrogen, data not shown).

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