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An Intranuclear Frame for Chromatin Compartmentalization and Higher-Order Folding

Paola Barboro,[1] Cristina D'Arrigo,[1,2] Michele Mormino,[2] Rosella Coradeghini,[1,3] Silvio Parodi,[1,3] Eligio Patrone,[2] and Cecilia Balbi[1]*

[1]Istituto Nazionale per la Ricerca sul Cancro, I-16132 Genova, Italy
[2]Istituto per lo Studio delle Macromolecole, Sezione di Genova CNR, I-16149 Genova, Italy
[3]Dipartimento di Oncologia, Biologia e Genetica, Università di Genova, I-16132 Genova, Italy

Abstract Recent ultrastructural, immunoelectron, and confocal microscopy observations done in our laboratory [Barboro et al. [2002] Exp. Cell. Res. 279:202–218] have confirmed that lamins and the nuclear mitotic apparatus protein (NuMA) are localized inside the interphase nucleus in a polymerized form. This provided evidence of the existence of a RNA stabilized lamin/NuMA frame, consisting of a web of thin (~3 and ~5 nm) lamin filaments to which NuMA is anchored mainly in the form of discrete islands, which might correspond to the minilattices described by Harborth et al. [1999] (EMBO. J. 18:1689–1700). In this article we propose that this scaffold is involved in the compartmentalization of both chromatin and functional domains and further determines the higher-order nuclear organization. This hypothesis is strongly supported by the scrutiny of different structural transitions which occur inside the nucleus, such as chromatin displacement and rearrangements, the collapse of the internal nuclear matrix after RNA digestion and the disruption of chromosome territories induced by RNase A and high salt treatment. All of these destructive events directly depend on the loss of the stabilizing effect exerted on the different levels of structural organization by the interaction of RNA with lamins and/or NuMA. Therefore, the integrity of nuclear RNA must be safeguarded as far as possible to isolate the matrix in the native form. This material will allow for the first time the unambiguous ultrastructural localization inside the INM of the components of the functional domains, so opening new avenues of investigation on the mechanisms of gene expression in eukaryotes. J. Cell. Biochem. 88: 113–120, 2003. © 2002 Wiley-Liss, Inc.

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In spite of the clear cut evidence of a highly organized internal nuclear matrix (INM) reported 25 years ago in the first extended study on this subject by Berezney and Coffey [1977], the subsequent developments of nuclear matrix research have registered an impressive sequence of ups and downs. Immediately after its discovery, the nuclear scaffold displayed an enormous morphological and biochemical complexity, that preluded extreme difficulties in the protein and structure characterization. The awareness of a possible dependence of the observed morphology on the isolation conditions led the students of nuclear architecture to use the precautionary expression "operationally defined structure" to denote the matrix for some years. Like Sisyphus, the giant condemned throughout eternity to roll uphill a boulder and then to watch it rolling back down, they experienced a continuing frustration, as successive lines of evidence of a permanent intranuclear lamin scaffold were unfailingly disregarded on the ground that serious isolation artifacts were involved. The visualization by resinless electron microscopy of a nucleoskeleton of 11 nm thick filaments [Jackson and Cook, 1988], confirmed by Penman's team 2 years later [He et al., 1990] came as a shock to everybody. However, in spite of the remarkable circumstance that the same nuclear substructure

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*Correspondence to: Dr. Cecilia Balbi, Istituto Nazionale per la Ricerca sul Cancro; Largo Rosanna Benzi, 10; I-16132 Genova, Italy. E-mail: cecilia.balbi@istge.it

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had been visualized in two different laboratories using very different methods for the extraction of the chromatin, such observations did not result in a unified model for the INM. He et al. [1990] favored the view that the core filaments contain structural RNA, based on the observation that they are removed by RNA digestion. This result has not been generally repeatable in subsequent experiments [Nickerson et al., 1995], although the occurrence of large RNA dependent rearrangements of the INM remains an uncontroverted point. Finally, immunoelectron microscopy experiments showing that the nucleoskeleton contains lamins [Hozák et al., 1995] were considered unconvincing [Nickerson, 2001]. In this way, no attempt was made to bring into harmony several elements of truth contained in the results from different laboratories. This state of affairs has stalemated the discussion of major structural and biochemical aspects till now.

In a recent paper [Barboro et al., 2002] we have presented a new model of the organization of the INM, highlighting the role of nuclear RNA in the stabilization of this labile nuclear subdomain. The recent identification of the major protein components has awakened our interest in apparently obsolete observations, and this reevaluation has provided additional support to the structural and biochemical model discussed in this article. In a truly heuristic approach even artifacts can be used to validate the object they distorted or obscured.

LAMIN/NuMA SCAFFOLD: A DYNAMIC STRUCTURE FOR THE COMPARTMENTALIZATION OF CHROMATIN AND FUNCTIONAL DOMAINS

The presence of lamins inside the nucleus and the existence of a stable intranuclear scaffold have been, since early studies on nuclear architecture, two strictly interrelated problems. We shall not attempt to review here the details of the experimental observations that, within the span of a few years, established the dogma of the confinement of lamins to the nuclear envelope. However, in consideration of the persistent skepticism about the reality of a lamin containing intranuclear scaffold, a few episodes are particularly telling. Four years after the isolation of the nuclear matrix [Berezney and Coffey, 1974], Gerace et al. [1978] using both immunofluorescence staining of K22 cells in interphase and electron microscopy immunoperoxidase localization on isolated rat liver nuclei, concluded that lamin antigens are located exclusively at the periphery of the interphase nucleus. In retrospect, we can immediately note that neither a suitable method for detecting low intensity intranuclear fluorescent staining was available at that time, nor these authors took into account the eventuality that, in their preembedding immunoelectron microscopy assay, lamin epitopes were to a large extent buried inside the bulk of condensed chromatin, as actually happens [Barboro et al., 2002]. Just 2 years later, Berezney [1980] in an essential paper reported a few analytical and morphological findings which strikingly preluded recent concepts of matrix organization. After sonication, a treatment that very likely degrades residual structural RNA, two matrix fractions were obtained, one consisting of ribonucleoproteins (RNPs), the other of lamins. In the electron microscope the latter showed a diffuse web of 3–5 nm fibrils and the presence of 8–10 nm globular structures. These observations directly pointed to a bipartite organization of the INM, in which a RNP network tightly interacts with another composed of lamins. The addition of 1 mM sodium tetrathionate (NaTT) [Berezney, 1980] at all stages of nuclear and matrix isolation to inhibit sulfhydryl dependent proteases, was however questioned by Kaufmann et al. [1981], who recorded a striking effect on the morphology of the INM. These authors concluded that NaTT induces disulfide bonds between neighboring INM proteins. Undoubtedly, the micrographs reported in Figure 1 of a subsequent paper [Kaufmann and Shaper, 1984] demonstrate very clearly that when nuclei are isolated in the presence of sulfhydryl blocking reagents the resulting matrix consists of the envelope and is almost devoid of intranuclear material, while incubation in NaTT induces a well developed internal network. This structure can be solubilized in 20 mM dithiothreitol, while the envelope is unaffected. As lamins were not detected in the soluble fraction, it was definitely concluded that they are not components of the intranuclear scaffold. Paradoxically enough, this statement builds on an artifact (more precisely, on a false negative). Indeed, lamins do form a thin fibrillar web throughout the nucleoplasm, but this substructure cannot be adequately contrasted in thin

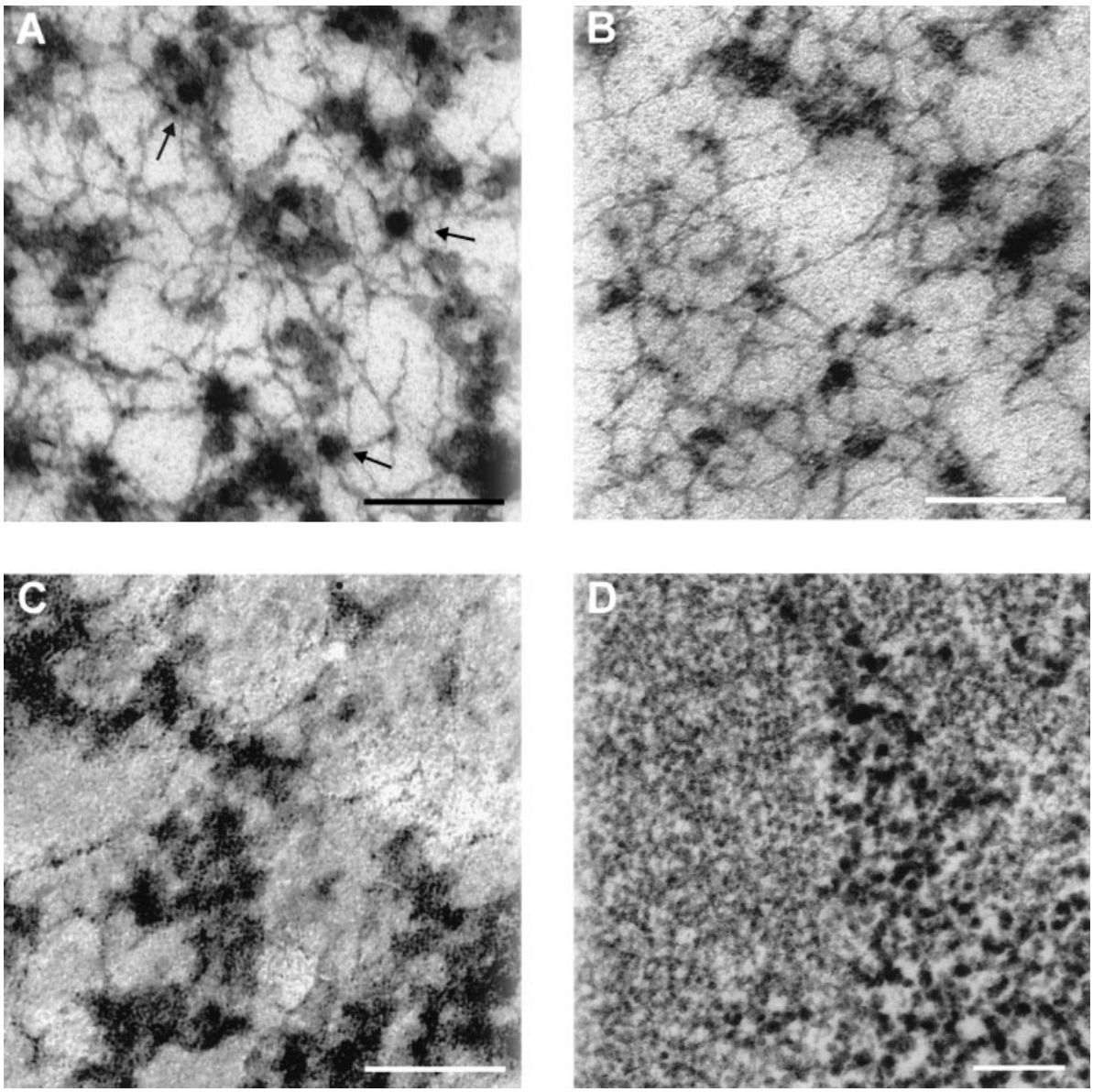

Fig. 1. Ultrastructural characterization of the lamin/NuMA fibrillar web in nuclear matrix samples isolated from rat hepatocytes in the presence (**A**) or absence (**B**) of 2 mM vanadyl ribonucleoside complex, an inhibitor of RNase. The samples were embedded in Poly/Bed 812 resin or in Epon (A and B, respectively) and stained with uranyl acetate and lead citrate. The contrast is high in either image, although as a rule Poly/Bed 812 does not stain as readily as Epon. The arrows in A indicate putative small aggregates of NuMA molecules. The ultrastructure of an aggregate of lamins A, B, and C reconstituted in vitro is shown for comparison in **C**. Lamins were isolated from rat liver nuclei according to Aebi et al. [1986] and reconstitution carried out following protocol *a* reported in the legend of Figure 4 of the paper quoted above. Note the close structural resemblance to the fibrillar web observed in vivo. The sample was embedded in Poly/Bed 812 and the surface of the thin section etched with alcoholic NaOH prior to staining in order to enhance the contrast. A well preserved matrix sample, isolated after prolonged treatment of rat liver nuclei with the vanadyl ribonucleoside complex is shown in the low magnification micrograph reported in **D**. The remnant of a chromatin domain and an interchromatin granule cluster are visible in the left hand and central part of the image, respectively. Poly/Bed 812 embedded sample. The bar is 100 nm in A, B and C, and 200 nm in D.

section of resin embedded matrices by standard staining procedures [Barboro et al., 2002]. The remainder of this story is better known. The somewhat heretical observation that under proper experimental conditions extraction of the chromatin uncovers a network of intermediate filament (IF) like fibers [Jackson and Cook, 1988] did not immediately stimulate immunocytochemistry and immunoelectron microscopy studies to identify eventual intranuclear

IF proteins; indeed, not until 1995 was presented evidence of the association of lamin epitopes with the nucleoskeleton [Hozák et al., 1995] and data on confirmation of the intranuclear localization of lamins have been reported only some years later [Neri et al., 1999; Moir et al., 2000; Barboro et al., 2002].

Another coiled coil, high molecular weight protein, the nuclear mitotic apparatus protein (NuMA) has been localized early inside the interphase nucleus [Lydersen and Pettijohn, 1980] and more recently in the core filaments of the nuclear matrix [Zeng et al., 1994]. Moreover, immunoelectron microscopy studies by Weber, Osborn and their colleagues have shown that NuMA is scattered throughout the nucleoplasm of detergent extracted HeLa cells, but overexpression induces a nuclear scaffold with a quasi-hexagonal architecture that fills the nucleus [Harborth et al., 1999]. Both the self-assembly and DNA binding properties [Ludérus et al., 1994] suggest that this protein is a good candidate for an INM component. This view bears a direct relation to the recent observation that NuMA is anchored through a fraction of nuclear RNA to a permanent web of thin lamin filaments [Barboro et al., 2002]. Thus, a lamin/NuMA substructure might play a specific functional role in the interphase nucleus.

To address this point in a straightforward way we have shown in Figure 1 two high resolution electron micrographs of nuclear matrix samples from rat hepatocytes isolated under conditions which prevent RNA degradation (A) or in the absence of RNase inhibitors (B); in this case, ~50% of the nuclear RNA is removed as a consequence of the activation of endogenous RNase(s). A characteristic morphology, consisting of clusters of electron dense spherical particles ~25 nm in diameter, connected with thin (~3 and ~5 nm) branched fibrils is observed in either sample, although in B less particles are present, and the structure of the web appears to be damaged (e.g., the edges of the particles are more irregular and not as sharp as in A). Using anti-lamin antibodies we were able to show that lamins are located both inside the clusters of dense particles and within the electron transparent regions, where the antibody decorates the thin web. An anti-NuMA antibody was strongly localized in the dense regions, and very few epitopes were detected outside. RNA digestion induces a strong (~65%) depletion of NuMA [Barboro et al., 2002]. These results show that a subset of NuMA molecules colocalizes with lamins inside the dense domains and is anchored to a permanent lamin scaffold through a fraction of nuclear RNA. The nuclear morphology of HeLa cells after transient overexpression of NuMA has been characterized in detail by Gueth-Hallonet et al. [1998]. In the high resolution electron micrographs presented by these authors an ordered quasi-hexagonal lattice can be seen, consisting of dark roundish bodies placed at the vertices of hexagons with an average spacing of ~170 nm; segments of ~6 nm filaments connecting the particles are also visualized. Harborth et al. [1999] have presented a detailed structural model for NuMA self assembly, starting from the multiarm oligomer previously characterized by careful reconstitution experiments in vitro. Getting back to Figure 1A, a few dark ~20 nm spherical particles (marked by arrows) connected with straight fibrils can be seen, which could correspond to the cores of oligomers containing a small number of NuMA molecules. However, the geometry of the web of thin fibrils does not conform to a simple hexagonal lattice, in line with the observation that the former consists of lamins. Harborth et al. [1999] have pointed out that in normal cells the lattice cannot fill the nucleus, and therefore NuMA molecules must be distributed into many "minilattices." The immunoelctron microscopy characterization recently carried out in our laboratory directly supports this view, as NuMA epitopes were found to form discrete islands, standing out against a diffuse background. Minilattices can increase (or decrease) in number and size depending on the functional state of the nucleus [Harborth et al., 1999]. However, our observations suggest that the nucleation of NuMA lattices involves a tight interaction of this protein with an underlying persistent lamin scaffold. As regards this hypothesis, it must be noted that the lamin net also shows extended domains of local order. In the immunoelectron microscopy images the gold particles are observed at distances multiple of ~25 nm, the axial periodicity of lamins filaments and fibers. Furthermore, Fourier filtering of selected clusters of particles shows that they are located at the nodes of a square lattice ~50 nm inside [Barboro, unpublished results]. Thus, ordered regions in the lamin web could contain the structural information for the specific binding of the large multiarm NuMA oligomer. Clearly, all these observations suggest the

existence of two distinct modes of association of NuMA with lamins. In one case, these proteins are intermingled in the dense regions connected with the thin lamin fibrils (Fig. 1A,B); this web could support and organize the chromatin domains. The factors stabilizing this composite structure are at present unknown. In the other case, NuMA gives rise to minilattices anchored to the lamin scaffold through a fraction of RNA which is readily degraded by RNase A or endogenous RNase(s). This superstructure could provide the functional domains with a structural frame; this hypothesis is supported by the observation that RNA digestion releases a fraction of NuMA together with a definite subset of RNP and induce rearrangements and partial depletion of the RNP network. Different molecular events could be involved in the dynamic assembly/disassembly of the lamin/NuMA scaffold. They include lamins and/or NuMA phosphorylation as well as the presence of competitors for the binding to RNA.

It must be stressed that the lamin/NuMA network does not merely represent a scaffold for the compartmentalization of the chromatin and of the functional domains. In addition, the transition between eu and heterochromatin is very likely modulated by local RNA and/or lamin and NuMA concentrations. Scrutiny of the experimental conditions under which pile up and displacement of nuclear chromatin are observed strongly supports this view. Chromatin aggregation into dense clumps occurs when HeLa cells are treated with actinomycin D, an inhibitor of transcription, or permeabilized and submitted to digestion with RNase A [Nickerson et al., 1989]. After digestion of rat liver nuclei with RNase A [Barboro, unpublished result] no change in the fraction of chromatin packaged into higher-order structure(s) was detected by differential scanning calorimetry, a sensitive tool for determining the average degree of condensation of nuclear chromatin [Balbi et al., 1989; Barboro et al., 1993]. Therefore, the morphological change reported above must be interpreted as the pile up of the chromatin driven by the collapse of the thin lamin web [Barboro et al., 2002], rather than as a true structural transition induced by the detachment of the chromatin loops from the scaffold [Balbi et al., 1999]. A more dramatic effect is induced in HeLa cells by overexpression of certain NuMA constructs truncated in the tail domain [Gueth-Hallonet et al., 1998]; nucleoli, DNA, and histone H1 are relocated to the nuclear rim, and the chromatin forms a uniform, electron dense layer on the inner surface of the nuclear envelope, a morphological change which is strongly reminiscent of apoptotic condensation. In apoptosis, chromatin condensation occurs prior to the onset of internucleosomal DNA cleavage [Allera et al., 1997] and is correlated with degradation of lamins A and C as well as of NuMA by caspase-6 [Robertson et al., 2000]. As regards the role of the lamin/NuMA scaffold in determining the higher-order structure of chromatin, we have recently shown that the stability of the highly condensed state inside the loop critically depends on the anchoring of the latter to the matrix [Balbi et al., 1999], although the molecular bases of this stabilizing effect are still poorly understood. Both lamins [Hakes and Berezney, 1991; Ludérus et al., 1992, 1994] and NuMA [Ludérus et al., 1994] show specific DNA binding properties. Moreover, LAP2α, a lamin associated polypeptide 2 isoform, is a component of the nucleoskeleton and it has been further suggested that complexes with type A lamins might be involved in the organization of chromatin structure [Dechat et al., 2000]. In consideration of these advances, immunoelectron microscopy methods can be successfully used to answer the question as to whether the intranuclear distribution of lamins and NuMA matches the topography of condensed and unfolded chromatin domains. As the same observations can be carried out at the resolution level of the light microscope, we expect that next experiments will satisfactorily clarify this point, so warranting the search for more mechanistic properties of the lamin/NuMA scaffold.

HIGHER-ORDER NUCLEAR STRUCTURE: HIGHLIGHTING THE ROLE OF LAMIN/RNA INTERACTIONS

In this section, we shall first compare the structural features of the lamin/NuMA network with the heuristic model of nuclear matrix structure presented by Penman and his colleagues [He et al., 1990]. Going on to a higher level of structural organization, we shall attempt to interpret recent experimental observations of the alterations induced in chromosome territories by RNA digestion and different salt extraction procedures [Ma et al., 1999].

In a recent paper [Barboro et al., 2002] we have shown that the INM actually does not

comprise extended void regions. If the surface of the thin section is etched with alcoholic NaOH and shadowed with heavy metals in order to enhance the contrast, the thin web shown in Figure 1A,B is sharply imaged, and in immunogold experiments using anti-lamin antibodies the gold particles decorate the fibrils in a characteristic bead on a string arrangement. In conventional electron micrographs of the matrix, however, the remnants of the chromatin domains appear as light areas devoid of structural details embedded in the bulk of the electron dense fibrogranular RNP network. The same morphology has been observed in thin sections of resin embedded nuclei or well preserved, crosslink-stabilized matrices stained for RNA by the EDTA-regressive staining to visualize the RNP network [Nickerson, 2001]. This landscape undergoes a sharp metamorphosis when the same matrix samples are visualized by resinless section electron microscopy. The void areas appear to be delimited by straight segments of 11 nm fibers, which extend inside the electron dense domains forming a structured, quasi-regular network. In the absence of crosslinks, the extraction of the matrices with 2 M NaCl removes almost completely the RNP aggregates and uncovers the network of 11 nm core filaments [He et al., 1990]. The biological reality of this nuclear substructure has been recently questioned. Pederson [2000], noting the dendritic pattern of the network, the propinquity of the vertices and number of extending filaments per unit volume, drew the conclusion that these characteristic morphological features are not compatible with the topography of the interchromatin space in the nucleus of living cells. In confirmation of Penman's model, however, more recent work has shown that synthetic sites are attached to a nucleoskeleton consisting of core filaments and of a diffuse skeleton containing many protein/nucleic acid complexes and NuMA [Hozák, 1996]. Our ultrastructural observations do support this basic principle of nuclear architecture, although, as expected [Hozák et al., 1995], we were unable to visualize the 11 nm filament network using thin section electron microscopy. On the other hand, the somehow unnatural appearance of the filaments should not be overlooked and suggests that stretching forces act at some stage of matrix isolation and/or mounting. As the same morphology has been observed using very different procedures for chromatin extraction [Jackson and Cook, 1988; He et al., 1990] we conclude that major morphological changes occur in the course of the mounting of the specimen. It is possible that during the dissolution of diethylene glycol distearate in butyl alcohol, which is carried out to obtain the final resinless section [Fey et al., 1986], the intranuclear network experiences strong shearing stress as the liquid–solid interface passes through the sample. The thin fibrillar web shown in Figure 1 is mainly composed of ~5 and ~3 nm thick lamin protofibrils and protofilaments, respectively, and reproduces, on a reduced scale, the morphological features of the core filament network. Therefore we can reason that during the extraction of diethylene glycol distearate the thin filaments break and merge together giving rise to a thicker network which maintains the characteristic dendritic topology. Thus, as we stated above, the explanation of an artifact can be used to validate the hidden structure in the distorted object.

As noted above, previous results from our laboratory have shown that the role of RNA in the stabilization of the INM is manifold. Digestion of RNA with endogenous RNase(s) releases several RNPs and ~65% of the nuclear content of NuMA. This large decrease is accompanied by the disappearance of extended NuMA islands. We conclude that a large fraction of NuMA molecules is associated with the functional domains. Instead, digestion of RNA inside the electron transparent regions of the INM, which contain very few NuMA molecules, induces dramatic morphological rearrangements, driven by the collapse of the fine lamin web. This result suggests that direct RNA–lamin interactions play a major role in the organization and stabilization of the INM, although we cannot absolutely rule out that additional proteins are involved. This attractively simple hypothesis deserves a careful consideration. In vitro reconstitution experiments of the fine web in the presence of different concentrations of nuclear RNA should be carried out in order to verify whether the latter specifically affects the self-assembly mode of lamins.

Chromosome territories determine the highest level of organization of chromatin in the interphase nucleus, and are identified by fluorescence in situ hybridization methods. Berezney and colleagues [Ma et al., 1999] were recently able to relate the ultrastructural characterization of the nuclear matrix isolated in situ with

the information obtained at the resolution level of the light microscope in the course of selective digestion and extraction experiments. Chromosome territory organization were disrupted after RNase A digestion and treatment with 2 M NaCl; treatment with 0.65 M $(NH_4)_2SO_4$ had no effect. In either experiment extensive extraction of electron dense intranuclear material was demonstrated by electron microscopy of the matrices; also a residual intranuclear structure, which was more evident in $(NH_4)_2SO_4$ extracted samples, was observed. By taking into account the finding that after exposure to 2 M NaCl the matrix still retains 37.0% of nuclear RNA, while a subset of acidic proteins, referred to as chromosome territory anchor proteins, is released, we conclude that a tight RNA/lamin complex, in association with proteins which probably bind in a specific fashion other INM components, is directly involved in the organization of chromosome territories. The sparse material seen inside the RNase A digested matrices after salt extraction very likely correspond to the thin lamin web, as also suggested by the composition of the final matrix pellet, which retains over 95% of nuclear lamins.

Consideration of the manifold involvement of RNA in the stabilization of nuclear structures of increasing complexity suggests that: for new structural features of the INM to be observed the integrity of RNA must be safeguarded as far as possible. For example, we recently noticed that when the matrix is isolated after careful equilibration of rat liver nuclei with vanadyl ribonucleoside complex, an inhibitor of RNase, the residual chromatin domains were found to be filled with a fine lattice of closely associated fibrils, showing beads on a string morphology; extremely well preserved interchromatin granule clusters were also visualized (Fig. 1D). Thus, isolated matrices can for the first time be reliably used in immunoelectron microscopy experiments for the exhaustive characterization and ultrastructural localization of the components of the functional domains.

CONCLUSION

Early attempts to relate nuclear matrix alterations to the modulation of aberrant gene expression in cancer conformed to a somewhat descriptive approach, centered on the characterization of differences in the expression of matrix proteins in tumors with respect to the normal tissue. These studies succeeded in identifying several tumor specific species which proved to be important tools for the diagnosis and management of cancer, but eluded the central problem of the role of the malignant alteration of the nuclear architecture [Nickerson, 1998]. Recent molecular biology reports [Fry and Peterson, 2002; Lomvardas and Thanos, 2002] have provided additional elements of the enormous molecular and biochemical complexity underlying the mechanisms of transcriptional activation. For this reason, the extension of investigation linking nuclear architecture to the biological and pathological control of gene expression [Stein et al., 1998] is critically needed. On the other hand, the search for the dependence of the trafficking of regulatory proteins on subnuclear organization [Stein et al., 2000] using immunolectron microscopy and ultrastructural methods involves serious experimental difficulties, and appears at present premature. However, both NuMA and lamins undergo definite changes in tumor cells, which deserve careful consideration. NuMA is overabundant in cancer cells [Keesee et al., 1996]; aberrant patterns of lamin expression have been described in tumors [Bosman, 1999]. Further studies should be carried out in order to establish whether these changes involve mechanistic aspects, or merely represent an epiphenomenon of the malignant transformation.

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