Proc. Natl. Acad. Sci. USA
Vol. 94, pp. 4446–4450, April 1997
Cell Biology

The nuclear matrix revealed by eluting chromatin from a cross-linked nucleus

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Contributed by Sheldon Penman, December 31, 1996

ABSTRACT **The nucleus is an intricately structured integration of many functional domains whose complex spatial organization is maintained by a nonchromatin scaffolding, the nuclear matrix. We report here a method for preparing the nuclear matrix with improved preservation of ultrastructure. After the removal of soluble proteins, the structures of the nucleus were extensively cross-linked with formaldehyde. Surprisingly, the chromatin could be efficiently removed by DNase I digestion leaving a well preserved nuclear matrix. The nuclear matrix uncovered by this procedure consisted of highly structured fibers, connected to the nuclear lamina and built on an underlying network of branched 10-nm core filaments. The relative ease with which chromatin and the nuclear matrix could be separated despite extensive prior cross-linking suggests that there are few attachment points between the two structures other than the connections at the bases of chromatin loops. This is an important clue for understanding chromatin organization in the nucleus.**

Nucleic acid metabolism is spatially organized in the cell nucleus. The application of powerful microscopy techniques has revealed an increasingly intricate domain organization within the nucleus (reviewed in ref. 1). Individual catalytic processes and the machinery they require are structurally constrained to spatial domains. The very intricate spatial organization of the nucleus presents an important research problem. Our goals are to identify the structure(s) maintaining the complex architecture of the nucleus and to characterize the molecular interactions that constrain components to specific locations.

Much of the domain organization of the nucleus remains after the experimental removal of chromatin (2–9). This suggests that chromatin itself is not the fundamental structure organizing the nucleus. In fact, chromatin may itself be architecturally organized in loop domains attached at their bases to an underlying structure (10, 11).

There is a second nucleic acid-containing structure distributed throughout the nucleus, an ribonucleoprotein (RNP)-containing network of fibrils and granules selectively stained by the EDTA-regressive method (12–14). This structure, identified in intact nuclei, corresponds to the nuclear matrix remaining after biochemical fractionation (15–17). The isolated nuclear matrix retains most nuclear RNA (15, 18), RNP proteins (17, 19), and may even require intact RNA for structural integrity (19, 20). It is this nuclear matrix to which chromatin loops are anchored (11, 21, 22).

Biochemical studies of the nuclear matrix and detailed ultrastructural studies of its architecture required the development of techniques to remove the larger mass chromatin while leaving the nuclear matrix undisturbed. Two very different and somewhat harsh fractionation protocols have uncovered a network of highly branched 10-nm filaments connected to the nuclear lamina and well distributed through the nuclear volume (18, 23). These may form the core structure upon which the RNP-containing nuclear matrix is constructed but are unlikely to represent the entire structure. Many proteins are artifactually removed from the structure by nuclear matrix preparation protocols because of the high ionic strength (18) or electrophoretic conditions (23) required for uncovering 10-nm filaments. The conditions employed, while preserving some core filaments, cause the spatial collapse of other elements of the RNP network into dense masses that are not observed in the unextracted nucleus.

Several procedures have been reported to "stabilize" the nuclear matrix (24–26). These employ treatment with either heat or sodium tetrathionate before the removal of chromatin and have been shown to increase protein yield (25). These do not, however, improve the ultrastructural preservation of the matrix (unpublished observations). In this paper, we report a technique for stabilizing the ultrastructure of the nuclear matrix by extensive cross-linking before the removal of chromatin. The matrix prepared by this technique has much improved preservation of fine structure. It is similar in appearance and spatial distribution to the RNP network of the intact nucleus. Most importantly, the stabilized nuclear matrix is built upon an underlying network of 10-nm filaments confirming our previous models of how the matrix is constructed (18, 23). The ease with which chromatin and nuclear matrix could be separated after extensive cross-linking, though initially surprising, is an important observation for understanding chromatin organization in the nucleus.

MATERIALS AND METHODS

Stabilized Nuclear Matrix Protocol. Cells were first washed in cold PBS. Soluble proteins were removed by extraction in cytoskeletal buffer [CSK; 10 mM Pipes, pH 6.8/300 mM sucrose/100 mM NaCl/3 mM $MgCl_2$/1 mM EGTA/20 mM vanadyl riboside complex/1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride] containing 0.5% Triton X-100 for 2 min at 4°C. The structure remaining was extensively cross-linked by treatment with 4% formaldehyde in CSK for 40 min at 4°C. After washing in CSK, DNA and it's associated proteins were removed by digestion with 400 units/ml DNase I for 50 min at 32°C in digestion buffer [10 mM Pipes, pH 6.8/300 mM sucrose/50 mM NaCl/3 mM $MgCl_2$/1 mM EGTA/20 mM vanadyl riboside complex/1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride]. Most of the DNA was removed at this step and additional cut DNA was removed by two washes. In most experiments the first wash was with 0.25 M ammonium sulfate extraction buffer [10 mM Pipes, pH 6.8/250 mM ammonium

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0027-8424/97/944446-5$2.00/0
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Abbreviations: RNP, ribonucleoprotein; DAPI, 4′,6-diamidino-2-phenylindole.

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sulfate/300 mM sucrose/3 mM $MgCl_2$/1 mM EGTA/20 mM vanadyl riboside complex, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride] and the second was with 2 M NaCl buffer [10 mM Pipes, pH 6.8/300 mM sucrose/2 M NaCl/3 mM $MgCl_2$/1 mM EGTA/20 mM vanadyl riboside complex/1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride]. The elevated ionic strength of these washes was not necessary. The remaining DNA could be removed almost as efficiently by two washes with CSK.

DNA release was measured by two techniques. In the first, cells were incubated overnight with 10 μCi/ml (1 Ci = 37 GBq) [^{3}H]thymidine in the medium and DNA release was measured in different fractions of the preparation by liquid scintillation counting. In the second, cells and isolated matrices were stained for epifluorescence microscopy with 5 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) which selectively stains DNA.

Electron Microscopy. Cells and nuclear matrices were prepared for transmission electron microscopy by two different techniques: resinless sectioning (27, 28) and conventional thin sectioning with EDTA regressive staining (14). Immunostaining of nuclear matrices was performed as described (29).

Reversal of Cross-Links for Polyacrylamide Gel Electrophoresis. Cells were labeled with 10 μg/ml [^{3}H]lysine overnight before fractionation. Samples were prepared for gel electrophoresis by reversal of formaldehyde cross-links. We adopted the strategy of others who had used heat and protein denaturing conditions (30, 31). Two variations on these protocols were used. In the first fractions of 100 μl were dialyzed against 0.01 M Tris·HCl (pH 7.4), 1 mM EDTA. 10 μl of 1% (wt/vol) SDS, and 5 μl of 2-mercaptoethanol were added and the samples incubated at 95°C for 30 min. Samples were degassed at 60°C under vacuum for 15 min. They were then either frozen or used directly for SDS/PAGE. In the second variation, samples were denatured by adding guanidine-HCl and 2-mercaptoethanol to final concentrations of 2 M and 0.5 M before heating at 95°C for 60 min. Both procedures were effective, as judged by the removal of histone dimers and higher order polymers with a corresponding increase in histone monomers. Histone gels were run by the procedure of Thomas (30) and proteins detected by fluorography.

RESULTS

In an effort to improve the preservation of nuclear matrix ultrastructure we tried a radical stabilization protocol. Soluble proteins were removed from cultured cells by extraction with 0.5% Triton X-100. The remaining nuclear structure was then extensively cross-linked by treatment with 4% formaldehyde in a buffer of approximately physiological pH and ionic strength. After cross-linking, chromatin was removed by DNase I digestion and two washes. The washes in initial experiments were at elevated ionic strength (0.25 M ammonium sulfate followed by 2 M NaCl) but this was not necessary for the removal of DNA. In some later experiments, washes at physiological ionic strength (CSK buffer) were sufficient to remove DNA after digestion.

The removal of DNA after formaldehyde cross-linking and DNase I digestion is demonstrated in Fig. 1. The CaSki cells pictured were grown on a glass slide. Soluble proteins were removed with 0.5% Triton X-100, and the structure remaining was cross-linked with 4% formaldehyde. Cells were stained with DAPI, a dye that specifically detects DNA, both before (Fig. 1*A*) and after (Fig. 1*B*) DNase I digestion of the cross-linked structure. DNA was very efficiently removed. In experiments not shown, cells were grown overnight with [^{3}H]thymidine and the release of DNA was evaluated by ^{3}H release. Those experiments showed that 93–99% of ^{3}H-labeled DNA could be removed following extensive formaldehyde cross-linking.

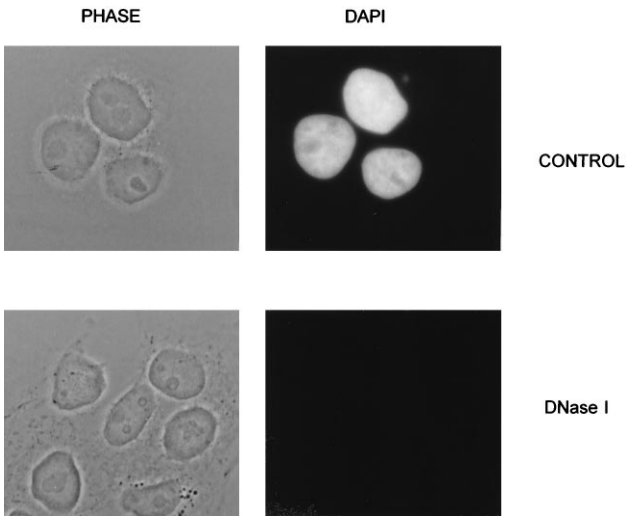

FIG. 1. DAPI staining shows that DNA is released from formaldehyde treated nuclei by DNase I digestion. CaSki cells were grown on coverslips and processed as described. (*Upper*) Cells were permeabilized with 0.5% Triton X-100 in CSK buffer before formaldehyde cross-linking and DAPI staining. (*Left*) Phase contrast image. (*Right*) Fluorescent image. (*Lower*) Cells were treated in the same way and then digested with 400 μg/ml DNase I for 50 min at 32°C before staining. The micrograph exposures and magnifications are identical to those in the *Upper* panels. Almost all detectable DNA was removed by the DNase I digestion despite the prior, extensive cross-linking of the structure.

DNA was effectively removed after cross-linking along with its associated histones as shown in Fig. 2. This gel of [^{3}H]lysine-labeled proteins shows that only a small fraction of histones are retained with the cross-linked nuclear matrix structure. This analysis required the development of techniques to reverse

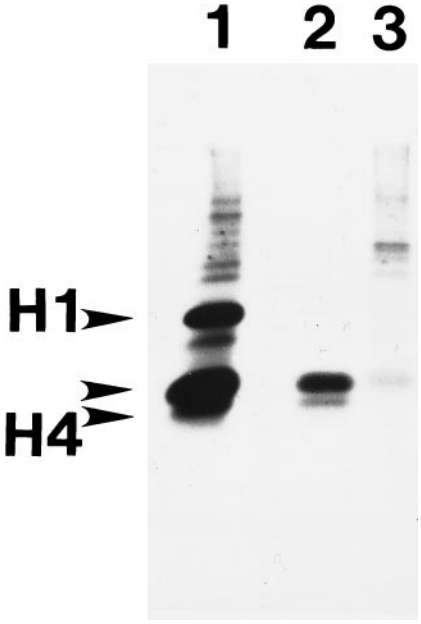

FIG. 2. Histones are released from formaldehyde-treated nuclei by DNase I digestion. CaSki cells were labeled with [^{3}H]thymidine before fractionation, reversal of cross-links, and electrophoretic analysis as described. Histones, because of their very high lysine content, strongly labeled. Most of the histones detectable in this experiment were released from the nucleus by DNase I digestion (lane 1). Few were released by postdigestion washes (lane 2). Little histone was retained with the cross-link stabilized nuclear matrix (lane 3). The positions of histones H1 and H4 on the gel are marked with histones H2A, H2B, and H3 running between them.

formaldehyde cross-links before electrophoresis. The methods employed were derived from the strategies of others (31) who heated cross-linked structures under protein denaturing conditions. The effective release of both DNA and histones from the nuclear matrix after extensive cross-linking is a surprising result and suggests that connections between the nuclear matrix and chromatin are relatively few. In one previous experiment (23) some *Hae*III-digested chromatin could be removed from the nucleus by electroelution following a light glutaraldehyde prefixation. The prefixation conditions and the fraction of chromatin removed were not reported.

The ultrastructure of the cross-link stabilized nuclear matrix is shown in the resinless section of Fig. 3. The nuclear matrix consists of the nuclear lamina (L) and an internal matrix connected to the lamina and filling the nuclear interior. This internal matrix is a network of irregular fibers with intricate fine structure (Fig. 3*A*). It is similar in spatial distribution and in ultrastructural detail to the RNP structure that can be seen in the intact nucleus (12–14). The structural remains of nucleoli can be clearly seen (Nu). Some subnucleolar compartments can still be observed in many nucleoli by resinless section electron microscopy. Empty regions observed within nucleoli in some resinless sections may correspond to centers from which nucleolar chromatin has been removed (32). Other matrix isolation protocols leave nucleolar remnants which are seen in resinless sections as dense masses with little observable internal structure.

At higher magnification (Fig. 3*B*) the fibers of the cross-link-stabilized nuclear matrix were observed to be intricately structured fibers with some integrated granular material. There is much better preservation of fine structure than any achieved with previous protocols. Careful examination reveals a network of branched filaments of about 10 nm that are at the structural core of irregular fibers. These filaments (marked by arrowheads) have been observed before and proposed as the core structure of the nuclear matrix (18, 23).

Few intermediate filaments remained attached to the outside of the nuclear lamina (Fig. 3*A*). This was surprising because nuclei had not been isolated before matrix preparation. Other nuclear isolation protocols leave an integrated nuclear matrix-intermediate filament structure with abundant and well preserved intermediate filament–nuclear lamina con-

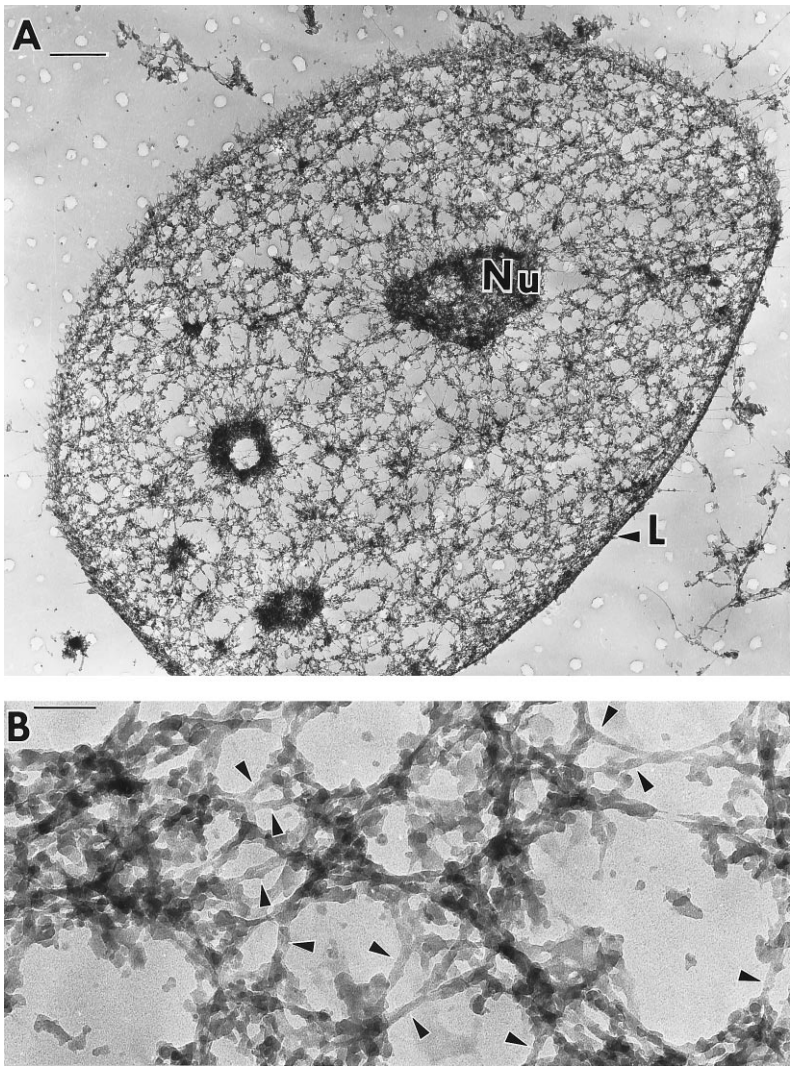

FIG. 3. The ultrastructure of the nuclear matrix revealed by resinless section electron microscopy. The nuclear matrix of a CaSki cell was prepared by the cross-link stabilized nuclear matrix preparation procedure and visualized by resinless section electron microscopy (27–29). (*A*) The nuclear matrix consisted of two parts, the nuclear lamina (L) and a network of intricately structured fibers connected to the lamina and well distributed through the nuclear volume. The matrices of nucleoli (Nu) remained and were connected to the fibers of the internal nuclear matrix. Three remnant nucleoli may be seen in this section. Few intermediate filaments were connected to the outside of the lamina. (*B*) Seen at higher magnification the highly structured fibers of the internal nuclear matrix seemed to be built on an underlying structure of 10-nm filaments that are occasionally branched. These were seen most clearly when, for short stretches, they were free of covering material (arrowheads). The irregular fibers, with granules well integrated into their structure, may be built on this filamentous core structure. [Bars = 1 μM (*A*) and 100 nm (*B*).]

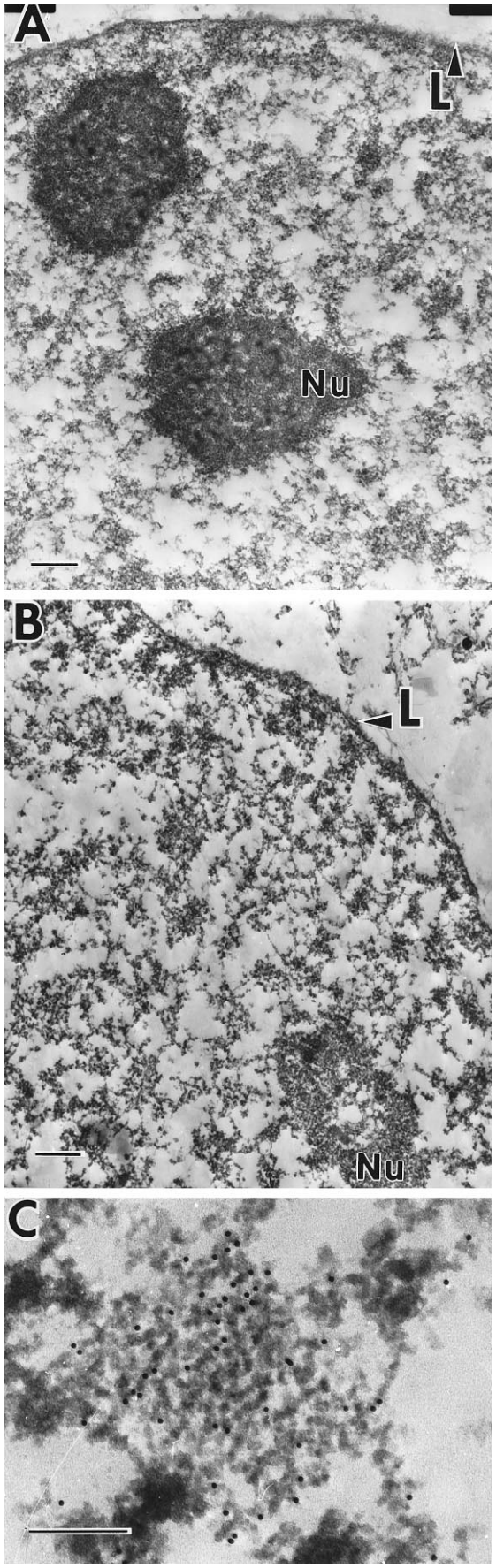

FIG. 4. The nuclear matrix retains the RNP network of the unextracted nucleus with good structural preservation. CaSki cells were prepared for conventional thin section microscopy before (*A*) and after (*B*) the removal of chromatin by the cross-linking/DNase I procedure. Thin sections were selectively stained for RNA by the EDTA regressive staining procedure (14) to visualize the RNP network that is an important part of the nuclear matrix. The nuclear lamina (L) forms the periphery of both nucleus (*A*) and nuclear matrix (*B*). The removal of chromatin after formaldehyde cross-linking did not substantially alter the structure or spatial distribution of the nuclear RNP network. (*C*) At higher magnification interchromatin granule clusters, sites of RNA-splicing factor concentration, could be seen with good preservation in the RNP network of the cross-link stabilized nuclear matrix. The CaSki nuclear matrix in this panel was counterstained with the B4A11 antibody which recognizes an RNA splicing factor and with a colloidal gold-conjugated second antibody. This comparison of RNP network ultrastructure in the nucleus and cross-link stabilized nuclear matrix showed good preservation of architecture. The RNP network was not substantially altered by the removal of chromatin if the structure was first extensively cross-linked. [Bars = 500 nm (*A* and *B*) and 200 nm (*C*).]

nections (18, 19, 23, 33). The reasons for this difference remain unclear.

Many nuclear matrix preparation protocols have been reported. It is important to develop criteria for evaluating these procedures. For studies where architectural preservation is important, protocols may be judged by how well they conserve structural features observable in the unextracted nucleus (15, 16). The nuclear matrix is more than a protein scaffolding; it is an RNA-containing structure corresponding in large part to the RNP network that can be visualized in the intact nucleus by selective staining for RNA (13–16, 28).

A good test for a matrix-isolation protocol would therefore be the degree of preservation in RNP network architecture. Fig. 4 shows a comparison of RNP network ultrastructure in the cross-link stabilized nuclear matrix (Fig. 4*B*) and in the chromatin-containing nucleus (Fig. 4*A*). The similarity in form, distribution, and fine structure of the RNP network is striking. The removal of chromatin by DNase I digestion after a prior cross-linking in formaldehyde did not markedly alter RNP network ultrastructure. This suggests that we had isolated a structure present in the nucleus and isolated it with a high degree of structural preservation. One important element of the RNP network observable at higher magnification in the unextracted nucleus is the interchromatin granule cluster where RNA splicing factors are concentrated (34, 35). These structures were retained with good structural conservation in the cross-link stabilized nuclear matrix as shown in Fig. 4*C*. This nuclear matrix was counterstained with a monoclonal antibody, B4A11, which recognizes an RNA splicing protein (2), and a colloidal gold-conjugated second antibody.

DISCUSSION

We have developed a technique for dissecting the nucleus, removing chromatin, and uncovering the nuclear matrix with unprecedented preservation of morphology. This technique is based on the initially surprising observation that DNA and its associated histones could be efficiently removed from detergent-extracted nuclei after extensive cross-linking with formaldehyde. The cross-linked and well preserved nuclear matrix consisted of several elements. Most of the nuclear volume contained a network of intricately structured fibers that were connected to the nuclear lamina. These fibers were built on an underlying network of branched 10-nm filaments that we and others have previously observed (18, 23). The 10-nm filaments could be seen beneath the structured fibers and in some locations as they emerged uncovered from the fibers. The observation of 10-nm filaments at the core of the cross-link stabilized nuclear matrix increases our confidence that the nuclear matrix is built on a network of branched 10-nm filaments connected to the nuclear lamina (18, 23).

The architecture of the cross-link stabilized nuclear matrix resembled that of the fibrogranular RNP network which can be visualized by selective staining of the intact nucleus (13, 14). This is an important criterion by which a nuclear matrix-preparation protocol should be judged. Using this criterion, we conclude that the method presented here affords a high degree of ultrastructural preservation.

Formaldehyde cross-linking has been used to map the structure of chromatin (31, 36–42). Histones are cross-linked to DNA and, within nucleosomes, to each other by formaldehyde treatment. We expect, therefore, that DNase I-digested DNA and histones are released from the nuclear matrix together in tightly cross-linked complexes.

The easy separation of DNA and histones from the nuclear matrix after extensive cross-linking suggests either that sites of nuclear matrix–chromatin interaction are few or that they are not cross-linked by formaldehyde. The great effectiveness of aldehydes as cross-linking reagents is the basis for their widespread use in microscopy and for mapping chromatin structure. We believe, therefore, that they should stabilize most matrix–chromatin interactions. If true, this means that nuclear matrix–chromatin interactions are relatively infrequent. This would be consistent with existing models describing how chromatin may be packaged in the nucleus (10, 21, 22). These propose that chromatin is organized in loops by attachments to the nuclear matrix. The average measured loop size is 70–100 kb of which only 1 kb of DNA at the base is protected against nuclease digestion (11). This 1 kb is likely to be the maximum length of chromatin that is in contact with the nuclear matrix at the loop base. If loop bases are the principal matrix attachments then most chromatin may not be sufficient proximate to the matrix to permit cross-linking by formaldehyde.

In these experiments DNA could be removed by DNase I digestion after formaldehyde cross-linking under isotonic conditions. The efficient removal of DNA in other matrix isolation protocols requires more than just nuclease digestion. A second step is required, usually involving salt extraction (i.e., 0.25 M ammonium sulfate, 0.4 M KCl, 2 M NaCl). One alternative protocol, yielding a nuclear matrix structure similar in overall construction to that shown in this report, removes DNA after digestion by electroelution under isotonic conditions (23). Digested chromatin may normally be held in place by electrostatic interactions that are broken down by salt treatment or overcome by electrical fields. Treatment of chromatin with formaldehyde in the procedure we report here may reduce those electrostatic interactions by modifying the many primary amines of histones, thus eliminating the need for salt washes or voltage gradients.

The formaldehyde cross-link stabilized nuclear matrix is easily prepared and architecturally well preserved. Formaldehyde-induced cross-links can be reversed by heat treatment under denaturing conditions. This allows some biochemical characterization of the structure. We expect, however, that its principal application will be in microscopy studies where ultrastructural preservation is most important.

We thank Drs. B. Blencowe and M. Mancini for helpful suggestions and discussions. This work was supported by grants from the National Institutes of Health (R01 CA67628 and P01 AR42262).

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