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Chromatin architecture and nuclear RNA

(nuclear matrix/nuclear structure/RNA synthesis inhibition/matrix RNA)

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ABSTRACT **The maintenance of normal chromatin morphology requires ongoing RNA synthesis. We have examined the role of RNA in chromatin organization, using selective detergent extraction of cells, RNA synthesis inhibitors, and enzymatic digestion of nuclear RNA. Comparison of extracted and unextracted cells showed that the important features of chromatin architecture were largely unchanged by the extraction procedure. Normally, chromatin was distributed in small heterochromatic regions and dispersed euchromatic strands. Ribonucleoprotein granules were dispersed throughout the euchromatic regions. Exposure to actinomycin led to the redistribution of chromatin into large clumps, leaving large empty spaces and a dense clustering of the remaining ribonucleoprotein granules. When the nuclei of extracted cells were digested with RNase A, there was a rearrangement of chromatin similar to but more pronounced than that seen in cells exposed to actinomycin. The inhibitor 5,6-dichloro-1-β-D-ribofuranosylbenzimidizole also inhibits RNA synthesis but by a different mechanism that leaves no nascent RNA chains. The drug had little effect on chromatin after brief exposure but resembled actinomycin in its effect at longer times. We also examined the structure of the nuclear matrix to which most heteronuclear RNA remains associated. Pretreatment of cells with actinomycin or digestion of the nuclear matrix with RNase A caused the matrix fibers to collapse and aggregate. The experiments show a parallel decay of chromatin and of nuclear matrix organization with the depletion of nuclear RNA and suggest that RNA is a structural component of the nuclear matrix, which in turn may organize the higher order structure of chromatin.**

The eukaryotic nucleus remarkably packages more than a yard of DNA into a 5-μm spheroid. The packaging of DNA into chromatin and of chromatin into the nucleus is highly ordered (1–6), with the coarse features of this organization correlating with transcriptional activity. The condensed chromatin (heterochromatin) is largely inactive, and transcription is localized in the extended, dispersed euchromatin. The basic packing structures, the nucleosomes, are arranged in polynucleosome chains, which are wound into 30-nm fibers (7). Much less is known about the packing of chromatin fibers into higher order structures in the nuclear interior.

There is a clear dependence of chromatin architecture on ongoing RNA metabolism; inhibition of RNA synthesis results in the retraction of chromatin from the nuclear lamina and its aggregation into massive clumps (8), whereas the spatial distribution of stained DNA in high-salt-extracted nuclei is changed by treatment with RNase (9). In what may be a related phenomenon, RNA synthesis inhibitors cause a retraction of polytene chromosome puffs (10, 11).

Many studies have described an internal structural framework in the nucleus called the nuclear matrix (12–17), which is associated with many important chromatin functions, including DNA replication (18, 19), RNA synthesis and processing (20–22), and hormone binding in the nucleus (23–25). Actively transcribed gene sequences may be selectively bound to the matrix, suggesting that the matrix plays a role in the regulation of gene expression (26, 27).

The nuclear matrix contains an RNA component packaged in nuclear ribonucleoprotein (RNP) particles (16, 17, 28–30). This nuclear RNP component of the matrix may be important for its organization, an idea supported by the results we report here. We suggest that the nuclear matrix may be the structural framework responsible for many features of higher order chromatin organization and that the rearrangement of the nuclear matrix after removal of its RNA component may be responsible for the concomitant collapse of chromatin.

MATERIALS AND METHODS

HeLa S3 cells were grown in suspension in Eagle's minimal essential medium supplemented with 7% (vol/vol) horse serum. For electron microscopy, cell pellets were resuspended in cytoskeleton buffer: 10 mM Pipes, pH 6.8/100 mM KCl/300 mM sucrose/3 mM $MgCl_2$/1 mM EGTA/4 mM vanadyl adenosine/1.2 mM phenylmethylsulfonyl fluoride/0.5% (vol/vol) Triton X-100. After 3 min at 4°C, the HeLa cytoskeletal frameworks were separated from soluble proteins by centrifugation at 600 × *g* for 3 min. Cells were usually fixed at this point for electron microscopy. In some experiments the cytoskeletal framework pellet was extracted in double-detergent buffer [10 mM Tris·HCl, pH 7.4/100 mM NaCl/10 mM $MgCl_2$/4 mM vanadyl adenosine/1.2 mM phenylmethylsulfonyl fluoride/1% (vol/vol) Tween 40/0.5% (vol/vol) sodium deoxycholate] for 5 min at 4°C and pelleted as before. This step strips away the cytoskeleton, leaving in the pellet nuclei with their attached intermediate filaments (17). The removal of chromatin with RNase-free DNase I was done as described (17). The removal of chromatin leaves a nuclear matrix–intermediate filament structure containing nuclear RNP complexes.

After fractionation, cells were fixed in 2.5% (vol/vol) glutaraldehyde in cytoskeleton buffer for 30 min at 4°C, washed three times in 0.1 M sodium cacodylate (pH 7.2), postfixed in 1% (wt/vol) OsO_4 in the wash buffer for 5 min at 4°C, dehydrated in ethanol, and transferred to 1-butanol before embedment in diethylene glycol distearate. Thin sections were cut, and the embedding medium was removed with 1-butanol. Sections were transferred to ethanol and dried through the CO_2 critical point.

Samples to be embedded in Epon were fixed, postfixed, and dehydrated in the same way. The transitional solvent was propylene oxide, and the sample was embedded in Epon–Araldite at 60°C for 2 days. Thin sections were cut as before and stained with uranyl acetate followed by lead citrate (31). Cells were similarly prepared for preferential staining of

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Abbreviations: DRB, 5,6-dichloro-1-β-D-ribofuranosylbenzimidizole; RNP, ribonucleoprotein.

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RNP-containing structures by the EDTA regressive method (32), except that the postfixation in OsO_4 was omitted. Thin sections were cut, stained with 5% (wt/vol) uranyl acetate for 1–3 min at room temperature, and then floated for 20–60 min in 0.2 M EDTA (pH 7.0). At this point the sections were stained for 1–3 min in lead citrate.

RESULTS

Nuclear Morphology in Detergent-Extracted Cells. Extraction with 0.5% Triton X-100 in cytoskeleton buffer dissolved the membrane lipids and released soluble proteins. When we compared stained Epon sections of extracted and unextracted cells, there were no visible differences in chromatin distribution within the nucleus (data not shown). The nuclear envelope had dissolved in the detergent, leaving the nucleus bounded by the nuclear lamina. The chromatin was more prominent against a clearer background, and numerous interchromatin granules and fibers containing RNPs were more conspicuous than in the unextracted cell.

The granules visible in Fig. 1*a* were identified as RNA-containing structures by using a regressive EDTA staining procedure (data not shown; ref. 32). The RNP-containing interchromatin granules were distributed in the spaces between masses of bleached heterochromatin (33, 34).

When cells were exposed to actinomycin for 2 hr (Fig. 1*b*), there was an extensive collapse of chromatin. Selectively stained RNP-containing structures were observed mostly as filaments bordering the condensed, destained chromatin masses or as small, dense clusters of granules (data not shown).

This effect of actinomycin suggested that RNA was essential for chromatin organization. An even more direct demonstration of the relation of RNA to chromatin organization was the collapse of chromatin after treatment of the extracted cell nuclei with RNase A (Fig. 1*c*). The enzyme had an effect on chromatin similar to but even more extensive than that of actinomycin. After digestion, chromatin appeared only as highly condensed aggregates, which were either localized perilaminarly or collapsed onto the modified nucleoli. Viewed by regressive staining, the digestion-resistant, selectively stained RNP-containing structures were granular and coalesced into a few large clusters adjacent to the bleached, condensed chromatin (data not shown).

Resinless Section Stereoscopic Microscopy. Embedded sections provide only a two-dimensional image of material stained at the surface of the section. A more complete view of chromatin organization in the entire section is available by preparing samples free of embedding resin (15, 35, 36). Embedment-free electron microscopy images the entire three-dimensional contents of the section, which is best seen in stereoscopic micrographs.

The micrographs of Fig. 2 illustrate the three-dimensional interrelationship between the chromatin network, the interchromatinic fibers and granules, and the nucleolus as seen in resinless sections of extracted cells. As shown in Fig. 2*a*, the condensed chromatin border lining the nuclear lamina appeared to be connected to the extensive network of filaments throughout the interchromatinic space. After treatment with actinomycin D (Fig. 2*b*), this filamentous network appeared to be disrupted. The chromatin had coalesced into several large clumps that appeared to be aggregated around remnant nucleoli and into smaller clumps and strands. The extended fibers of chromatin had largely disappeared, leaving large empty spaces. A few thick fibers still interconnected the masses of clumped chromatin. Granules, possibly corresponding to the RNP granules seen in Fig. 1 (33, 34), were still present and associated with chromatin clumps. The resinless section of Fig. 2*c* shows in greater detail that the effect of RNase digestion of the extracted nucleus was similar to but

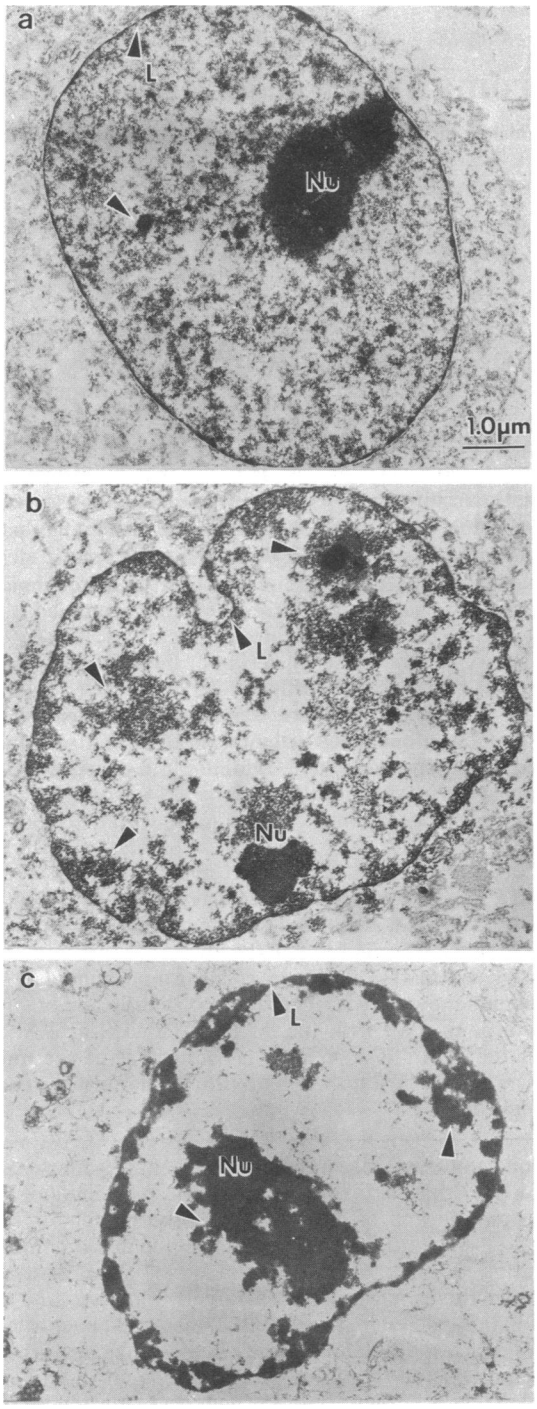

FIG. 1. The distribution of chromatin and RNP-containing structures in the nucleus of extracted control, actinomycin D-treated, or RNase A-treated cells. HeLa cells were extracted with 0.5% Triton X-100. All of the micrographs in this figure are of Epon-embedded sections stained with uranyl acetate followed by lead citrate, and all are shown at the same magnification. (*a*) Untreated control cells. The nucleus contained a nucleolus (Nu) and uniformly distributed chromatin fibers with patches of aggregated chromatin (arrowheads), which were both perilaminar and scattered in the nuclear interior. (*b*) Cells treated for 2 hr with 5 μg of actinomycin D per ml. The nuclear lamina (L) had become invaginated, and there was a dramatic redistribution of the chromatin with clumping and condensation into aggregates (arrowheads). The nucleolus (Nu) was rearranged; the two components—fibrillar and granular—were segregated. (*c*) Cells treated with 5 μg of RNase A per ml for 20 min after extraction in 0.5% Triton X-100. Treatment with RNase caused the clumping of chromatin (arrowheads) both onto the nuclear lamina (L) and onto the nucleolus (Nu).

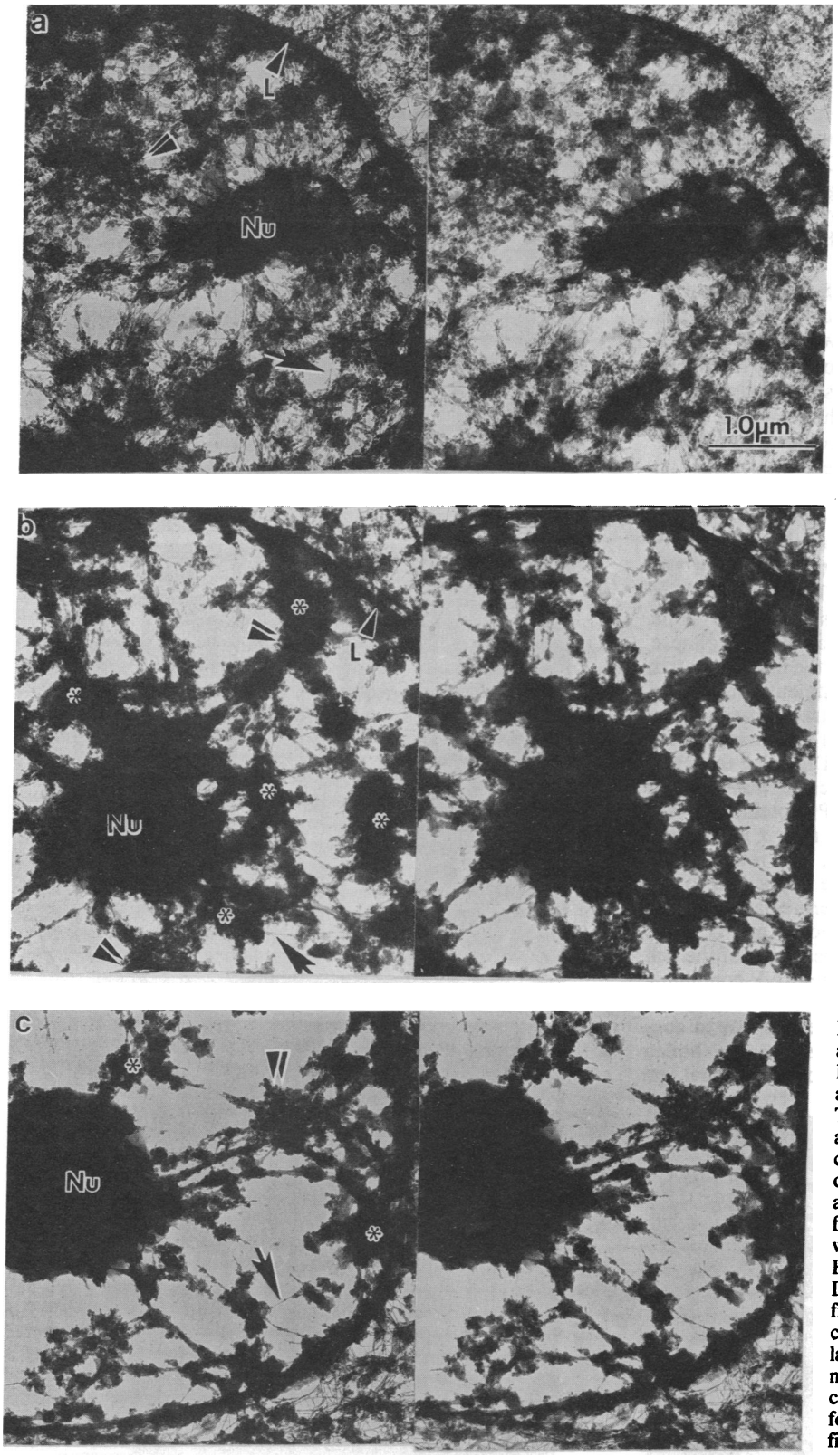

FIG. 2. Effect of actinomycin D or RNase A digestion on nuclear morphology as seen in stereoscopic resinless sections. HeLa cells were incubated with or without actinomycin D for 2 hr, extracted with 0.5% Triton X-100 in cytoskeletal buffer, fixed, and prepared for resinless-section microscopy as described. L, lamina; Nu, nucleolus; double arrowheads, aggregates of granules; asterisk, condensed chromatin; arrow, thin filaments. (*a*) Control HeLa cells not treated with actinomycin D before extraction. (*b*) HeLa cells treated with 5 μg of actinomycin D per ml for 2 hr before extraction and fixation. Dense areas (marked by asterisk) of condensed chromatin, some of which is collapsed onto nucleoli, were visible in the nuclear interior. (*c*) Nuclear matrices from control cells treated with 100 μg of RNase A for 20 min after extraction and before fixation.

more extensive than that of actinomycin treatment of the cell (Fig. 2*b*).

Effects of Protein and RNA Synthesis Inhibitors on Nuclear Morphology. The effect of RNA synthesis inhibitors on chromatin organization was direct and was not caused by a secondary decrease in protein synthesis due to mRNA depletion. Inhibitors of protein synthesis, such as cycloheximide, had little or no effect on gross chromatin morphology even after 90 min (data not shown). The effects of RNA synthesis inhibitors were independent of the mode by which the agent inhibits RNA synthesis. The inhibitor 5,6-dichloro-1-β-D-ribofuranosylbenzimidizole (DRB) had long-term effects similar to those of actinomycin, although its mode of action is completely different.

Unlike actinomycin, DRB does not intercalate and either inhibits the initiation of many RNA transcripts (37, 38) or causes premature RNA termination (39, 40). Whichever is its mode of action, DRB should rapidly strip nascent RNA

chains from their site of synthesis on DNA. Fig. 3 *Left* is a resinless section showing that chromatin morphology was essentially normal after 5 min of exposure to DRB—sufficient time for the drug to have entered and terminated a major fraction of nascent molecules (40), indicating that nascent RNA chains are not important in maintaining chromatin organization. Longer exposure (2 hr) to DRB had a similar effect to that of actinomycin (Fig. 3 *Right*), although the chromatin rearrangement was less extensive, perhaps reflecting the less complete (60% of control) inhibition of heteronuclear RNA synthesis.

Effect of Actinomycin D and RNase A on the Nuclear Matrix. The resinless-section micrograph of Fig. 2*a* suggests that the thick chromatin strands are associated with a network of thinner fibers. These fibers might be involved in the maintenance of chromatin structure and constitute part of the nuclear matrix. In a previous study (17), the treatment of the nuclear matrix with RNase A caused a collapse of nuclear matrix structure, showing that the nuclear matrix has an RNA component that is essential to its organization.

Fig. 4*a* shows the nuclear matrix revealed by the removal of chromatin. We have named this complex the RNP-containing nuclear matrix because it retains most of the heteronuclear RNA in the form of RNP. The interior fibers are studded with many 20-nm particles (15). The same treatment of cells with actinomycin that caused the collapse of chromatin also effected a breakdown of the nuclear matrix structure (Fig. 4*b*). After actinomycin treatment, the interior fibers of the RNP-containing nuclear matrix collapsed into dense aggregates localized in the middle of the nucleus while still anchored to the lamina by filaments. Digesting the RNA component of the RNP-containing nuclear matrix with RNase A releases the heteronuclear RNP proteins but otherwise leaves the proteins of the nuclear matrix unchanged (17). This RNP-depleted structure (Fig. 4*c*) retained the exterior intermediate filaments that were connected to the nuclear lamina in a nuclear matrix–intermediate filament scaffold. The amount of aggregation of nuclear matrix fibers was not as extensive after actinomycin treatment of the cell as it was after RNase treatment of the RNP-containing matrix, but the overall change was similar. The effect of RNase A was due to nucleolytic cleavage and not to contaminating proteolytic activity. The enzyme was boiled before use and, when analyzed by two-dimensional gel

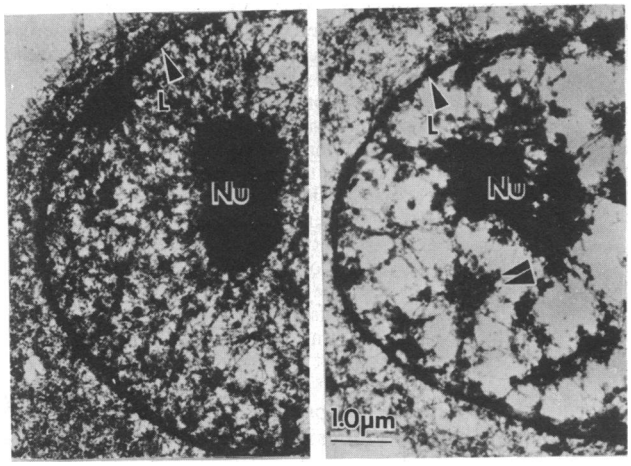

FIG. 3. Effect of DRB on nuclear morphology as seen in resinless sections. (*Left*) Cells incubated with 25 μg of DRB for 5 min before extraction with 0.5% Triton X-100. The DRB treatment caused little change in chromatin morphology. The symbols are the same as in Fig. 2. (*Right*) Cells incubated for 2 hr in 25 μg of DRB per ml before extraction. This longer incubation caused a collapse of chromatin.

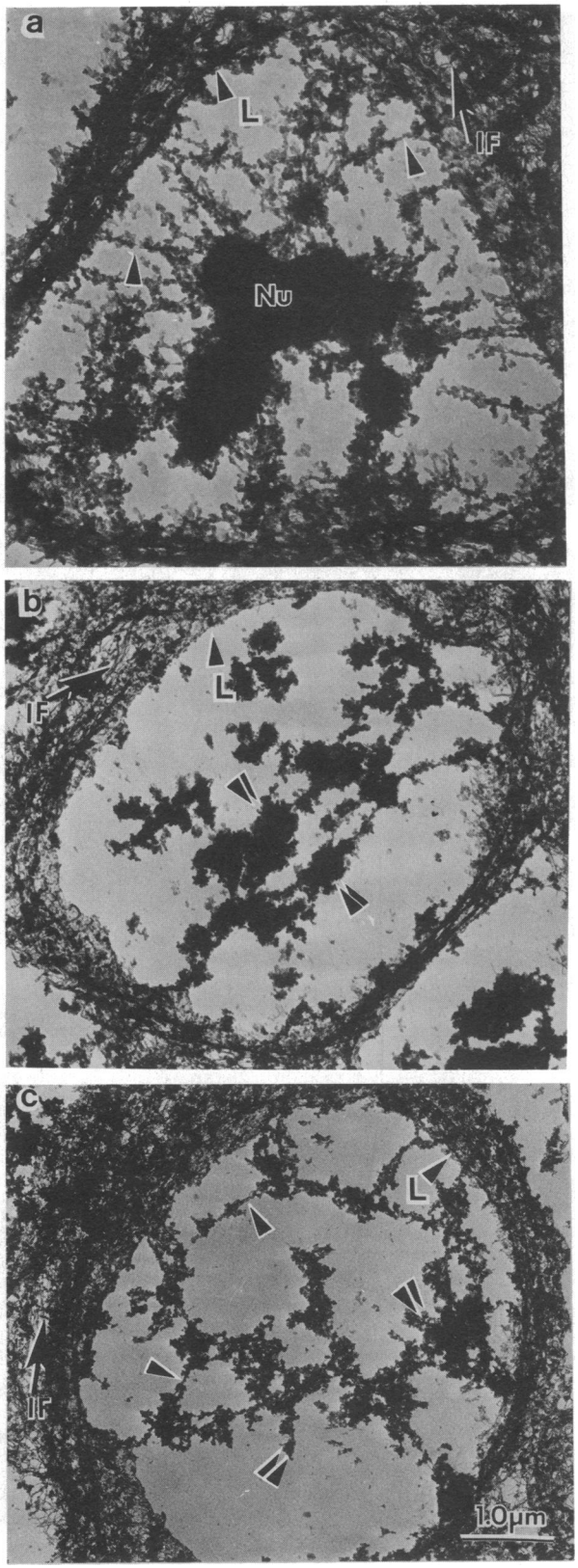

FIG. 4. Effect of actinomycin D or RNase A digestion on nuclear matrix structure. This figure compares the structure of the RNP-containing nuclear matrix, the RNP-containing nuclear matrix of an actinomycin D-treated cell, and the nuclear matrix after treatment with RNase. All cells were extracted and digested with DNase I as described. (*a*) RNP-containing nuclear matrix. The nuclear region is bounded by the nuclear lamina (L) and contains the remnants of a nucleolus (Nu). The filaments of the nuclear matrix (arrow) appear

electrophoresis, the proteins of the nuclear matrix showed no degradation (data not shown).

DISCUSSION

Nuclear RNA is associated with the nuclear matrix and plays a role in nuclear structure. Observing chromatin in Triton X-100-extracted cells allows comparison of the effect on living cells of RNA synthesis inhibitors with the direct effect of RNase A on nuclei. Both treatments resulted in a marked collapse and clumping of chromatin. These effects are consistent with a role for nuclear RNA in the maintenance of chromatin organization.

Although the effect of actinomycin on chromatin structure is similar to that of RNase, the resemblance could be coincidental. Actinomycin intercalates into DNA and might cause alterations in chromatin by changing its helicity. However, other inhibitors of RNA synthesis, working by different mechanisms, affect chromatin organization in a similar way. In particular, DRB is a drug that does not intercalate into DNA and yet had much the same effect, after long exposures, as actinomycin. Since the effect of RNA synthesis inhibitors is not a direct effect on DNA, the data suggest that an unstable RNA is an important component of chromatin structure. The lack of a marked DRB effect at short times (Fig. 3 *Left*) also suggests that nascent RNA chains are not responsible for the actinomycin effect. Inhibition of protein synthesis with cycloheximide for 2 hr did not cause major structural changes to chromatin, showing that the need for ongoing RNA synthesis did not reflect a requirement for continuing protein synthesis.

The nuclear matrix preparation procedure used in this paper leaves 73% of the heteronuclear RNA with its full complement of associated proteins bound to the RNP-containing nuclear matrix (17). We have shown here and in previous studies (17) that RNA not only is associated with the nuclear matrix but also may be an important structural component. The nuclear matrix was severely deranged after treatment of cells with actinomycin D for 2 hr (Fig. 4*b*) or by digestion of the isolated RNP-containing nuclear matrix with RNase A (Fig. 4*c*). The same treatment of cells with actinomycin that caused the collapse of chromatin also caused a breakdown of the nuclear matrix, an effect similar to that seen after treatment with RNase. Thus, the disruption of nuclear RNA, either *in situ* with nuclease or *in vivo* with actinomycin, leads to a collapse of nuclear matrix structure. The breakdown of the nuclear matrix caused by the removal of RNA paralleled a simultaneous collapse of chromatin and suggested that the two phenomena might be related. This correlation of effects leads us to propose that changes in chromatin architecture and in nuclear matrix morphology are related and that RNA is an essential component of the nuclear matrix, which in turn is required for normal chromatin organization.

to connect the nucleolus to the nuclear lamina. In the cytoplasmic region, intermediate filaments (IF) can be seen radiating out from the nuclear lamina. (*b*) The RNP-containing nuclear matrix of a cell incubated with 5 μg of actinomycin D per ml for 2 hr before extraction. A large rearrangement of the RNP-containing nuclear matrix is apparent. Some filaments of the nuclear matrix are still visible (arrow), while others may have collapsed into dense aggregates of filaments (double arrowheads). (*c*) An RNP-depleted nuclear matrix prepared by digesting the RNP-containing nuclear matrix with 25 μg of RNase A per ml for 20 min. The filaments of the nuclear matrix can no longer be seen but had collapsed together into dense aggregates of filaments (marked by double arrowheads). The intermediate filaments (IF) radiating out from the lamina (L) can be seen better in this panel. All panels in this figure represent cells at the same magnification.

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