

# A Nucleolar Skeleton of Protein Filaments Demonstrated in Amplified Nucleoli of *Xenopus laevis*

WERNER W. FRANKE, JUERGEN A. KLEINSCHMIDT, HERBERT SPRING, GEORG KROHNE, CHRISTINE GRUND, MICHAEL F. TRENDELENBURG, MICHAEL STOEHR, and ULRICH SCHEER

*Division of Membrane Biology and Biochemistry, Institute of Cell and Tumor Biology, and Division of Pathomorphology, Institute of Experimental Pathology, German Cancer Research Center, D-6900 Heidelberg, Federal Republic of Germany*

**ABSTRACT** The amplified, extrachromosomal nucleoli of *Xenopus* oocytes contain a meshwork of ~4-nm-thick filaments, which are densely coiled into higher-order fibrils of diameter 30–40 nm and are resistant to treatment with high- and low-salt concentrations, nucleases (DNase I, pancreatic RNase, micrococcal nuclease), sulfhydryl agents, and various nonionic detergents. This filamentous “skeleton” has been prepared from manually isolated nuclear contents and nucleoli as well as from nucleoli isolated by fluorescence-activated particle sorting. The nucleolar skeletons are observed in light and electron microscopy and are characterized by ravel of filaments that are especially densely packed in the nucleolar cortex. DNA as well as RNA are not constituents of this structure, and precursors to ribosomal RNAs are completely removed from the extraction-resistant filaments by treatment with high-salt buffer or RNase. Fractions of isolated nucleolar skeletons show specific enrichment of an acidic major protein of 145,000 mol wt and an apparent pI value of ~6.15, accompanied in some preparations by various amounts of minor proteins. The demonstration of this skeletal structure in “free” extrachromosomal nucleoli excludes the problem of contaminations by nonnucleolar material such as perinucleolar heterochromatin normally encountered in studies of nucleoli from somatic cells. It is suggested that this insoluble protein filament complex forms a skeleton specific to the nucleolus proper that is different from other extraction-resistant components of the nucleus such as matrix and lamina and is involved in the spatial organization of the nucleolar chromatin and its transcriptional products.

In studies of the organization of the interphase nucleus, considerable progress has been made in the elucidation of the arrangement of chromatin components and transcriptional products. However, relatively little is known about the composition and function of another category of nuclear structures, the nonnucleoproteinaceous architectural components that are insoluble in solutions of low and high ionic strength, despite numerous studies dedicated to this problem. Such structures include (a) the nuclear envelope and its pore complexes (1, 15, 18, 23, 37, 41), (b) a peripheral layer of insoluble protein (“lamina”; 1, 15, 22, 23, 59), (c) certain skeletal proteins related to the chromosome “scaffold” described by Laemmli and co-workers (see references 2 and 3), and (d) ill-defined tangles of fibrillar structures of the nuclear interior that are collectively described as residual “matrix” (6, 21; for reviews, see references

4 and 12). The latter, preparatively defined insoluble structures that in most preparations included residual nucleolar material (7, 9, 13, 31, 46), showed a remarkable heterogeneity of both electron microscopically identified components and polypeptides. In nuclear matrix fractions from several somatic cells of higher organisms, an enrichment of three characteristic major polypeptides of between 60,000 and 80,000 mol wt was observed that, however, appeared to be similar if not identical to the major proteins present in fractions of the peripheral lamina–nuclear pore complex structures (7, 17, 23, 37, 59).

As far as the topology of such insoluble protein components is concerned, positive evidence exists only in the case of the three major polypeptides associated with the isolated nuclear membrane (“lamins” *sensu* reference 22) that have been localized by antibody techniques in the nuclear periphery (17, 23,

36, 59). Studies on possible skeletal components of the nucleolus are usually hampered, in somatic cells, by the nucleoli being intimately associated with chromosomal material, especially the perinucleolar heterochromatin, as well as with lamina components and nuclear membrane fragments (cf. references 51 and 58). In view of these limitations it is not surprising that attempts to identify possible insoluble (skeletal) components of nucleolar fractions from rat liver have revealed the predominance of the same nonnucleolar proteins positively localized in the peripheral lamina associated with both condensed chromatin and the nuclear envelope. To examine the existence and significance of skeletal structures of the nucleolus proper, we have therefore used the amphibian oocyte. The nucleus of this cell type contains numerous amplified extrachromosomal nucleoli (>1,000 in *Xenopus laevis*; 8), which can be easily separated from both the nuclear envelope and chromosomes (e.g., 30), thus providing a considerable natural enrichment of nucleolar material over other nuclear structures. In the present study we describe the isolation of a framework of nucleolar filaments resistant to high-salt buffer and detergent from oocyte nuclei of *Xenopus laevis*. This "skeletal" framework is composed of protein filaments enriched in a characteristic acidic protein of 145,000 mol wt.

## MATERIALS AND METHODS

### Isolation of Oocyte Nuclei, Nucleoli, and Nuclear Contents

Nuclei of full-sized (stages V and VI) oocytes of *Xenopus laevis* were isolated either manually in buffered "5:1 isolation medium" (83 mM KCl, 17 mM NaCl, 10 mM Tris-HCl, pH 7.2) containing 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and then transferred to the same medium with additional 10 mM MgCl<sub>2</sub> (37, 56) or by the large-scale procedure described by Scalenghe et al. (55). Mass-isolated nuclei were sedimented through a cushion of 5:1 isolation medium containing 10 mM MgCl<sub>2</sub>, 2.5 mM dithiothreitol, and 0.5 mM PMSF, instead of Eagle's medium as used by Scalenghe et al. (55). The nuclear envelopes of the individual nuclei were removed manually under a dissecting microscope, and the "gelled nuclear contents" (37) were washed in 5:1 isolation medium and finally collected in an Eppendorf reaction tube (Eppendorf Geraetebau, Hamburg, W. Germany). Alternatively, individual nucleoli were collected manually with micropipettes attached to a micromanipulator under observation with an inverted microscope (26).

### Fluorescence-activated Sorting of Fluorochrome-stained Nucleoli

Mass-isolated nuclei (in 5:1 isolation medium containing 5 mM MgCl<sub>2</sub>, 2.5 mM dithiothreitol, and 0.5 mM PMSF) were gently homogenized by sucking the solution several times up and down in an Eppendorf plastic tip and were stained, in same solution, simultaneously with propidium iodide (PI, 20 µg/ml; Serva, Heidelberg, Germany) and diamidinophenylindole (DAPI, 3 µg/ml; Serva). Using the UV line of an argon ion laser for excitation illumination, PI-stained particles (nucleoli) fluoresced deeply red, whereas other particles, to which more DAPI was bound, fluoresced white-blue. Debris and follicle cell nuclei showed intermediate blue-red fluorescence. Criteria for electronic sorting with a flow cell sorter were derived from window settings in the two-dimensional distribution of blue vs. red fluorescence, thereby selecting particles that exhibited a large amount of red fluorescence with a minor degree of blue fluorescence. Nucleoli were sorted, counted, and collected in 5:1 isolation medium containing 2.5 mM dithiothreitol, 1 mM MgCl<sub>2</sub> and 0.5 mM PMSF by pelleting at 9,000 g for 5 min. Supernatant solutions were saved and either precipitated in cold 5% TCA or used for extractions (see above).

### Extraction Procedures

Gelled nuclear contents, nucleoli isolated by fluorescence-activated particle sorting, and, in some experiments, nucleoli manually collected by pipetting were suspended in 1–1.5 ml of the following solutions by sucking the material several times into an Eppendorf plastic tip (all buffers contained 0.5 mM PMSF): (a) 1 M KCl, 1% Triton X-100, 10 mM Tris-HCl, pH 7.4; (b) 1.5 M KCl, 1% Triton X-

100, 10 mM Tris-HCl, pH 7.4; (c) 1.5 M KCl, 10 mM Tris-HCl, pH 7.4; (d) 1 mM Tris-HCl, pH 7.2; or (e) 0.1 mM sodium borate buffer, pH 9.0. In some experimental series, 20 mM dithiothreitol was added to each of these solutions. Incubation under gentle agitation was carried out for 30 min at room temperature or at 4°C. After centrifugation at 9,000 g for 4 min or 3,500 g for 30 min, the pellets were resuspended in wash buffer (10 mM Tris-HCl, pH 7.4; 10 mM Sørensen phosphate buffer, pH 7.4; or borate buffer as described above) and centrifuged once more.

### Gel Electrophoresis of Proteins

One-dimensional slab gel electrophoresis in the presence of SDS was carried out essentially according to Laemmli (40) in 10% or 12.5% polyacrylamide gels. Some samples were radioactively labeled in vitro with [<sup>3</sup>H]dansylchloride as described for proteins of other subfractions from *Xenopus* oocyte nuclei (37). For two-dimensional gel electrophoresis (48), samples were solubilized according to Kelly and Cotman (33). Gels were stained with Coomassie Blue or with the silver method described by Switzer et al. (60).

### RNA Analyses

Nuclei were manually isolated from *Xenopus laevis* oocytes and immediately transferred to ice-cold 70% ethanol. A batch of 150 nuclei was drained of ethanol and then suspended in 0.3 ml of 50 mM Tris-HCl buffer (pH 8.4) containing 20 mM EDTA, 1% Sarkosyl NL-97 and 0.5 mg/ml proteinase K (Merck, Darmstadt, Germany; preincubated for 30 min at 37°C). After ~6 h at 37°C, 0.3 g of solid CsCl was added, and the solution was layered upon a cushion of 0.2 ml of 5.7 M CsCl, 0.1 M EDTA, 10 mM Tris-HCl, pH 7.2 (24) in a small nitrocellulose nitrate tube. The tube was overlaid with liquid paraffin and centrifuged in a SW65 rotor using special adaptors (Beckman Instruments, Munich, Germany) for 12 h at 40,000 rpm and 20°C. The bottom of the tube was cut off and placed upside-down on a piece of filter paper to drain most of the liquid, and then the "invisible" RNA pellet was resuspended in 50 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.2), transferred to an Eppendorf reaction tube, precipitated by adding 2.5 vol of ice-cold ethanol, and stored overnight at -20°C. The solution was then centrifuged (3,500 g for 30 min), the pellet dried *in vacuo* and resuspended in 20 µl of 4 mM Tris-HCl, 4 mM NaCl, 0.5 mM EDTA (pH 8.0). Electrophoresis was carried out in horizontal 1.5% agarose (Seakem, Marine Colloids Div., Rockland, Maine) slab gels (9 × 10 cm). Molecular weight markers (tobacco mosaic virus [TMV] RNA: 2.07 × 10<sup>6</sup>; *Xenopus laevis* rRNAs: 1.5 and 0.7 × 10<sup>6</sup>) were run in adjacent slots. After electrophoresis (~120 min; 7 V/cm), the gel was placed for 15 min in electrophoresis buffer (20 mM Tris-HCl, 20 mM NaCl, 2 mM EDTA, pH 8.0) containing 1 µg/ml ethidium bromide and then photographed under UV illumination.

RNA analyses of high-salt-extracted fractions were performed as follows: Nuclear contents and nucleolar fractions from *Xenopus laevis* oocytes were collected in ice-cold 5:1 isolation medium and suspended in 1 ml of buffer containing 1 M KCl, 1% Triton X-100, and 0.5 mM PMSF and gently homogenized by sucking the solution several times up and down in an Eppendorf plastic

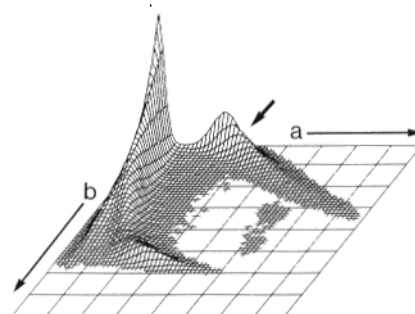


FIGURE 1 Preparation of nucleoli from oocyte nuclei of *Xenopus* isolated by dual fluorescence staining and particle sorting. Mass-isolated germinal vesicles were ruptured and simultaneously stained with propidium iodide (a, red fluorescence) and diamidinophenylindole (b, blue fluorescence). The figure presents the two-parameter frequency profile obtained by flow cytometric analysis using a UV laser for fluorescence activation. The population of the variously sized nucleoli (arrow) can be separated and discriminated from other particles, including contaminating yolk platelets and follicle cell nuclei, because of their red fluorescence. The method also allows counting of the nucleoli per preparation, and separation of different size classes of nucleoli.

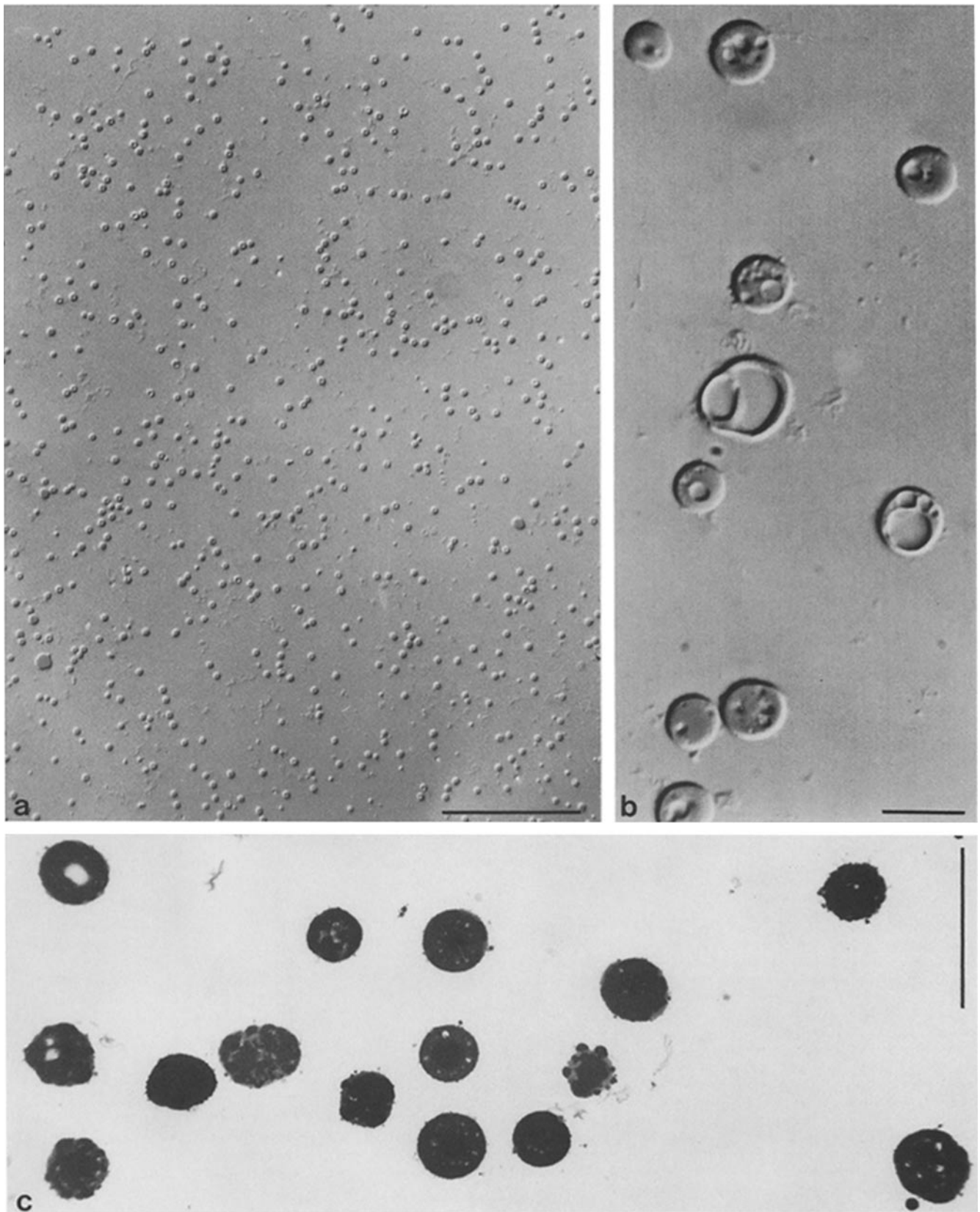
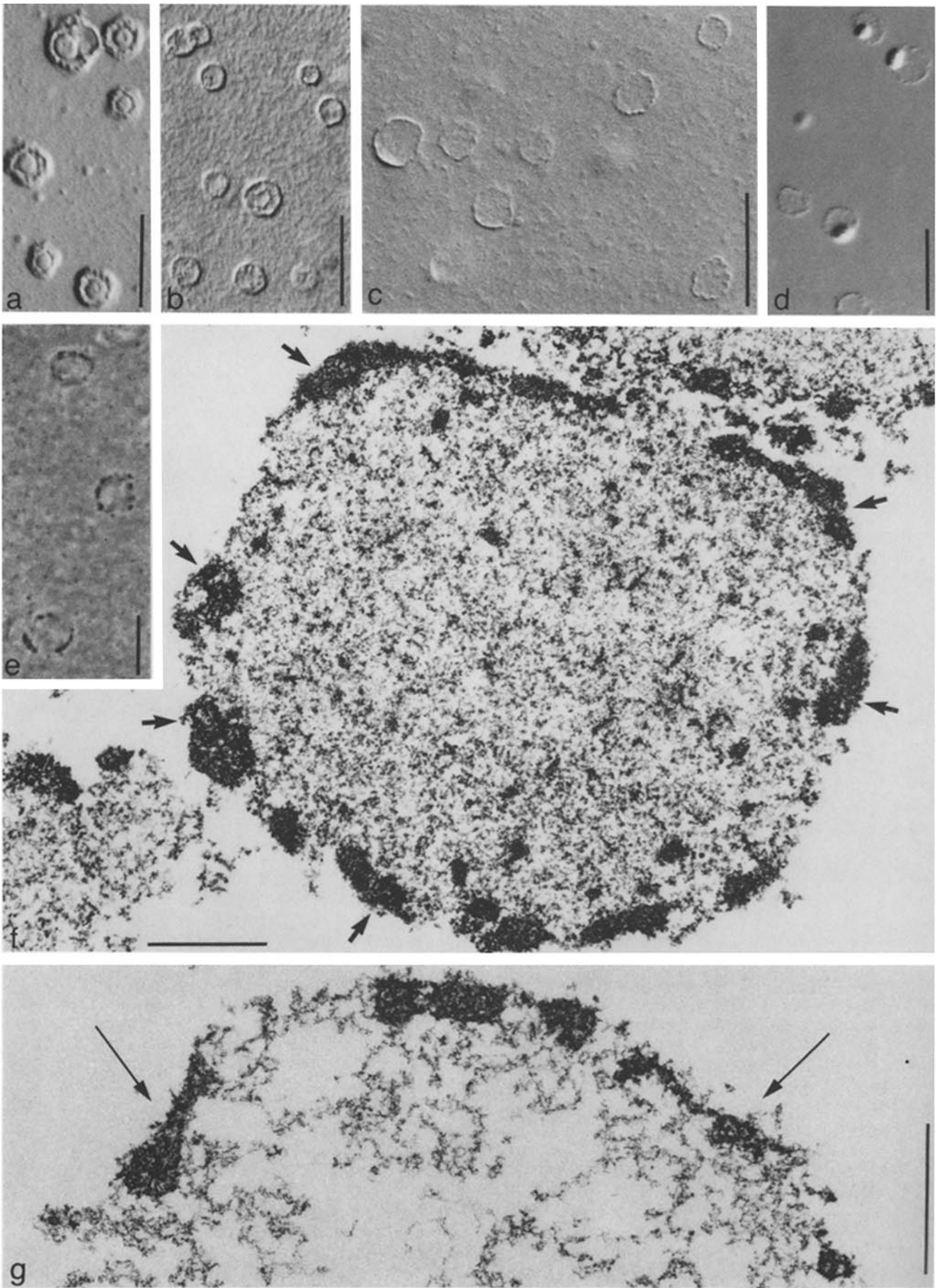


FIGURE 2 Fractions of nucleoli from *Xenopus* oocytes isolated by fluorescence-activated particle sorting as seen in a survey light micrograph (a, interference contrast) showing the purity of this nucleolar subfraction (mean nucleolar diameter, 4.5  $\mu\text{m}$ ; range, 3.5–6.5  $\mu\text{m}$ ). b presents a higher magnification of a purified subfraction of larger nucleoli (mean diameter, 8.2  $\mu\text{m}$ ; range, 7–14  $\mu\text{m}$ ), showing some morphological heterogeneity, including the occurrence of “vacuolated” nucleoli. c is a low-power electron micrograph of the nucleolar fraction shown in a. Bars, 100  $\mu\text{m}$  (a) and 10  $\mu\text{m}$  (b and c).  $\times 250$  (a),  $\times 1,500$  (b), and  $\times 2,900$  (c).



tip. After 30 min of incubation at 4°C, the solution was centrifuged (3,500 g for 30 min) at 4°C. The pellet was resuspended in 1 ml of the same solution and, after 30 min at 4°C, centrifuged again. Both supernates were pooled. Nucleic acids and nucleoprotein material were precipitated from the supernate as described by Dessev and Grancharov (14). In brief, to the 2 ml of supernatant, 50  $\mu$ l of 0.2 M sodium phosphate buffer (pH 7.6) was added, followed by 12  $\mu$ l of 1 M MgCl<sub>2</sub> and 1.44 ml of 96% ethanol. After 30 min at -20°C, the solution was centrifuged for 10 min at 3,500 g. The pelleted material was finally resuspended in 0.4 ml of the proteinase K solution specified above. The pellet of the first centrifugation was also taken up in 0.4 ml of the proteinase K solution. Digestion time was 3–4 h at 37°C. Then 20  $\mu$ g of tRNA was added to each tube as carrier, followed by 2.5 vol of ethanol. After centrifugation, pellets were analyzed by gel electrophoresis as described above.

To quantitate the RNA distribution in the sedimentable and nonsedimentable fractions, we radioactively labeled the RNA. A piece of *Xenopus laevis* ovary was incubated in Barth's medium containing all four tritiated nucleosides (100  $\mu$ Ci/ml each; Amersham Radiochemical Centre, Buckinghamshire, England) for 17 h at 20°C. Isolated nuclear contents and nucleoli were treated with high-salt buffer followed by centrifugation as described above. Nucleic acids in the pellet and supernate were precipitated in 10% ice-cold TCA, collected on Whatman GF/C glass fiber filters, and dried from ethanol. The radioactivity was then determined with a liquid scintillation counter.

### Treatments of Isolated Nucleoli and Nucleolar Residues with Nucleases

Nuclear contents and isolated nucleoli were treated with one or several of the following enzymes: DNase I (Worthington Biochemical Corp., Freehold, N. J.; 100 U/ml), micrococcal nuclease (Worthington Biochemical Corp., 400 U/ml); and pancreatic RNase (Serva; 50  $\mu$ g/ml). Enzyme treatments were carried out for 30 min at room temperature in 5 mM Tris-HCl (pH 7.4), 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>. The material was then thoroughly washed several times in 1 mM Tris-HCl (pH 7.2) with or without 0.5 mM EDTA and was processed for observation by light or electron microscopy or biochemical analysis. In another series of experiments, isolated nucleoli were digested in 5:1 isolation medium containing 1 mM MgCl<sub>2</sub>, 0.5 mM PMSF, and 2.5 mM dithiothreitol, with DNase I and pancreatic RNase (concentrations as above: ~20  $\mu$ g of each enzyme per 10<sup>6</sup> nucleoli) for 30 min at room temperature, pelleted (5 min, 9,000 g), and either analyzed directly or extracted further with low-salt buffer (10 mM Tris-HCl, pH 7.4). In some experiments, these nuclease-digested nucleolar fractions were further extracted with high-salt buffers with or without dithiothreitol as described above. Alternatively, nuclear contents isolated in 5:1 isolation medium containing 2 mM MgCl<sub>2</sub> were treated with both DNase and RNase (same concentrations as above) in isolation medium for 30 min and then directly adjusted to high-salt concentration by addition of an equal volume of 2.0 M KCl, 10 mM Tris-HCl (pH 7.4), 2 mM MgCl<sub>2</sub>, 10 mM dithiothreitol, with or without 2% Triton X-100. After another 30-min incubation, the residual material was pelleted at 3,000 g for 20 min. The pellet was washed in 20 mM Tris-HCl or Sørensen phosphate buffers (both pH 7.4) and used for gel electrophoresis or microscopy.

### Light Microscopy

Preparations were made in microscope slide chambers (for technical details see references 10 and 20) and centrifuged at 2,000 g for 10 min to attach the material firmly to the cover slip forming the bottom of the chamber. Photographs were taken with the inverted microscope IM 35 (Carl Zeiss, Oberkochen, Germany) using phase contrast or differential interference contrast (Nomarski optics).

### Electron Microscopy

Isolated nucleoli and nuclear contents obtained after the various extraction procedures were fixed at room temperature or on ice for 30 min, with 2.5% glutaraldehyde in 0.05 M sodium cacodylate buffer (pH 7.2) or in the buffer used in the specific extraction procedure. In some experiments, high-salt-extracted fractions were fixed in 2.5% glutaraldehyde made up in 10 mM phosphate buffer (pH 7.4) containing 1.0 or 1.5 M KCl, to avoid rearrangements during reduction of ionic strength. Then the material was washed thoroughly by several changes of cold cacodylate buffer and postfixed in 2% osmium tetroxide for 60 min in the cold. After several washes in distilled water the samples were incubated overnight at 4°C in an aqueous 0.5% solution of uranyl acetate. After dehydration in a graded ethanol series and a passage through propylene oxide, the material was embedded in Epon 812 (Serva). Ultrathin sections obtained with a Reichert OmU3 ultramicrotome (Reichert, Vienna, Austria) were double-stained according to conventional procedures and observed in the electron microscope (Elmiskop 101, Siemens, West Berlin, Germany; EM 10A, Carl Zeiss).

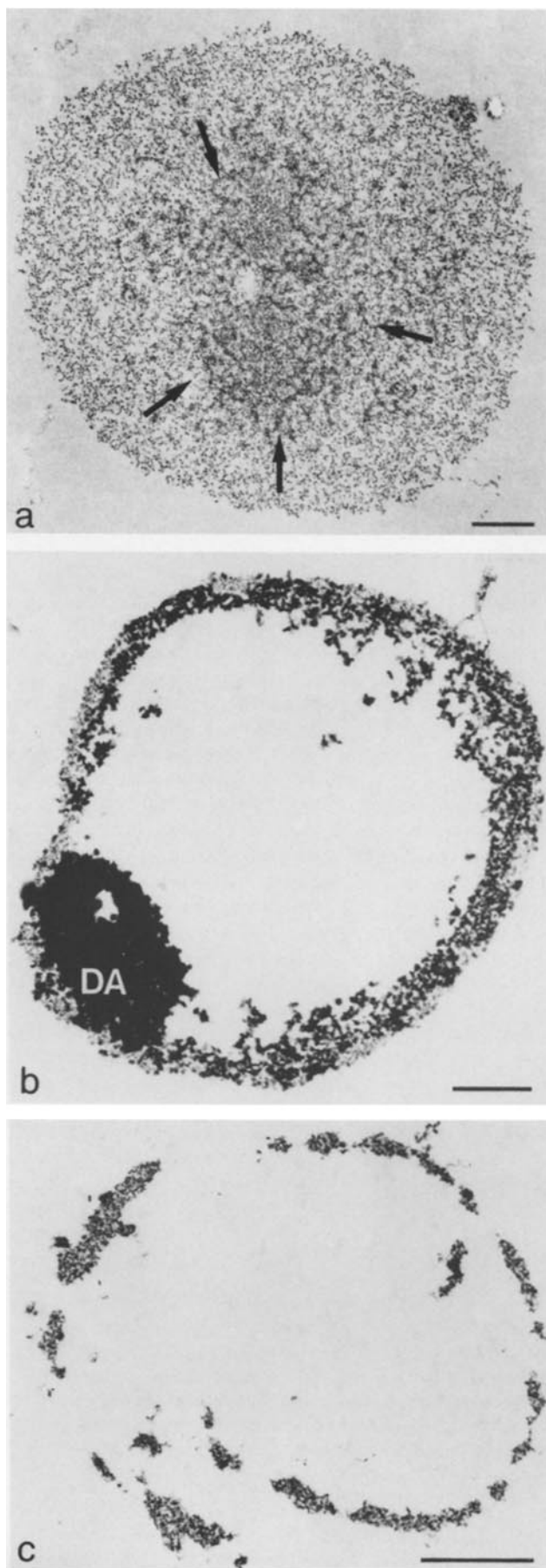
Spread preparations were made essentially according to the procedure described by Miller and Bakken (45), using the following modification: The material treated as described above was layered on top of a cushion consisting of 1% glutaraldehyde, 0.1 M sucrose, 0.5 mM sodium borate buffer of differing pH values (7.4, 8.0, 9.0) in a centrifugation chamber and centrifuged onto freshly glow-discharged carbon-coated grids (3,500 g for 30 min). The grids were rinsed in distilled water and negatively stained with 1% uranyl acetate. Some preparations were also positively stained with ethanolic 1% phosphotungstic acid and dried from 100% ethanol (45).

## RESULTS

### Isolation of Nucleoli

The nucleus ("germinal vesicle") of the maturing oocyte of *Xenopus laevis* contains ~1,000 extrachromosomal nucleoli that are relatively closely spaced and represent, by far, the most frequent structural components present in these nuclei. Nucleoli were isolated by one of the following procedures: (a) Nucleolar residual material (skeletons) was directly enriched from manually isolated germinal vesicles by first preparing gelled nuclear contents in the presence of millimolar concentrations of MgCl<sub>2</sub> (37, 56) followed by extraction in high-salt buffers. (b) Nuclei isolated by the mass-isolation procedure (55) were homogenized, and nucleoli were separated from other particles, including contaminating yolk platelets, mitochondria, and nuclei of follicle epithelial cells, by fluorescence-activated particle sorting using a UV laser (Fig. 1). With this method, either the whole nucleolar population was collected or different size classes of nucleoli were fractionated. By this procedure several million nucleoli could be isolated and counted in one experiment. The purity of the nucleolar fractions obtained by this procedure as well as the good morphological preservation is demonstrated in Fig. 2. Electron microscopy of spread preparations of such fractions revealed typical arrays of RNA polymerase-covered nucleolar chromatin intercepts separated

FIGURE 3 Light microscopy (a–d, interference contrast; e, phase contrast) and electron microscopy (f and g, ultrathin sections) of residual ("skeletal") structures of amplified nucleoli manually isolated from *Xenopus* oocytes obtained after various treatments. (a) Nucleoli isolated in buffered 5:1 isolation medium containing 2 mM MgCl<sub>2</sub>, after treatment with pancreatic RNase, followed by washing in low-salt buffer (1 mM Tris): a central dense aggregate is surrounded by a ringlike cortical structure. (b) Nucleoli similarly isolated and then treated with a mixture of DNase I, micrococcal nuclease, and pancreatic RNase: the dense central aggregate of the nucleoli has been reduced and, in some nucleoli, has completely disappeared, whereas the residual cortical shell has been retained. (c) Nucleolar residues obtained after isolation in the presence of 2 mM MgCl<sub>2</sub> and subsequent treatment with DNase and repeated washes in low-salt buffer: the dense aggregate has disappeared, the only structure resistant is the cortical skeletal component appearing as a ring. (d) Nucleoli isolated in buffered 5:1 isolation medium containing 2 mM MgCl<sub>2</sub> and then washed and incubated in 1 mM Tris-HCl buffer (pH 7.2). A ringlike cortical structure is seen, which surrounds a dense, often eccentrically located aggregate. (e) Nucleolar skeletons obtained after isolation in buffered 5:1 isolation medium containing 2 mM MgCl<sub>2</sub> and treatment with buffer containing 1 mM KCl and 1% Triton X-100. (f) Electron micrograph of nucleolar skeleton after treatment as described for e, showing a loosely packed filament meshwork and peripheral densely stained aggregates. (g) Higher magnification of a preparation similar to that shown in f, illustrating the difference in filament density in central and cortical skeleton components. Some of the peripheral skeleton aggregates are denoted by arrows in f and g. Bars, 10  $\mu$ m (a–c and e), 20  $\mu$ m (d), and 1  $\mu$ m (f and g).  $\times$  1,700 (a and b),  $\times$  2,100 (c),  $\times$  750 (d),  $\times$  1,100 (e),  $\times$  22,000 (f), and  $\times$  32,000 (g).



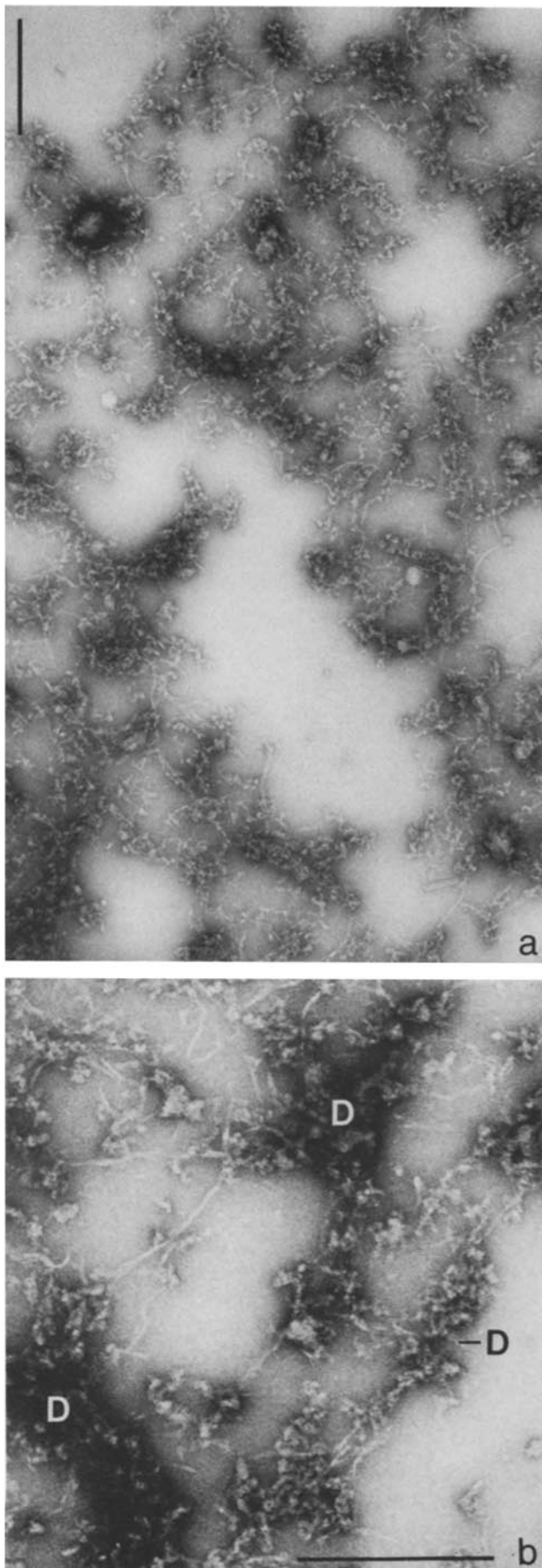
by spacer regions (M. F. Trendelenburg and J. Kleinschmidt, unpublished data). (c) Nuclei isolated in 5:1 isolation medium without  $MgCl_2$  were opened with a fine needle, and individual nucleoli were collected by use of a micropipette attached to a micromanipulator under observation in an inverted phase-contrast microscope (for technical details, see reference 26). With this method, ~200 nucleoli could be collected per hour.

#### Morphology of Residual Nucleolar Structures Obtained after Treatment with Nuclease and Low-salt Buffer

When isolated nucleoli from oocyte nuclei of *Xenopus* were digested with pancreatic RNase, rapid loss of some material occurred but a residual structure consisting of a shell-like nucleolar cortex and a central spheroidal aggregate was left (Fig. 3a). Electron microscopy showed that this central intranucleolar body contained densely aggregated and heavily stained fibrillar material, mostly nucleolar chromatin (not shown here). During digestion with DNase, micrococcal nuclease, and nuclease mixtures, the central aggregate body was gradually removed but the cortical shell component was still seen as a distinct "nucleolar ghost" demarcating the contour of the original nucleolus (Fig. 3b and c; in optical sections this structure usually appeared as ring). Prolonged treatment with nucleases for up to an hour as well as subsequent washes in low-salt buffers did not change the appearance of these residual nucleolar ghosts. Similar nucleolar ghost structures surrounding an often eccentrically located internal dense body were also observed when nucleoli isolated in 5:1 isolation medium with 2 or 10 mM  $MgCl_2$  were incubated in very low salt buffer (Fig. 3d) such as 1 mM Tris-HCl buffer (pH 7.2) or borate buffer at pH 9.0. Electron microscopy of such low-salt-extracted,  $Mg^{2+}$ -stabilized nucleoli showed two predominant structures, a dense aggregate and a cortical skeleton (Fig. 4b). Inclusion of 20 mM dithiothreitol or 2-mercaptoethanol in the various solutions did not result in significant changes of morphology. Such nucleolar skeleton shells were not induced by treatment with low-salt buffer or Triton X-100 alone because digestion with nucleases, followed directly by high-salt treatment, with (Fig. 4c) and without (not shown here) Triton X-100, also resulted in the appearance of these structures.

Pretreatment of the isolated nuclei and/or nucleoli with 2–10 mM concentrations of  $MgCl_2$  was critical for the appearance

FIGURE 4 Electron micrographs of ultrathin sections of manually isolated nucleoli from *Xenopus* oocytes after different extraction procedures. (a) Nucleolus isolated in 5:1 isolation medium without added divalent cations and subsequently treated with very low salt buffer at elevated pH (0.1 mM borate buffer, pH 9.0). After this treatment the slightly swollen nucleolus presents a central aggregate of densely stained fibrillar arrays (arrows) surrounded by an outer sphere of less densely packed fibrillar granular material. (b) Nucleolus isolated in 5:1 medium containing 2 mM  $MgCl_2$  and subsequently incubated in 1 mM Tris-HCl buffer (pH 7.2), presenting a cortical shell structure still associated with a dense, heavily stained aggregate (DA) that is often eccentrically located. (c) Nucleolus isolated in 5:1 medium containing 1 mM  $MgCl_2$ , treated with DNase I and RNase (see Materials and Methods), extracted in low-salt buffer (procedure described in Materials and Methods), washed several times in low-salt buffer (20 mM phosphate, pH 7.4), showing the preservation of the cortical shell structure of densely aggregated filaments. Bars, 1  $\mu m$ .  $\times 9,500$  (a),  $\times 12,000$  (b), and  $\times 18,000$  (c).



of a distinct, separate cortical skeleton in the nucleolus. Fig. 4a presents the morphology of a nucleolus from a nucleus exposed to isolation medium without  $MgCl_2$  and extracted in low-salt buffer. The central dense aggregate containing the transcriptionally active rDNA chromatin fibrils is distinguished from the peripheral material but the skeletal components are not detached and separated into a distinct cortical shell.

#### Morphology of Nucleolar Skeletons Obtained after Treatment with High-salt Buffers and Detergents

When gelled nuclear contents or isolated nucleoli from *Xenopus* oocytes were extracted in high-salt buffers (1.0 or 1.5 M KCl) containing 1% Triton X-100, without or with dithiothreitol, characteristic residual structures were found (Fig. 3e-g). Such residual nucleolar structures were roughly spheroidal and exhibited essentially the same range of variation of diameters (3.5–14  $\mu m$ ) as the intact nucleoli from which they were derived. In thin sections the basic structural components present in these nucleolar skeletons appeared as a three-dimensional meshwork of filaments (Fig. 3f and g). Two types of filament organization could be distinguished: (a) The interior of the nucleolar skeletons was formed by relatively loosely packed tangles of filaments  $\sim 4$  nm thick, which appeared mostly to be arranged in higher-order coils of diameters 30–40 nm. (b) In the periphery of the nucleolar skeletons intensely stained aggregates of various sizes were seen that represented local ravelings of filament packing (Fig. 3f and g) and revealed many filament continuities with internal filament elements. The condensed filament coil aggregates present in the periphery were also visible in the light microscope (Fig. 3e). In thin sections, the tightly coiled organization of these skeletal filaments often gave the impression of 25- to 30-nm large granules (Fig. 3g) but closer inspection, especially of nucleolar skeletons washed in low-salt buffers after high-salt treatment, revealed the filament-coil nature of both components, the internal meshwork, and the peripheral aggregates. The residual nucleolar cortex structure was also observed when nucleoli had been treated with DNase I and RNase, before extraction in high-salt buffers with (Fig. 4c) or without (not shown here) Triton X-100.

In negatively-stained spread preparations of nucleolar skeleton-enriched fractions obtained by combined treatment with high-salt buffer and Triton X-100, the filamentous composition was also apparent (Fig. 5). The best resolution of the individual filaments was obtained when the nucleolar residue material prepared in high-salt buffer was first washed in low-salt buffer (10 mM phosphate buffer, pH 7.2), followed by brief incubation in 0.5 mM borate buffer of pH 9.0 before spreading. In such preparations various degrees of coiling and/or aggregation of the constitutive filaments were observed, including the occur-

FIGURE 5 Electron microscopy of negatively stained residual fractions of *Xenopus* oocyte nucleoli manually isolated in 5:1 isolation medium containing 2 mM  $MgCl_2$ , extracted as described in procedure c of Materials and Methods, washed in 10 mM phosphate buffer (pH 7.4), and visualized after spread preparation by negative staining with uranyl acetate. The characteristic filamentous composition is recognized (a). At higher magnification (b), two types of residual filament structures are distinguished, one type being coiled and often densely aggregated (D), the other type of filament appearing more rigid and smoothly contoured and extending in between the dense knots. Bars, 0.2  $\mu m$ .  $\times 87,000$  (a),  $\times 150,000$  (b).

rence of "nodules" or densities ( $D$  in Fig. 5 *b*) that seemed to correspond to the peripheral dense aggregates described above in thin sections. In these negatively stained preparations, the diameters of the individual filaments were found to exhibit some variation, ranging from 3 to 6 nm.

### Absence of RNA in High-salt-extracted Nucleolar Skeletons

Because the transcriptionally active nucleoli of amphibian oocytes are very rich in RNA, we examined the question of the retention of nucleolar RNA in the high-salt-extracted nucleolar skeletons. As can be seen from Fig. 6, nucleolar RNA, i.e., 40S pre-rRNA and the nuclear form of 28S rRNA, was completely recovered in the supernate of high-salt extractions of nuclear content material (Fig. 6 *b*, slot 2). Correspondingly, no substantial amount of RNA was retained in the pelleted nucleolar skeletons (Fig. 6 *b*, slot 3). The nuclear form of 18S rRNA (see Fig. 6 *a*) was predominantly nucleoplasmic and not retained in significant amounts in the gelled nuclear contents and isolated nucleoli used as starting material, in agreement with reports of reduced amounts of 18S rRNA in isolated nuclear structures from various cells (16, 19, 38, 49; see there for further references).

The association of RNA with high-salt-extracted nucleolar skeletal structures was also examined in experiments in which oocyte RNA was extensively labeled. In three different exper-

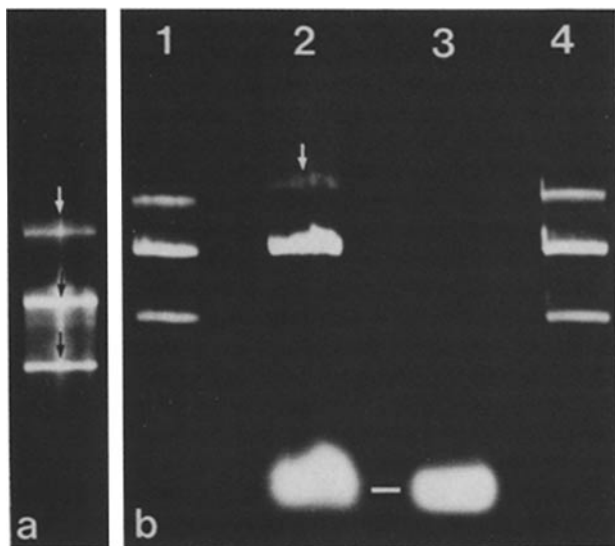


FIGURE 6 Absence of RNA in high-salt-extracted nucleolar skeletons as demonstrated by gel electrophoresis of RNA (1.5% agarose gels) from manually isolated nuclei (*a*) and high-salt-extracted nuclear contents (*b*) of *Xenopus laevis* oocytes. (*a*) RNA extracted from oocyte nuclei (150 nuclei) is concentrated mainly in three bands (arrows) representing (from top to bottom) 40S pre-rRNA, nuclear 28S rRNA, and nuclear 18S rRNA. (*b*) High-salt-extracted nuclear contents were separated in pelletable and supernatant material, and the RNA content was analyzed separately (pellet, slot 3; supernate, slot 2). Marker RNAs were run in adjacent slots (1 and 4) and represent, from top to bottom: TMV RNA and 28S and 18S rRNA of *Xenopus laevis*. The pre-rRNA (arrow, slot 2) and nuclear 28S rRNA species are almost quantitatively recovered in the supernatant fraction (slot 2), whereas no RNA is detected in the pellet containing the nucleolar skeletons, except the carrier tRNA added (indicated by the bar). Note that the 18S rRNA species is almost completely absent from these nuclear subfractions.

iments, always <1% of the total radioactive RNA was found to be associated with the nucleolar residue fraction obtained after extraction with high-salt buffer; practically all RNA was recovered in TCA-precipitates of combined supernatant fractions.

### Proteins of Nucleoli and High-salt-extracted Nucleolar Skeletons

Fractions of isolated nucleoli as they have been used as starting materials for the preparations of nucleolar skeletons contained a number of polypeptides, most of them >35,000 mol wt (Fig. 7 *a*). The complexity of polypeptide bands as revealed by one-dimensional electrophoresis on polyacrylamide gels (Fig. 7 *a*) was similar to that described in nucleolar fractions from early oocytes (stages II and III) isolated (30, 52) by centrifugation in Metrizamide (Nyegaard, Oslo, Norway). Characteristically, washed isolated nucleoli and gelled nuclear contents were largely depleted of soluble nucleoplasmic proteins such as the most predominant nuclear protein of 30,000 mol wt (cf. Fig. 7 *a*, *b*, and *e*; for characterization of this phosphoprotein, see references 34, 35, 47). All nucleolar fractions isolated by particle sorting or manually still contained considerable amounts of nuclear actin (Fig. 7), in agreement with observations of other authors (e.g., 11, 37, 44). The polypeptide patterns of large and small nucleolar subfractions were practically identical.

When fractions enriched in high-salt-resistant nucleolar skeletons were examined by one- and two-dimensional gel electrophoresis of proteins, a remarkable reduction of complexity of polypeptide composition was found (Figs. 7 and 8). Treatment with high-salt buffers and Triton X-100 resulted in the enrichment of one major polypeptide of an apparent molecular weight of 145,000 (denoted by arrows and arrowheads in Figs. 7 and 8). Enrichment of this protein was most conspicuous in nucleolar skeleton fractions prepared by extraction in buffers containing 1 M KCl, whereas nucleolar residue fractions extracted with solutions containing lower or higher (1.5 M KCl) salt concentrations usually showed the presence of some other proteins in variable amounts (Figs. 7 and 8), including a component of ~65,000 mol wt and various amounts of residual actin (e.g., Fig. 7). However, both the 65,000 mol wt polypeptide and actin were clearly not specific to the nucleolus because they were also recovered, in larger proportions, in other nuclear subfractions, including pooled supernatant fractions obtained during preparations of nucleoli (data not shown). The protein was well visualized after staining with Coomassie Blue (Fig. 7 *a-c*), silver ions (Fig. 8 *b*) and by radioautography after [<sup>3</sup>H]dansylation in vitro (Fig. 7 *d* and *e*). On two-dimensional gel electrophoresis, the 145,000 mol wt protein enriched in the nucleolar skeleton fractions appeared as a single isoelectric component with an isoelectric pH value of ~6.15 (Fig. 8 *b*).

We conclude that this protein, which was also identified as a moderately frequent component of purified whole nucleoli (Fig. 7 *a*, *b*, and *e*), is a major constitutive component of the high-salt-resistant skeletal filament meshwork of the nucleolus. This protein was only a minor protein of whole nuclei (Fig. 7 *b*, slot 2, and Fig. 8 *a*) and was not detected at all in native (not shown) or high-salt-buffer-extracted (Fig. 7 *f*) nuclear supernatant fractions obtained after pelleting nucleoli at each step of nucleolar isolation. Interestingly, we also noted an enrichment of a polypeptide of 130,000–140,000 mol wt when we reexamined the results of an experiment by Higashinaka-gawa et al. (30), in which these authors had digested isolated



*Xenopus* oocyte nucleoli with pancreatic RNase, treated the digest with low-salt concentrations, and pelleted the residual material.

The gel electrophoretic analyses of the nucleolar skeletal proteins also demonstrated the absence of detectable amounts of typical ribonucleoproteins of the nucleolar and ribosomal type as well as of histones in these preparations (compare also reference 30).

## DISCUSSION

Our observations demonstrate the existence of a fibrous meshwork (nucleolar skeleton) in the nucleolus, that is resistant to

treatments with low- and high-salt concentrations, DNases and RNases, and nonionic detergents such as Triton X-100 and Nonidet P-40. This residual filament structure is also observed after treatment with solutions containing relatively high (20 mM) concentrations of sulfhydryl agents such as dithiothreitol and 2-mercaptoethanol, which indicates that disulfide bonds are not critically important for the maintenance of this skeletal complex. Thus, preparatively, the nucleolar skeleton of *Xenopus* oocytes falls into the class of nuclear substructures described in somatic cells that is commonly referred to under the collective term "residual nuclear matrix" (for synonyms and reviews, see references 4 and 12).

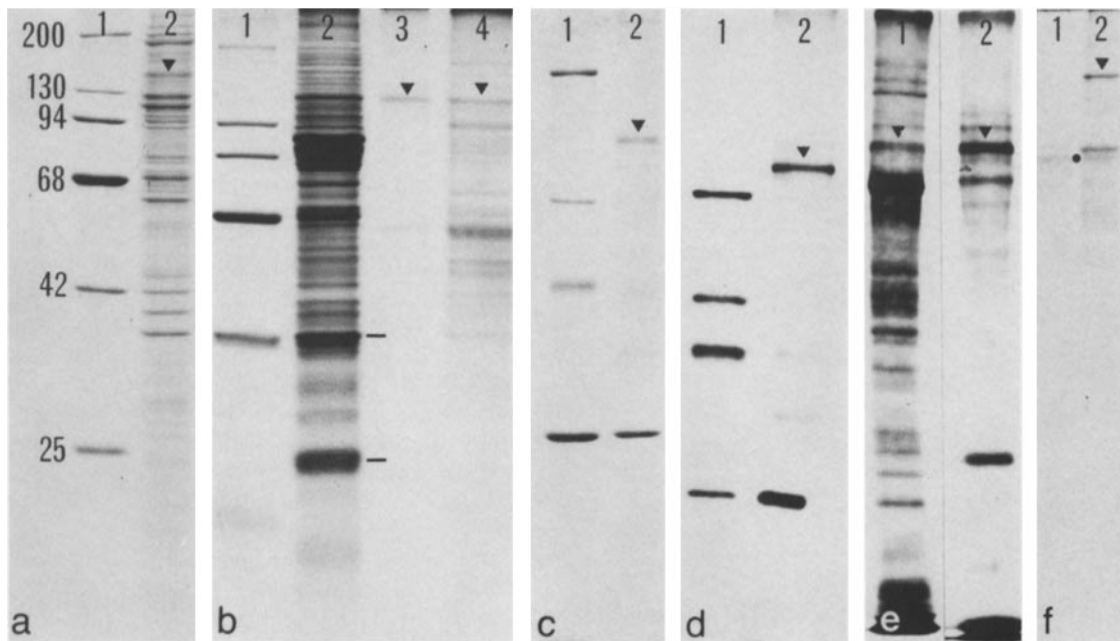


FIGURE 7 SDS-polyacrylamide gel electrophoresis showing the enrichment of the polypeptide of 145,000 mol wt (denoted by arrowheads) in nucleolus-rich fractions and high-salt-buffer-extracted nucleolar skeleton preparations from *Xenopus* oocytes. (a) Polypeptide pattern of nucleoli isolated by fluorescence-activated particle sorting (slot 2; similar patterns have been found for the various nucleolar size classes separated by this technique), in comparison with reference proteins (slot 1, molecular weight references in  $10^3$  units are indicated in the left; from top to bottom: myosin heavy chain,  $\beta$ -galactosidase, phosphorylase *a*, bovine serum albumin, rabbit skeletal muscle actin, chymotrypsinogen). (b) Reference polypeptides (slot 1) and polypeptides present in whole manually isolated oocyte nuclei (slot 2; bars denote the position of actin and the soluble phosphoprotein of  $M_r$  30,000) are compared with polypeptides of nucleoli isolated by fluorescence-activated particle sorting and extracted once with high-salt buffer (slot 4, from  $2 \times 10^6$  sorted nucleoli) and polypeptides of nucleolar skeleton-rich fractions obtained after two extractions in high-salt-detergent buffer according to procedure a described in Materials and Methods (slot 3). (c) Nucleolar skeleton-rich material prepared from gelled nuclear contents by extraction in high-salt buffers (extraction procedure b as described in Materials and Methods, from a total of 150 nuclei), in comparison with reference proteins (slot 1, as in a but without  $\beta$ -galactosidase and chymotrypsinogen). Gels shown in a–c have been stained by Coomassie Blue. (d) Comparison of reference polypeptides (slot 1;  $\beta$ -galactosidase, transferrin, BSA, and actin) with polypeptides present in nucleolar skeleton-rich fraction (similar preparation as that shown in c, slot 2) visualized by autoradiography after electrophoresis of in vitro  $^3\text{H}$ -dansylated proteins (see Materials and Methods). (e) Polypeptides present in whole nucleoli isolated by fluorescence-activated particle sorting in 5:1 medium containing  $\text{MgCl}_2$  that have been treated with DNase and RNase, washed in 10 mM Tris-HCl buffer (pH 7.4), denatured, and  $^3\text{H}$ -dansylated (slot 1), in comparison with polypeptides of nucleolar skeletons obtained therefrom by extraction with high-salt buffer (1 M KCl) containing 1% Triton X-100 (see procedure b of Materials and Methods), denatured, and  $^3\text{H}$ -dansylated (slot 2). (f) Proteins of soluble nuclear material treated with high-salt buffer (slot 1) and Triton X-100 in comparison with pelletable nuclear material obtained after extraction with high-salt buffer containing Triton X-100 (slot 2). In this type of experiment oocyte nuclei (500–800) isolated according to the procedure of Scalenghe et al. (55) were homogenized, in 5:1 isolation medium, by sucking up and down in a pipette (see Materials and Methods), and the homogenate was centrifuged for 6 min at 9,000 g. The pellet was directly extracted for 30 min at room temperature in high-salt buffer (1 M KCl, 10 mM Tris-HCl, pH 7.4) containing 1% Triton X-100 and showed, as judged by microscopy, enrichment of residual nucleolar skeletons and nuclear membrane-associated structures (slot 2 shows the  $M_r$  145,000 polypeptide associated with nucleolar skeletons, and the  $M_r$  68,000 polypeptide that is the major component of nuclear envelope residues; cf. reference 37). The supernate was adjusted to 1 M KCl and 1% Triton X-100, incubated for 30 min, and recentrifuged (same conditions). Only trace amounts of the  $M_r$  65,000 polypeptide were recovered in this pellet (slot 1, dot), indicating that no sedimentable structure has been induced to form from supernatant proteins during the incubation with high-salt buffer and detergent.

Preparations of whole nuclear matrix fractions described in the literature have included residual nucleolar structures, but obviously this material represents only a minor proportion of the total "insoluble" material used in these studies (4-7, 9, 13, 31, 46, 62). Our mode of preparation of extrachromosomal nucleolar skeletons excludes most of the contaminations frequently encountered in preparations from somatic cells such as chromosomal nonhistones of the "scaffold-type" (2, 3), peripheral lamina-pore complex material (1, 22, 23; for the related preparation from *Xenopus* oocytes, see reference 37), and matrix-associated ribonucleoproteins containing mRNA sequences (29, 42, 46).

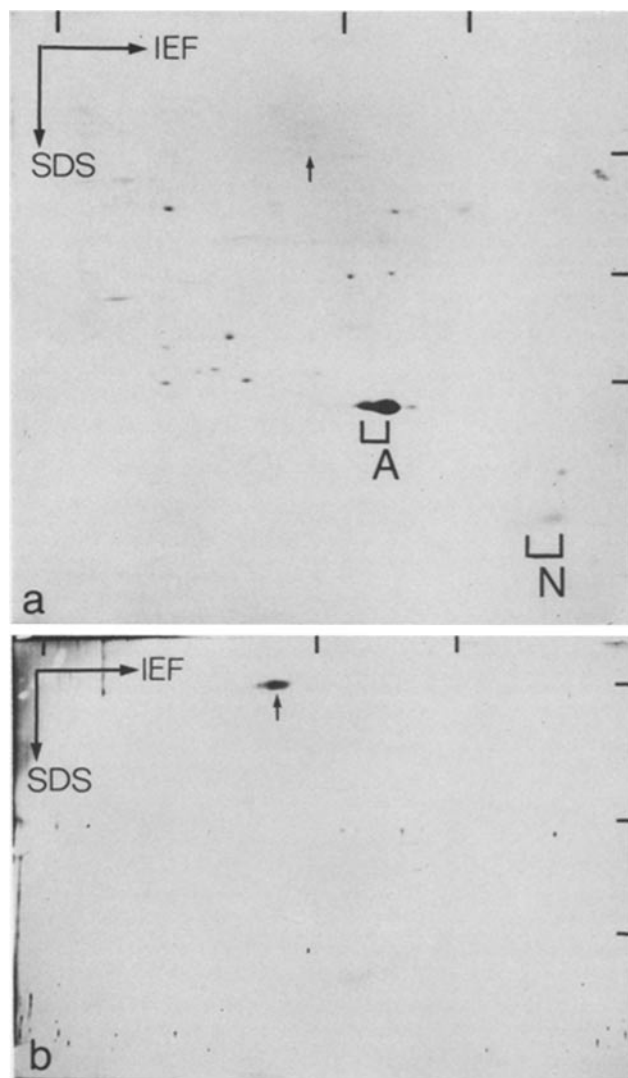


FIGURE 8 Two-dimensional gel electrophoresis of total nuclear content proteins (a, Coomassie Blue) and proteins of nucleolar skeletons from  $1.7 \times 10^6$  nuclei extracted in high-salt buffer (1 M KCl, 1% Triton X-100, 10 mM Tris-HCl, pH 7.4) as seen after staining with silver (b). The high-salt resistant nucleolar protein of  $M_r$  145,000 focuses at pH 6.15 (arrow). Note that this polypeptide is present only as a minor protein of the nuclear contents. The  $M_r$  145,000 polypeptide was not identified in similar gel electrophoretic analyses of total proteins from nuclear supernatant fractions as used in the experiment described in Fig. 7f (not shown here). A, actin; N, nucleoplasmin, the  $M_r$  30,000 phosphoprotein. The horizontal scale (top) presents pH values (from left to right: 7.0, 6.0, 5.0), the vertical scale (right margin) presents estimated molecular weights (from top to bottom: 150,000, 70,000, 45,000).

The resistant nucleolar filament meshwork (skeleton) described in this study is clearly different, by structural and biochemical criteria, from all other preparations of high-salt-resistant nuclear components described so far. (a) Its stainability, in thin sections, with uranyl and lead salts is much less than that of ribonucleoprotein fibrils, matrix material, nuclear pore complex structures, and high-salt-extracted chromosomal residues from oocytes and other cells. This is in agreement with the reduced staining seen in nucleolar residue structures compared with that in matrix and lamina material of the same nucleus (see references 2, 13, 31, 62; see also Figs. 7 and 8 of reference 7). (b) The organization of the nucleolar skeleton in  $\sim 4$ -nm filaments tightly coiled into higher-order fibrils of  $\sim 30$ -40 nm as well as the pattern of nodular aggregates of the nucleolar cortex is different from the appearance of high-salt-extracted residual filaments present in matrix and nuclear envelope-derived fractions (e.g., 5, 13, 15, 57). (c) It is not associated with RNA in a way resistant to extraction in high-salt buffers. These observations are in agreement with reports of a release of pre-rRNA-containing particles under relatively mild conditions, i.e., at low or physiological salt concentrations, from nucleoli of amphibian oocytes (54), from macronuclei of *Tetrahymena* (53), and from various vertebrate cells (e.g., 39, 43). These findings, as well as ours, seem to be in contrast to a report (28) claiming that large amounts of rRNA precursors are not released by high-salt and DNase treatment from the macronuclear matrix of *Tetrahymena* (their data, including the observed retention of some 17S rRNA after high-salt treatment, could be the result of inefficient extraction). Moreover, our results, obtained after nuclease treatment of nucleoli, show that neither RNA or DNA is a constituent of the nucleolar skeleton. (d) The protein composition of the nucleolar skeleton fraction described here is different from that of all other high-salt-resistant structures described in amphibian oocytes (37) and in somatic cells from a broad range of organisms, including whole matrix preparations (7, 13, 28, 42, 50), chromosomal and nuclear scaffolds (2, 3), nuclear membrane and pore complex-lamina fractions (1, 22, 23, 32, 37, 41, 59), and nucleolus-enriched fractions from rat liver (7, 61). Our finding of the enrichment of one protein of 145,000 mol wt in nucleolar skeleton fractions of *Xenopus* oocytes also finds some support in the analyses of RNase-treated nucleoli from early *Xenopus* oocytes described by Higashinakagawa et al. (30).

We conclude that nucleoli in their pure extrachromosomal form, and possibly also chromosome-bound nucleoli, contain a skeletal meshwork of filaments resistant to various extractions that is specific to the nucleolus and characterized by a relatively simple protein composition. Evidence available at present suggests that the nucleus contains several distinct and different architectural units that can be classified as "skeletal" by both their insolubility and maintenance of the specific morphology of a nuclear substructure: examples of such structures are the chromosome scaffolds, the lamina structures, and the pore complexes. We speculate that the nucleolar skeleton protein(s) represent a self-assembly structure that can associate, with high specificity, with the rDNA-containing chromatin and/or with its transcriptional products. That fibrillar meshworks indistinguishable from the nucleolar skeleton structures described here are also observed in anucleolate (0-nu) mutants of *Xenopus laevis*, forming so-called "pseudo-nucleoli" (27), further suggests that such skeletal proteins can assemble into spheroidal meshwork structures independent of the presence of nucleolar chromatin.

The electron microscopy of thin sections of extracted nucleoli shows that the skeletal filament meshwork extends throughout both pars fibrosa and pars granulosa, apparently with greater density of filament packing in the outermost portion of the pars granulosa, and probably also the "fibrillar centers" (for reviews on nucleolar morphology, see references 9, 25, and 58). Hopefully, future studies aiming at localization of the major nucleolar skeleton protein by antibody techniques will clarify the distribution of the skeletal filament components within the intact nucleolus. From the demonstrated location of ribosomal precursor particles in the same nucleolar regions that contain the filament meshwork, it is tempting to hypothesize that the nucleolar skeleton serves as a filament support for the attachment and storage of these particles.

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