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

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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 ravels 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 coworkers (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 nuclear 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; 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 ("laminas" sensu reference 22) that have been localized by antibody techniques in the nuclear periphery (17, 23,
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 MgCl₂ for extractions (see above).

Fluorescence-activated Sorting of Fluorochrome-stained Nucleoli

Mass-isolated nuclei (in 5:1 isolation medium containing 5 mM MgCl₂, 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 were: nucleoli fluoresced red, with a minor degree of blue fluorescence. Nucleoli were collected manually with micropipettes attached to a micromanipulator under observation with an inverted microscope.

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 M 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 [3H]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 dried in 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 mM 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 microcentrifuge nitrate tube. The tube was overlayed with liquid paraffin and centrifuged in an SW65 rotor using special adaptors (Beckman Instruments, Munich, Germany) for 12 h at 40,000 rpm and 4°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 x 10 cm). Molecular weight markers (tobacco mosaic virus [TMV] RNA: 2.07 x 10⁶; *Xenopus laevis* rRNAs: 1.5 and 0.7 x 10⁶) 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 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 μm; range, 3.5–6.5 μm). b presents a higher magnification of a purified subfraction of larger nucleoli (mean diameter, 8.2 μm; range, 7–14 μm), 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 μm (a) and 10 μm (b and c). × 250 (a), × 1,500 (b), and × 2,900 (c).
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 supernatants were pooled. Nuclear acids and nucleoprotein material were precipitated from the supernatant as described by Deves and Grancharov (16). In brief, to the 2 ml of supernatant, 50 µl of 0.2 M sodium phosphate buffer (pH 7.6) was added, followed by 12 µl of 1 M MgCl₂ 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 pellet 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 µg of RNA 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 µCi/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. Nuclear 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 µg/ml). Enzyme treatments were carried out for 30 min at room temperature in 5 mM Tris-HCl (pH 7.4), 1 mM CaCl₂, 1 mM MgCl₂. 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 MgCl₂, 0.5 mM PMSE, and 2.5 mM diethiothreitol, with DNase I and pancreatic RNase (concentrations as above: ~20 µg of each enzyme per 10⁴ 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 diethiothreitol as described above. Alternatively, nuclear contents isolated in 5:1 isolation medium containing 2 mM MgCl₂ 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 M KCl, 10 mM Tris-HCl (pH 7.4), 2 mM MgCl₂, 10 mM diethiothreitol, with or without 2% Triton X-100. After 30 min of incubation, the residual material was pelleted at 3,000 g for 15 min. The pellet was washed in 20 mM Tris-HCl or Sorensen phosphate buffers (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 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 (Elmiscop 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 ethanol 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 MgCl₂ (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 intercets separated...
by spacer regions (M. F. Trendelenburg and J. Kleinschmidt, unpublished data). (c) Nuclei isolated in 5:1 isolation medium without MgCl₂ 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 MgCl₂ 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, Mg²⁺-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 MgCl₂ was critical for the appearance of

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**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 MgCl₂ 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 MgCl₂, treated with DNase I and RNase (see Materials and Methods), extracted in high-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 μm. X 9,500 (a), X 12,000 (b), and X 18,000 (c).
of a distinct, separate cortical skeleton in the nucleolus. Fig. 4 a presents the morphology of a nucleolus from a nucleus exposed to isolation medium without MgCl₂ 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. 3 e–g). Such residual nucleolar structures were roughly spheroidal and exhibited essentially the same range of variation of diameters (3.5–14 μm) 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 ~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 ravels 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. 3 e). 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-

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**FIGURE 5** Electron microscopy of negatively stained residual fractions of *Xenopus* oocyte nucleoli manually isolated in 5:1 isolation medium containing 2 mM MgCl₂, 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 μm. × 87,000 (a), × 150,000 (b).
reference of "nodules" or densities (D in Fig. 5b) 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. 6b, slot 2). Correspondingly, no substantial amount of RNA was retained in the pelleted nucleolar skeletons (Fig. 6b, slot 3). The nuclear form of 18S rRNA (see Fig. 6a) 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 experiments, 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. 7a). The complexity of polypeptide bands as revealed by one-dimensional electrophoresis on polyacrylamide gels (Fig. 7a) 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. 7a, b, and c) 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 nuclear 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. 7a–c), silver ions (Fig. 8b) and by radioautography after [3H]dansylation in vitro (Fig. 7d 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. 8b).

We conclude that this protein, which was also identified as a moderately frequent component of purified whole nucleoli (Fig. 7a, 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. 7b, slot 2, and Fig. 8a) and was not detected at all in native (not shown) or high-salt-buffer-extracted (Fig. 7f) 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 Higashinakagawa 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 sulphydryl 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](image-url)
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).

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 nucleolar components described so far. (a) Its stability, in thin sections, with uranyl and lead salts is much less than that of ribonucleoprotein fibrils, matrix material, nucleolar 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 ~4-nm filaments tightly coiled into higher-order fibrils of ~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 nor DNA is a constituent of the nucleolar skeleton. (d) The protein composition of the nucleolar 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 nucleolus 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 skeletal 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.

**Figure 8** Two-dimensional gel electrophoresis of total nuclear content proteins (a, Coomassie Blue) and proteins of nucleolar skeletons from 1.7 × 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 Mr 145,000 focuses at pH 6.15 (arrow). Note that this polypeptide is present only as a minor protein of the nuclear contents. The Mr 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, nucleoplasm, the Mr 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 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 "fibricular 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 intact nucleolus, it is tempting to hypothesize that the nucleolar skeleton serves as a filament support for the attachment of these particles and storage of these particles.

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