Immunological Localization of a Major Karyoskeletal Protein in Nucleoli of Oocytes and Somatic Cells of *Xenopus laevis*

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ABSTRACT Oocyte nuclei of *Xenopus laevis* contain two major karyoskeletal proteins characterized by their resistance to extractions in high salt buffers and the detergent Triton X-100, i.e. a polypeptide of 68,000 mol wt which is located in the pore complex-lamina structure and a polypeptide of 145,000 mol wt enriched in nucleolar fractions. Both proteins are also different by tryptic peptide maps and immunological determinants. Mouse antibodies were raised against insoluble karyoskeletal proteins from *Xenopus* oocytes and analyzed by immunoblotting procedures. Affinity purified antibodies were prepared using antigens bound to nitrocellulose paper. In immunofluorescence microscopy of *Xenopus* oocytes purified antibodies against the polypeptide of 145,000 mol wt showed strong staining of nucleoli, with higher concentration in the nucleolar cortex, and of smaller nucleoplasmic bodies. In various other cells including hepatocytes, Sertoli cells, spermatogonia, and cultured kidney epithelial cells antibody staining was localized in small subnucleolar granules. The results support the conclusion that this "insoluble" protein is a major nucleus-specific protein which is specifically associated with—and characteristic of—nucleoli and certain nucleolus-related nuclear bodies. It represents the first case of a positive localization of a karyoskeletal protein in the nuclear interior, i.e. away from the pore complex-lamina structure of the nuclear cortex.

The cell nucleus contains various structures that are insoluble in high and low salt buffers and nonionic detergents and are assumed to provide karyoskeletal support to other nuclear components. These karyoskeletal elements include diverse components such as the peripheral nuclear lamina, the nuclear pore complex, nucleolar fibrils, a "scaffold" structure described for both metaphase chromosomes and interphase nuclei, and fibrillar, less defined masses extending through the nuclear interior which are often referred to under the collective term "nuclear matrix" (for references see 1–3, 8, 14–16, 19–21, 33). We have recently described, in fractions enriched in amplified nucleoli from oocytes of *Xenopus laevis,* a meshwork of filaments resistant to various extractions in high salt buffers which contains only one predominant protein characterized by a polypeptide of 145,000 mol wt (14). This protein has not been found in pore complex-lamina fractions or in "nuclear sap" from the same nucleus, nor in frog erythrocyte nuclei, which are known to be devoid of nucleoli. Therefore, we have suggested that it might be specifically associated with nucleoli and nucleoluslike fibrillar bodies (14, 26). In the present study we report that this protein is chemically and immunologically different from other karyoskeletal "insoluble" nuclear proteins and can be localized, by antibody techniques, to nucleoli of oocytes and other cells.

MATERIALS AND METHODS

Animals and Cells

Clawed toads (*Xenopus laevis*) of both sexes obtained from the South African Snake Farm (Fish Hoek, South Africa) were kept in water tanks. Cultured kidney epithelial cells of *X. laevis* were grown as described (4, 12).

Isolation of Oocyte Nuclei and Nuclear Subfractions

Nuclei of full-sized oocytes (stages V and VI) from *X. laevis* were manually isolated in "5:1-medium" (cf. 19) or by a modification (14) of the mass-isolation technique of Scalenghe et al. (29). Gelled nuclear contents were prepared as described (20). For enrichment of karyoskeletal proteins isolated oocyte nuclei were incubated for 30 min in cold (4–8°C) 10 mM Tris-HCl buffer (pH 7.2) containing 1.0 M KCl and 1% Triton X-100. Residual material pelleted by centrifugation at 9,000 *g* for 5 min was washed once more with this "high-salt–Triton buffer" and then twice with 10 mM Tris-HCl (pH 7.2).

Gel Electrophoresis and Tryptic Peptide Analysis

SDS PAGE was as described in references 14 and 34. Analysis of tryptic peptides derived from electrophoretically separated and radioiodinated polypeptides was performed using the method of Elder et al. (10).

Antisera and Immunological Detection of Antigens using Protein Blotting

For the production of antibodies to karyoskeletal proteins of *Xenopus* oocytes the material resistant to extraction in "high-salt–Triton buffer" (see above) was used. Mice were immunized as described (34).

Human antibodies against nucleolar antigens were obtained from the serum of a patient suffering from scleroderma (kindly supplied by Dr. B. Platzer, Department of Dermatology, School of Medicine, University of Innsbruck, Austria). IgG was prepared by chromatography on DEAE-cellulose (4). Controls included preimmune sera from the mice used as well as various antibodies to nonnucleolar nuclear proteins (cf. reference 4).

Polypeptides separated by electrophoresis in gels containing 10% acrylamide were transferred to nitrocellulose paper and processed as described (34).

Affinity Purification of Antibodies against Oocyte Karyoskeletal Proteins

Monospecific antibodies were purified following the strategy of Olmsted (27). 15–20 slots (slot width 3.5 mm) of 8% polyacrylamide gels (1.5-mm thick) were loaded with the antigens (protein concentration as shown in Fig. 1, slot *5*). The electrophoretically separated polypeptides were renatured by incubating the gel for 2 h in 4 M urea (10 mM Tris-HCl pH 7.5, 2 mM EDTA, 0.1 mM dithioerythritol). Then the gel was equilibrated for 30 min in transfer buffer (0.25 M Tris, 0.192 M glycine), and the polypeptides were transferred electrophoretically (3 h at 400 mA and 10–15 V; for details see reference 38) to nitrocellulose paper (BA 85, 0.45-μm pore size; Schleicher & Schuell, Dassel, FRG). One strip of the nitrocellulose was stained with amido black (38) to identify the position of the antigens with 68,000 and 145,000 mol wts. The horizontal strips (0.3–0.6 cm wide) corresponding to 68,000 and 145,000 mol wts were cut out and incubated for 2–12 h at 4°C in phosphate buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 7 mM Na_2HPO_4, 1.5 mM KH_2PO_4) containing 1% bovine serum albumin (BSA) and 0.02% NaN_3 to block the remaining protein binding capacity of the nitrocellulose. Each nitrocellulose strip was incubated for 3 h at room temperature in a sealed plastic envelope with 2 ml of diluted serum (1:100 in PBS containing 0.1% BSA) containing antibodies against the two oocyte karyoskeletal polypeptides. The nitrocellulose strips were then washed for 30 min in PBS. For elution of the bound antibodies the nitrocellulose strips were incubated for 5 min at room temperature in a sealed plastic envelope containing 3 M KSCN in PBS (final pH 6.0 to 6.5). The eluted antibodies were immediately diluted with PBS to 1 M KSCN and concentrated by vacuum dialysis against PBS to a final volume of 100–150 μl using collodion bags (No. SM 13200; Sartorius, Göttingen, FRG). The affinity purified antibodies were directly taken for immunofluorescence microscopy. Each nitrocellulose strip was used at least three times for antibody purification, without recognizable reduction of binding capacity. Nitrocellulose strips were stored in sealed plastic envelopes containing PBS plus 0.05% NaN_3.

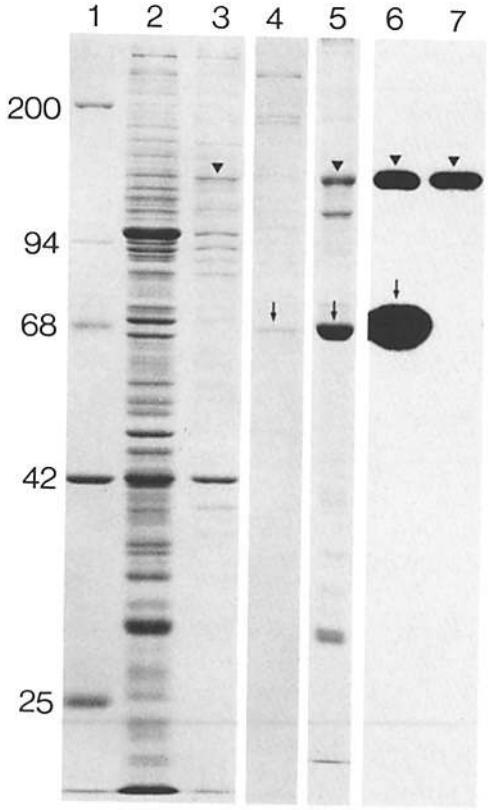

FIGURE 1 Gel electrophoresis of total nuclear proteins from *Xenopus* oocytes and subnuclear fractions (slots *1–5*, Coomassie Blue) and antibody reactions on polypeptide blots obtained therefrom (slots 6 and *7*, autoradiography). Slot *1*, reference proteins (from top to the bottom): myosin heavy chain (*200*), phosphorylase-a, BSA, actin, and chymotrypsinogen (*25*); slot *2*, seven whole nuclei; slot *3*: 25 gelled nuclear contents extensively washed in "5:1 medium;" slot *4*, 70 nonextracted nuclear envelopes; slot *5*; residues of mass-isolated oocyte nuclei after extraction with high salt and Triton; slot *6*, reaction of murine antibodies with the karyoskeletal polypeptides of *Xenopus* oocytes (same as in slot 5) as visualized by autoradiography; slot *7*, same reaction on polypeptides from 220 gelled nuclear contents (same preparation as in slot *3*). The 145,000 mol wt polypeptide is marked by an arrowhead and the 68,000 mol wt polypeptide by a vertical arrow. Molecular weight designations at left are $\times 10^3$.

Immunofluorescence Microscopy

Cryostat sections of frozen tissue and cells grown on cover slips were processed for indirect immunofluorescence microscopy as described (12, 21, 34). Cover slips and cryostat sections were reacted for 15 min with diluted sera (1:50 in PBS) or purified IgG, washed twice for 5 min with PBS, incubated with the FITC-

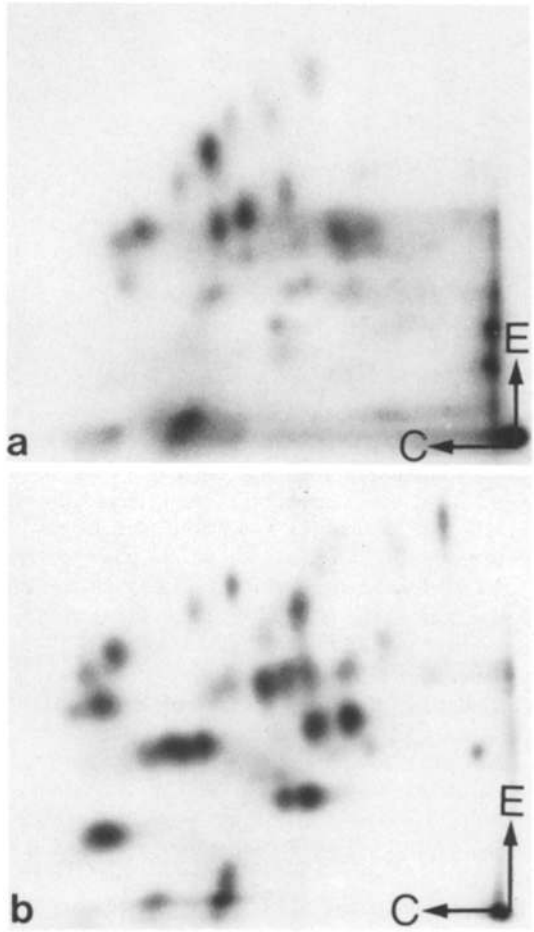

FIGURE 2 Comparison of the two-dimensional pattern of [^{125}I]iodine-labeled tryptic peptides derived from the radioiodinated major karyoskeletal polypeptides of *Xenopus* oocytes that had been separated on SDS PAGE and individually excised. *a*: polypeptide of 145,000 mol wt; *b*, polypeptide of 68,000 mol wt. *E*, first dimension by electrophoresis; *C*, second dimension by chromatography.

conjugated antibodies for 10 min and washed twice in PBS for 5 min each. Specimens were dehydrated in 95% ethanol and mounted with Mowiol 4.88 (Hoechst AG, Frankfurt, FRG) containing *p*-phenylenediamine (1 mg/ml Mowiol) to reduce fading (18). For immunofluorescence microscopy on isolated nuclear contents oocyte nuclei were isolated in "5:1-medium" without divalent cations and the nuclear envelope was pulled away with needles. The nuclear contents were then transferred to a microcentrifugation chamber which was sealed at the bottom with a cover slip. After centrifugation (10 min at 500 *g*) the cover slip was removed and processed as described for cultured cells, omitting the fixation with methanol and acetone and the drying step after the fixation.

RESULTS

When whole oocyte nuclei (germinal vesicles) of *X. laevis* were extracted in buffer containing 1 M KCl and 1% Triton X-100 certain structures remained in a form pelletable by centrifugation at low speed. Most of this resistant material could be identified by electron microscopy as nuclear pore complex residues interconnected by the nuclear lamina, a loose fibril meshwork at the level of the inner nuclear membrane (cf. 16, 19, 20, 21, 22, 31, 33, 34) and as spheroidal fibril masses of a tumbleweedlike organization derived from nucleoli and/or "nucleoluslike bodies" ("micronucleoli") described in oocytes as well as in somatic cells (14, 25, 26, 28, 36). Recently, we have shown by gel electrophoretic examination that this karyoskeletal material from whole oocyte nuclei contains only two major polypeptides (22, 34), one of 145,000 mol wt, present in nuclear contents and enriched in nucleolar fractions (14), and the other of 68,000 mol wt which is exclusively located in the nuclear pore complex-lamina structure (19, 33, 34).

A murine antiserum raised against the total oocyte karyoskeletal protein was found to contain antibodies directed against the 68,000-mol wt protein as well as antibodies against the 145,000-mol wt protein (Fig. 1), as detected by reaction of polypeptides blotted on nitrocellulose paper (Fig. 1, slot *6*). However, when such immunoblotting experiments were done on karyoskeletal or total polypeptides from gelled nuclear contents, i.e. after manual removal of the nuclear envelope (cf. 19, 20), only one band showed antibody binding, i.e. the protein of 145,000 mol wt (e.g., Fig. 1, slot *7*).

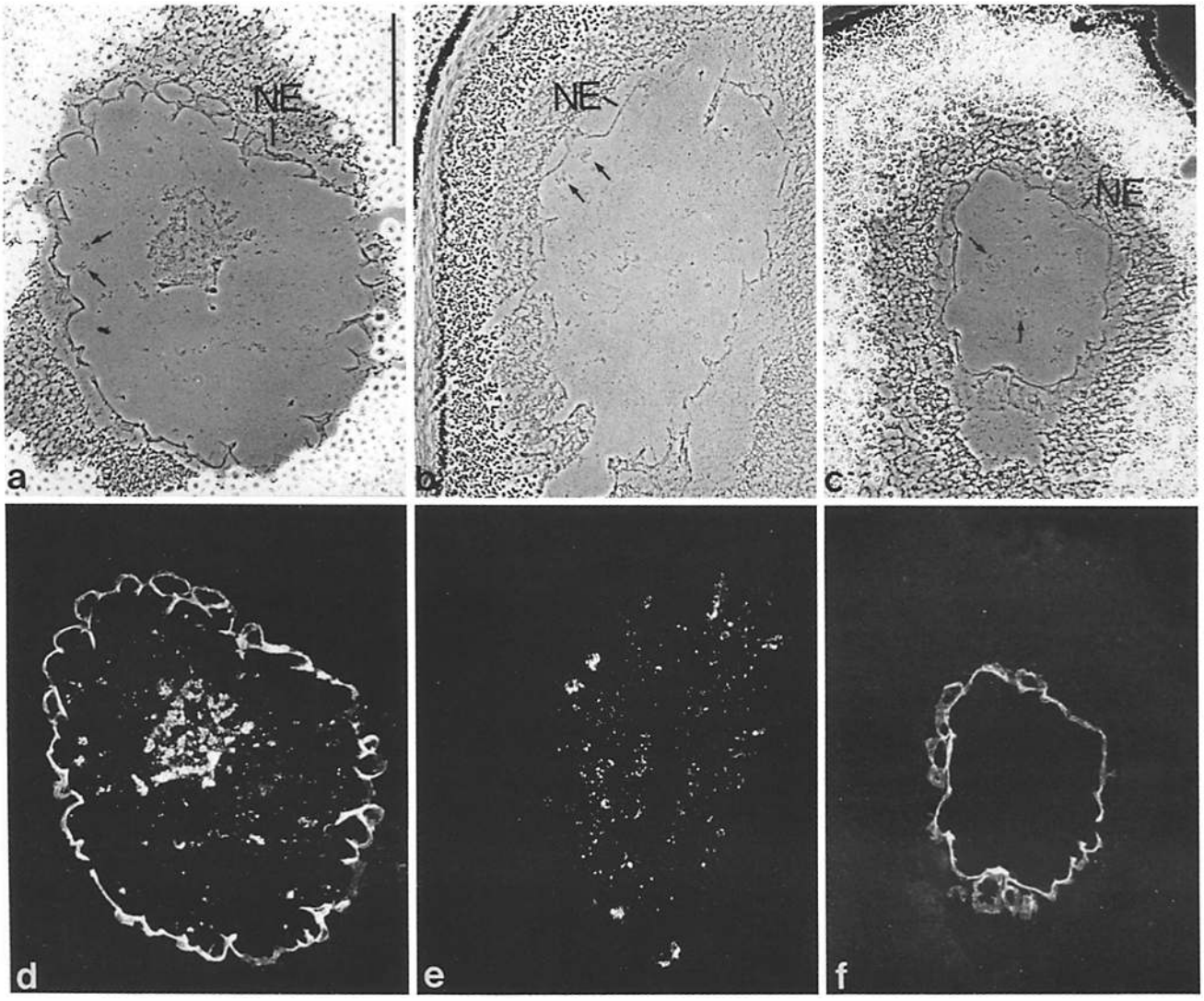

FIGURE 3 Indirect immunofluorescence microscopy on frozen sections through vitellogenic oocytes of *Xenopus* showing the nucleus. Sections were incubated with the diluted murine serum raised against the total skeletal proteins of *Xenopus* oocyte nuclei (*a*, *d*) and with the affinity-purified antibodies against the polypeptide with 145,000 mol wt (*b*, *e*) and the 68,000 mol wt polypeptide (*c*, *f*). Phase contrast optics: *a–c*; epifluorescence optics: *d–f*. Some amplified nucleoli are denoted by arrows (*a–c*). *NE*, nuclear envelope. Bar, 100 μm. × 190 (all).

We have used two different approaches to demonstrate the biochemical and immunological difference between these two karyoskeletal polypeptides. (*a*) When radioiodinated peptides obtained after trypsin treatment were examined, the great majority of cleavage products from the 145,000 mol wt protein were different from those from the nuclear envelope-associated karyoskeletal protein of 68,000 mol wt as demonstrable in parallel fingerprint separations (Fig. 2) as well as in fingerprints of mixtures of the trypsin digest from both proteins (not shown). (*b*) We have prepared affinity purified antibodies against each of the two karyoskeletal proteins, using gel electrophoretically separated antigens after the transfer to nitrocellulose paper for antibody binding. When the purified antibodies were examined by indirect immunofluorescence microscopy on frozen sections through *Xenopus* ovaries, it was found that antibodies against the karyoskeletal polypeptide of 68,000 mol wt showed exclusive reaction with the oocyte nuclear membrane and did not react with structures in the nuclear interior (Fig. 3 *c, f*). This result is in agreement with our recent immunological studies on the pore complex-lamina of *Xenopus* oocytes (for details see reference 34). By contrast, affinity blot-purified antibodies against the polypeptide of 145,000 mol wt exclusively stained the amplified nucleoli and a class of frequent smaller spheroidal bodies (Fig. 3 *b, e*) described as medusoid fibril bodies ("micronucleoli"; for references see 25, 26, 28). When sections through amplified nucleoli of *Xenopus* oocytes were observed at higher magnification the distribution of the 145,000-mol wt polypeptide was not homogeneous throughout the large nucleoli but was concentrated, in punctate arrays, in the nucleolar cortex (Fig. 4).

When isolated nuclear contents centrifuged on cover slips were examined by immunofluorescence microscopy again nucleoli and micronucleoli were positively stained (Fig. 5 *a, b*). The specificity of the staining of the small nucleoluslike bodies was demonstrated in various controls; in particular these particles were not stained when nucleolus-specific antibodies from other sources were applied, including human IgG from a patient suffering from scleroderma (Fig. 5 *c, d*).

Antibodies against the karyoskeletal protein of 145,000 mol wt also reacted with nucleoli in frozen sections through tissues of adult *Xenopus* frogs such as in hepatocytes (Fig. 6 *c–f*) and testicular cells, including Sertoli cells and spermatogonia (Fig. 7 *c, d*). The staining with the purified antibodies was always restricted to the periphery of the nucleoli and appeared in distinct spots,usually 2–3 per nucleolus. This specific punctate decoration of the nucleolar cortex was best seen in cells with large nucleoli such as hepatocytes (Fig. 6 *e, f*, right cell) and Sertoli cells (Fig. 7 *c, d*). The accessibility of the nucleolar interior of somatic cells for the immunoglobulins was demonstrable by staining of frozen sections through *Xenopus* liver (Fig. 6 *a* and *b*) and testis (Fig. 7 *a* and *b*) with other nucleolus-specific antibodies (human IgG from a patient suffering from scleroderma). The comparison of Fig. 6 *b* and *f* as well as Fig. 7 *b* and *d* shows that the karyoskeletal protein of 145,000 mol wt is detectable only in a restricted area in the cortex of the large nucleoli of hepatocytes and Sertoli cells. The same results were obtained when cultured kidney epithelial cells from *Xenopus* were decorated with the antibodies against the 145,00-mol wt polypeptide (data not shown).

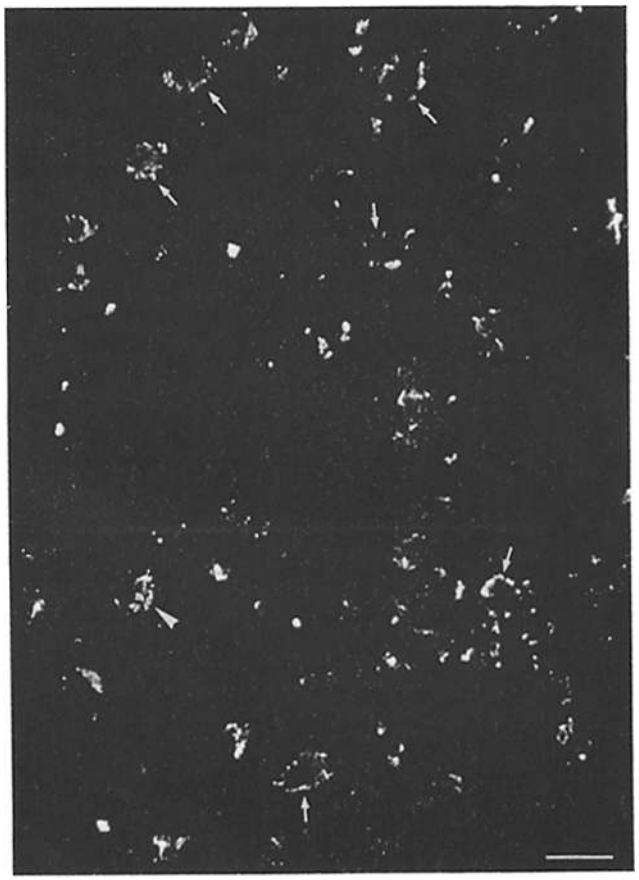

FIGURE 4 Frozen section of *Xenopus* ovary showing one part of an oocyte nucleus with numerous nucleoli after incubation with affinity-purified antibodies against the 145,000 mol wt polypeptide. The cortex of the amplified nucleoli is intensely stained, often in punctate arrays. Arrows, cross-sectioned nucleoli; arrowhead, tangentially sectioned nucleolus. Bar, 10 μm. × 900.

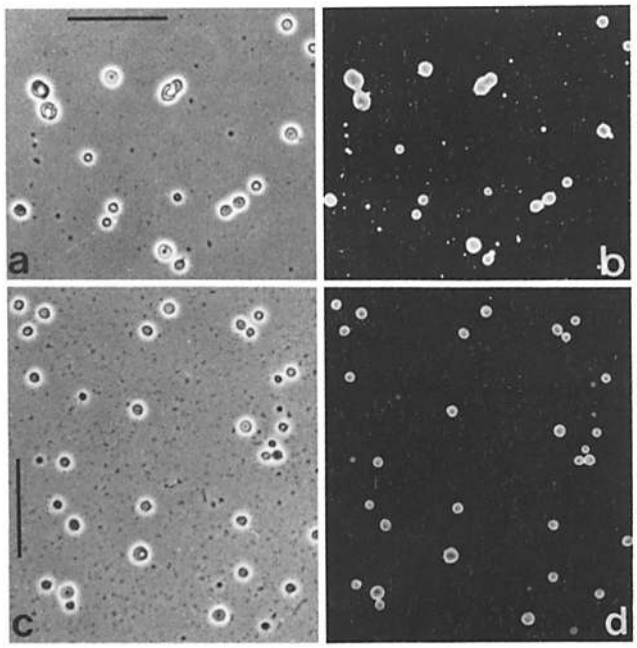

FIGURE 5 Indirect immunofluorescence microscopy on sedimented isolated nuclear contents of oocytes (a–*d*) after incubation with antibodies to the oocyte karyoskeletal proteins (*a, b*) and with human auto-antibodies to nucleolar antigen (*c* and *d*). Phase contrast optics: *a, c*; epifluorescence optics: *b, d*. Note positive reaction in nucleoli (*b, d*); the antibodies to the karyoskeletal proteins also react with small nucleoplasmic bodies (*b*) whereas the human antibodies do not (*d*). Bars, 50 μm. × 180 (*a, b*), × 260 (*c, d*).

The antibodies against this karyoskeletal protein did not stain the nuclei of *Xenopus* cells lacking nucleoli such as erythrocytes and late stages of spermiogenesis (Fig. 7 *c*, *d*), indicating that this protein is not a structural component of the nucleolus organizer chromatin but is a part of the active nucleolus. Controls using antibodies to other nuclear proteins such as RNA polymerase II left the nucleolus selectively unstained (not shown here; cf. 4).

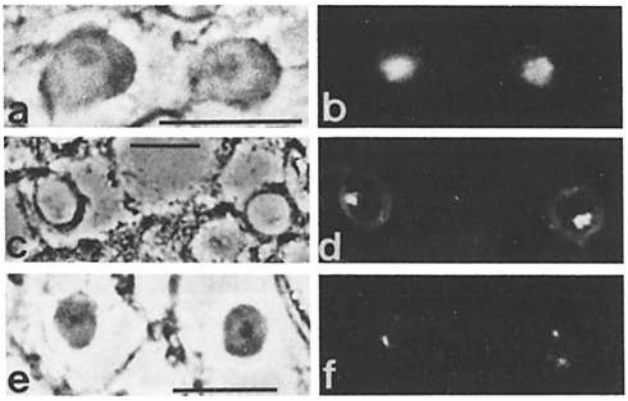

FIGURE 6 Immunofluorescence microscopy on frozen sections through *Xenopus* liver after staining with human auto-antibodies to nucleolar antigens (*a*, *b*), diluted mouse serum raised against the oocyte karyoskeletal proteins (*c*, *d*), and with affinity-purified antibodies against the 145,000 mol wt polypeptide (*e*, *f*). The human autoantibodies stain the entire nucleoli (*b*) whereas staining with antibodies to the 145,000 mol wt polypeptide is mostly restricted to a few small areas of the nucleolar cortex (*f*, right cell). Phase contrast optics: *a*, *c*, *e*; epifluorescence optics: *b*, *d*, *f*. Bars, 10 μm. × 1850 (*a*, *b*); × 900 (*c*, *d*); × 1,400 (*e*, *f*).

DISCUSSION

Various antigens localized to nucleoli by microscopical techniques have been described (for review see reference 39), using human autoantibodies directed against specific RNAs (e.g., 23, 35), antibodies raised against ribosomal proteins (e.g. reference 7), RNA polymerase I (17), ribonucleoprotein complexes (37), or other high-salt–extractable proteins. Some of these antigens seem to occur in tumors but not in other cells (9). In addition, antigens preferentially located in perinucleolar heterochromatin have been reported (6). The present study localizes a karyoskeletal protein to the cortex of the nucleoli of oocytes and somatic cells, supporting our suggestion that the 145,000-mol wt protein of *Xenopus* oocytes is associated with nucleoli (14).

Karyoskeletal proteins resistant to extractions in high salt buffers and nonionic detergents have been described in various nuclear subfractions. However, only in the case of the lamina proteins has a positive localization been achieved, and it has been shown that recognition of antibodies to these proteins is restricted to the nuclear periphery (16, 21, 33, 34). For none of the residual proteins assumed to be constituents of the nuclear matrix or chromosomal scaffolds has an intranuclear location (i.e. away from nuclear envelope and lamina) been reported. Thus, our localization of the 145,000-mol wt protein represents the first positive localization of a karyoskeletal protein to a truly intranuclear, nonlamina structure.

In sections and, more clearly, in spread preparations of nucleoli, masses of filaments have been observed which surround the nucleolar chromatin and often appear to be associated with particles resembling pre-rRNP particles (24, 26; see also Fig. 4.14 in reference 5). When such isolated nucleoli are extracted in high salt buffers and/or treated with nucleases

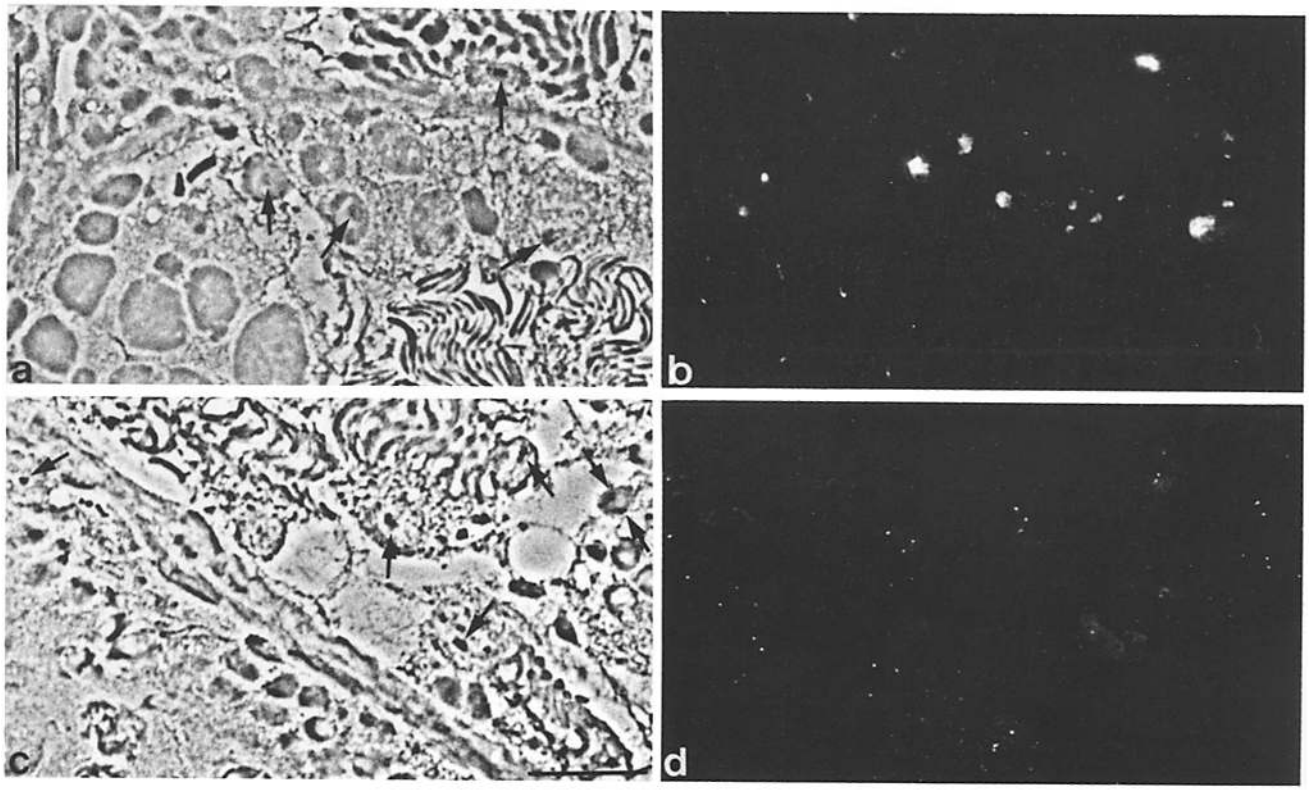

FIGURE 7 Frozen sections through *Xenopus* testis after staining with human autoantibodies to nucleolar antigens (*a*, *b*) and with purified antibodies against the 145,000 mol wt polypeptide (*c*, *d*). Arrows denote nuclei of Sertoli cells. Phase contrast optics: *a*, *c*; epifluorescence optics: *b*, *d*. Bars, 20 μm. × 780 (*all*).

(14) aggregates of filaments remain that form meshworklike aggregates, especially in the nucleolar cortex. The concomitant enrichment of tangles of ~4- to 8-nm filaments and the 145,000-mol wt protein in high salt- and detergent-treated nucleolar subfractions and the specific localization of the 145,000-mol wt protein by immunofluorescence microscopy to the nucleolar cortex suggests that this protein might be a major constituent of these filaments. Immunoelectron microscopic studies on spread preparations of nucleolar filament meshworks are underway to decide whether the 145,000-mol wt protein is exclusive to these filaments.

In addition, our study shows that the 145,000-mol wt protein is not confined to the nucleolus proper but also occurs, especially in the oocyte nucleus, in distinct small spheroidal units (micronucleoli) that seem to be identical to the "nucleoluslike bodies" described in various cells as fibrillar masses that can be associated with newly synthesized RNA but do not contain DNA (e.g. 30, 32, 36). The function of the karyoskeletal filaments and the 145,000-mol wt protein in both the nucleolar cortex and the "nucleolus-related bodies" is still unclear although a role as skeletal support for pre-rRNP particles is suggestive.

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