A Novel Karyoskeletal Protein: Characterization of Protein NO145, the Major Component of Nucleolar Cortical Skeleton in *Xenopus* Oocytes

Sandra Kneissel,* Werner W. Franke,* Joseph G. Gall,† Hans Heid,* Sonja Reidenbach,* Martina Schnölzer,‡ Herbert Spring,§ Hanswalter Zentgraf,‖ and Marion S. Schmidt-Zachmann* ¶

†Division of Cell Biology, ‡Protein Analysis Facility, §Biomedical Structure Analysis Group, ‖Applied Tumor Virology Program, German Cancer Research Center, D-69120 Heidelberg, Germany; and †Department of Embryology, Carnegie Institution of Washington, Baltimore, Maryland 21210

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The nucleolus is a ubiquitous, mostly spheroidal nuclear structure of all protein-synthesizing cells, with a well-defined functional compartmentalization. Although a number of nonribosomal proteins involved in ribosome formation have been identified, the elements responsible for the shape and internal architecture of nucleoli are still largely unknown. Here, we report the molecular characterization of a novel protein, NO145, which is a major and specific component of a nucleolar cortical skeleton resistant to high salt buffers. The amino acid sequence of this polypeptide with a SDS-PAGE mobility corresponding to \( M_r 145,000 \) has been deduced from a cDNA clone isolated from a *Xenopus laevis* ovary expression library and defines a polypeptide of 977 amino acids with a calculated mass of 111 kDa, with partial sequence homology to a synaptonemal complex protein, SCP2. Antibodies specific for this protein have allowed its recognition in immunoblots of karyoskeleton-containing fractions of oocytes from different *Xenopus* species and have revealed its presence in all stages of oogenesis, followed by a specific and rapid degradation during egg formation. Immunolocalization studies at the light and electron microscopic level have shown that protein NO145 is exclusively located in a cage-like cortical structure around the entire nucleolus, consisting of a meshwork of patches and filaments that dissociates upon reduction of divalent cations. We propose that protein NO145 contributes to the assembly of a karyoskeletal structure specific for the nucleolar cortex of the extrachromosomal nucleoli of *Xenopus* oocytes, and we discuss the possibility that a similar structure is present in other cells and species.

**INTRODUCTION**

Ever since the discovery (1835–1838) by R. Wagner, G. Valentin, and M. Schleiden of the nucleolus as a large and constitutive nuclear organelle common to all biosynthetically active animal and plant cells (reviewed by Franke, 1988; Gerbi, 1997; Pederson, 1998), this distinct, mostly spheroidal structure has attracted the special interest of cell biologists. It has also been noted early on that the number of nucleoli per nucleus can vary greatly, from one or a few located in chromosomal loci, termed nucleolar organizers, to more than a thousand amplified extrachromosomal nucleoli in certain amphibian oocytes (Hadjiolov, 1985). Although the nucleoli have been known for some decades as the sites of rRNA genes and their expression, followed by the assembly of ribosomal precursor structures (Hadjiolov, 1985; Reeder, 1990; Scheer and Weisenberger, 1994), more recent evidence has indicated additional functions such as the assembly, modification, storage, and transport of a series of non-rRNA ribonucleoprotein particles, the buildup of locally enriched enzyme pools, and as a compartment for intranuclear sequestration and regulated inactivation of proteins (Pederson, 1998; Carmo-Fonseca et al., 2000; Olson et al., 2000; Pederson and Politz, 2000; Visintin and Amon, 2000). Therefore, it is not surprising that the nucleolus contains, in addition to proteins involved in ribosome formation, nu-
Figure 1. Isolation of karyoskeletal proteins from X. laevis oocyte nuclei. (A) Schematic representation of the experimental procedure for the isolation and fractionation of mass-isolated X. laevis oocyte nuclei (see MATERIALS AND METHODS). The essential protein fractions are shown in bold letters. (B) Coomassie Blue staining of SDS-PAGE-separated karyoskeletal proteins present in the LSP fraction after high salt/detergent extraction. The three major polypeptides identified by MALDI mass spectrometry are marked by arrows. R, reference proteins: 205, 116, 97.4, 66, 45, and 29 kDa (from top to bottom).

Numerous other proteins engaged in any of the other functions mentioned (Shaw and Jordan, 1995; Busch, 1997).

Morphologically, the nucleolus displays three major structural components and this is true for both nucleoli on chromosomal nucleolar organizer and extrachromosomal amplified rDNA copies: 1) the fibrillar center (FC), surrounded by 2) the dense fibrillar component (DFC) and 3) the granular component (GC). Localization studies with the use of antibodies and hybridization probes have also indicated that the biosynthesis and assembly of ribosomal particles is a vectorial process, in which nascent preribosomes move from the DFC region to the more peripherally located GC (Scheer and Hock, 1999; Thiry et al., 2000).

Although the initial formation of a nucleolus appears to require the transcription of rDNA by RNA polymerase I, it is still controversial whether continued transcriptional activity is needed to maintain the near-spheroidal shape and the dense and complex three-component organization (Oakes et al., 1993; Dousset et al., 2000; Verheggen et al., 2000) for the “pseudonucleoli” in embryos of the 0-nu mutant of the clawed toad, Xenopus laevis, lacking functional rRNA genes, see Hay and Gurdon, 1967; Steele et al., 1984). Obviously, the specific architecture is dependent on some intrinsic nucleolar activities or factor(s) because several inhibitors of transcription result in dramatic rearrangements, the best studied of which is the actinomycin D-induced condensation and hemisphere segregation of FC, DFC, and GC (Hadjiolov, 1985). Moreover, the dense-packed arrangement of the nucleolar components into a spheroidal structure is by no means a trivial consequence of rDNA transcription as is perhaps best illustrated by the effect of the RNA polymerase II inhibitor 5,6-dichloro-β-d-ribofuranosylbenzimidazole, resulting in a spectacular unraveling of the transcribed rDNA chromatin and the distribution of the nucleolar components over the nucleoplasm so that the nucleolus as a distinct body is no longer seen (“necklace formation”; Granick, 1975a,b; Scheer et al., 1984; Desnoyers et al., 1996; Le Panse et al., 1999).

The extrachromosomal nucleoli formed by amplified rDNA copies in oocytes of various species provide an especially “pure” form of nucleolar material. In particular, the nucleoli present in amphibian oocyte nuclei (“germinal vesicles”, GV) present an excellent model system for studies of the biochemical composition and structural organization of the nucleoli and the regulation of nucleolar activities, due to their enormous size, high rDNA copy content, and high transcriptional activity, the massive accumulation of primary and secondary gene products, and show a structural organization remarkably similar to that of somatic nucleoli (Gall, 1968; Buongiorno-Nardelli et al., 1972; Mais and Scheer, 2001).

Several years ago, in studies of amplified nucleoli of advanced stages of oogenesis in X. laevis, a further structural component has been described as a layer of tangles of filaments and knot-like aggregates confined to the very nucleolar cortex (Franke et al., 1981; Krohne et al., 1982; Benavente et al., 1984). In the present study we report on the identification and molecular characterization of the major protein of this cortical nucleolar structure.

MATERIALS AND METHODS

Biological Material

Clawed toads (X. laevis) were purchased from the South African Snake Farm (Krynau, Republic of South Africa). Toads (X. borealis, X. tropicalis, Bombina orientalis), newts (Triturus cristatus), and salamanders (Pleurodeles waltl) were reared in our laboratory.

Procedures for snap-freezing of tissue samples as well as culture conditions for X. laevis kidney epithelium (XLKE, line A6) and mammalian cells have been described (Krohne and Franke, 1980; Zirwes et al., 2000).

Large-Scale Isolation and Fractionation of X. laevis Oocyte Nuclei

Large numbers of mature X. laevis oocyte nuclei (stages IV–VI; Dumont, 1972) were obtained by mass isolation, a large-scale procedure described by Scalenghe et al. (1978) and modified by Klein-Schmidt and Franke (1982). Subsequent fractionation of nuclear contents by differential centrifugation was performed as described in detail by Hügle et al. (1985), resulting in fractions (Figure 1A) termed low-speed pellet (LSP), high-speed pellet (HSP), and high-speed supernatant (HSS) procedures for snap-freezing of tissue samples as well as culture conditions for X. laevis kidney epithelium (XLKE, line A6) and mammalian cells have been described (Krohne and Franke, 1980; Zirwes et al., 2000).
speed supernatant (HSS). For enrichment of karyoskeletal proteins isolated LSPs were extracted as described (Krohne et al., 1982).

**Small-Scale Oocyte Isolation, Microinjections, Spreads of GV Contents, and Preparation of Cell Lysates**

Small ovary pieces were removed from anesthetized animals, defolliculated by collagenase treatment, and the individual oocytes were kept at 18°C for several days. Stage I–VI oocytes were grouped based on size, rinsed with OR2 buffer (Wallace et al., 1973), and stored at −80°C until use. Microinjection of in vitro translation products as well as the manual isolation of nuclei, oolplasms, nuclear contents, and nuclear envelopes from oocytes were as described (Krohne et al., 1989; Cordes et al., 1991).

Stage VI oocytes were induced to mature in vitro by addition of 40 μM progesterone (Sigma, Munich, Germany). For some experiments oocytes were incubated in the presence of actinomycin D (Serva, Heidelberg, Germany) at a final concentration of 10 μg/ml for 4 h.

Spreads of GV contents were performed according the experimental procedure described in detail before (Gall et al., 1991, 1999; Gall, 1998). Total cellular lysates of *X. laevis* cultured cells (XLKE, line A6) were prepared as described (Schmidt-Zachmann et al., 1998).

**Mass Spectrometry and Amino Acid Sequence Analysis**

Protein bands of interest were excised from the gel and digested with sequencing grade modified trypsin in 40 mM NH₄CO₃ overnight at 37°C. The reaction was stopped by freezing. Matrix-assisted laser desorption ionization (MALDI), spectrometric analysis, database searches, and amino acid sequence analysis were performed as described (Kuhn et al., 2001).

**Isolation of cDNA Clones and Polymerase Chain Reaction (PCR) Products**

Total DNA from a *X. laevis* ovary (Stratagene, Heidelberg, Germany) was used for PCR with the library-specific 17 primer as antisense primer and a degenerated sense primer deduced from the amino acid sequence DFWEDQY. Small ovary pieces removed from anesthetized animals, defolliculated by collagenase treatment, and the individual oocytes were kept at 18°C for several days. Stage I–VI oocytes were grouped based on size, rinsed with OR2 buffer (Wallace et al., 1973), and stored at −80°C until use. Microinjection of in vitro translation products as well as the manual isolation of nuclei, oolplasms, nuclear contents, and nuclear envelopes from oocytes were as described (Krohne et al., 1989; Cordes et al., 1991).

**Expression and Purification of His-tagged Protein NO145**

To express an amino-terminal His₄-tagged version of protein NO145, the blunt-ended XbaI-XhoI fragment derived from clone pBT-NO145-211 was subcloned into the vector pQE-31 (QIAGEN, Hilden, Germany), previously cut with Smal. The recombinant protein was purified under denaturing conditions following the manufacturer’s protocol.

**Gel Electrophoresis and Immunoblotting**

Protein fractions were analyzed by SDS-PAGE (cf. Kleinschmidt and Franke, 1982). The polypeptides were transferred to nitrocellulose membranes and visualized by Perkin-Elmer’s CBB stain. The nitrocellulose membranes were blocked in Tris-buffered saline containing 0.05% Tween 20 (TBST) and 5% nonfat dry milk for 1 h at room temperature and then incubated at room temperature with affinity-purified or monoclonal antibodies for 1 h in TBST and 5% nonfat...
dry milk. Bound antibodies were detected by chemiluminescence with the use of the ECL system (NEN, Dreieich, Germany) after incubation with hors eradish peroxidase-coupled secondary antibodies (Dianova) diluted 1:10 000 in TBST and 5% nonfat dry milk for 1 h.

**Immunofluorescence Microscopy**

For immunofluorescence microscopy, cells grown on coverslips were fixed (Zirwes et al., 2000), washed twice in phosphate-buffered saline (PBS), and incubated with purified guinea pig antibodies (1:200 diluted in PBS) or mAbs (culture supernatant, undiluted) for 30 min at room temperature. After several washes in PBS, cells were incubated for 30 min with the appropriate secondary antibodies (1:100–1:500 in PBS), washed in PBS, dehydrated in ethanol, air-dried, and mounted in Fluoromount (Biozol, Eching, Germany).

Cryosections (~5 μm) of frozen tissues were either fixed with acetone (10 min, −20°C) or in PBS containing 2% formaldehyde (15 min, room temperature). Formaldehyde-fixed samples were washed once in PBS containing 50 mM NH4Cl for 5 min and then twice for 5 min in PBS before incubation with the antibodies.

GV spreads were fixed after centrifugation in 2% formaldehyde/1 mM MgCl2 in PBS for 1 h. After fixation, preparations were rinsed in PBS, blocked with 10% horse serum, in PBS, and incubated with the appropriate antibodies as outlined above. The preparations were examined with the use of a Zeiss Axioshot, a Zeiss confocal laser scanning microscope (LSM 510; Zeiss, Oberkochen, Germany), or a Leica TCS NT (Leica, Nussloch, Germany).

**Immunoelectron Microscopy**

Cyrosections of 5 μm of Xenopus ovaries were fixed for 10 min in PBS containing 2% formaldehyde, 2% sucrose, 1 mM MgCl2, washed in NH4Cl and PBS, and finally blocked in 5% goat serum in PBS. Incubation with the primary antibody (affinity-purified serum NO145-H, diluted 1:100) was performed in a wet chamber for 1 h at room temperature. After three washes with PBS for 5 min each, bound antibodies were reacted for 1 h at room temperature with anti-guinea pig IgG-conjugated nanogold (Nanoprobes, Stony Brook, NY) diluted 1:50 in PBS. After several washes with PBS for 5 min each, the tissue was fixed with 2.5% glutaraldehyde in 0.05 M cacodylate buffer for 15 min at 4°C. Silver enhancement was according to Uchida et al. (1996). Subsequently, the tissue was postfixed with 2% osmium tetroxide solution and processed for flat embedding in Epon (Franke et al., 1978).

**RESULTS**

**Isolation and Analysis of a cDNA Clone Encoding a Novel Type of Structural Nucleolar Protein**

When *X. laevis* oocyte nuclei were isolated (Scalenghe et al., 1978), homogenized, and further fractionated by differential centrifugation (Figure 1A), some well-characterized protein fractions were obtained (cf. Hu¨gle et al., 1985). LSP fractions, known to be highly enriched in nucleoli, nuclear envelopes, and chromosomes, were subsequently extracted with a high salt/detergent-containing buffer to enrich for karyoskeletal structures. The proteins of the resulting residual pellet were subjected to SDS-PAGE and revealed only three major polypeptides of mobilities corresponding to 145, 100, and 68 kDa (Figure 1B). These polypeptides were excised and subjected to “in-gel” tryp tic digestion. The eluted fragments were then analyzed by MALDI mass spectrometry, and the peptide mass fingerprints were compared with predicted peptides of the NCBI-nr database by the ProFound search algorithm. Although the digestion profile

**Figure 2.** Amino acid sequence of Xenopus protein NO145 and comparison with the rat SCP2. (A) Amino acid sequence of protein NO145 of *X. laevis* deduced from the cDNA clone pBT-NO145-211 (EMBL accession no. AJ249963). The peptide sequences determined by protein sequencing are shaded gray. The putative nuclear localization signal is indicated by a box and sequences used for generating antibodies are underlined. (B) Partial sequence comparison between *X. laevis* protein NO145 (aa 90–319) with the rat protein SCP2 (aa 95–323; cf. Offenberg et al., 1998). The symbols between the two primary sequences are defined as follows: vertical bar, identical amino acids; 1–5, conservative exchanges (1, aliphatic, nonpolar; 2, aliphatic, polar; 3, aromatic; 4, basic; 5, acidic).
of the 100-kDa polypeptide matched the peptide fingerprints of lipovitellin I (Wiley and Wallace, 1981), a yolk protein of Xenopus ovary tissue. RNA-size markers of 9.5, 7.4, 4.4, 2.4, 1.4, and 0.24 kb corresponding to a c probe. Note the reaction of a single band labeled NO145-specific probe. The sequence information obtained from the resulting PCR fragments did not extend beyond the 5′ end of the isolated cDNA clone (our unpublished data).

The most conspicuous feature of the encoded protein is the extraordinarily high content of potential phosphorylation sites, including 26 for protein kinase C, 15 for casein kinase II, and 2 for tyrosine kinase. Presently, we do not know the actual degree of phosphorylation. Thus, we cannot exclude that post-translational modifications might account for the observed difference in molecular mass estimated for the polypeptide from the cDNA-derived sequence (111 kDa) or from its SDS-PAGE mobility (145 kDa; see below). This protein has been designated protein NO145.

We have also noted a putative nuclear localization signal (NLS) between aa positions 782–799 [KKK(3×)KPRK, denoted by a box in Figure 2A; Dingwall and Laskey, 1991]. Other notable features, e.g., sequence elements involved in nucleic acid binding or protein–protein interactions, have not been detected.

In X. laevis database searches we have noticed two expressed sequence tags of 656 and 555 nt in length, which correspond to the 5′ and 3′ end, respectively, of clone pBT-NO145-211 (accession no. BE680667 and BE678123). Although NO145 is a novel protein, it displays a striking similarity to SCP2, a rat synaptonemal complex (SC) protein of 173 kDa (EMBL accession no. Y08981; Offenberg et al., 1998), with an overall amino acid sequence identity of 26% and a similarity, including conservative exchanges, of 38%. Notably, the N-terminal region of both proteins contains a domain of 229 aa with a remarkably high sequence homology (43% identity and 59% similarity; Figure 2B).

**Molecular Characterization of the cDNA Encoding the Xenopus Protein NO145**

The completeness of the isolated cDNA clone was demonstrated by three different types of experiments illustrated in Figure 3.

**Experiment 1** In vitro transcription and translation of pBT-NO145-211 in a reticulocyte lysate yielded a polypeptide with a SDS-PAGE mobility corresponding to ~145 kDa, clearly different from the predicted M<sub>r</sub> of 111,000. Similar deviations of estimates based on SDS-PAGE from predicted M<sub>r</sub> values have also been reported for several other proteins, in particular some carrying a very high negative charge, be it due to a very low isoelectric point (e.g., N1/N2, nucleoplasmin, NO38, NO29) or to a high degree of phosphorylation (Nopp140; Kleinschmidt et al., 1986; Dingwall et al.,...
Experiment 2 Total RNA and poly(A)\(^{+}\) RNA from *X. laevis* ovary tissue as well as from *X. laevis* A6 cells were probed in Northern blot experiments with a 0.95-kb random prime-labeled cDNA fragment derived from clone pBT-NO145-211. A strong signal corresponding to a mRNA of \(\sim 3.2\) kb was detected in the ovary, indicating that the pBT-NO145-211 clone was of full or nearly full length. Due to the large amount of rRNAs present in the total RNA sample, the mRNA coding for NO145 shows a slightly decreased electrophoretic mobility. No signal was obtained on mRNAs isolated from *X. laevis* kidney epithelial cells (XLKE, line A6), suggesting that expression of the gene encoding NO145 is cell type specific (Figure 3B). However, an mRNA of similar size could be demonstrated in ovaries from other *Xenopus* species (*X. borealis* and *X. tropicalis*), whereas we did not detect NO145-specific mRNA in ovaries from *P. waltl* and *T. cristatus* (our unpublished data).

Experiment 3 When protein NO145 synthesized in vitro was microinjected into *Xenopus* ooplasm, it was subsequently recovered exclusively in the manually isolated nuclei by autoradiography as well as by immunoblotting with the use of NO145 antibodies. The antibodies reacted with one single polypeptide band, indicating that the injected and the endogenous protein have the same size (our unpublished data). This result suggests that NO145 is a nuclear protein that is accumulated in oocyte nuclei, presumably by an active process.

**Biochemical Characterization of Protein NO145 and Its Synthesis**

To study the intracellular distribution and location of endogenous protein NO145, a panel of polyclonal antibodies against peptides deduced from the cDNA sequence of pBT-NO145-211 (Figure 2A) as well as several mAbs against the recombinant protein expressed in *Escherichia coli* were generated. The presence of NO145 in different nuclear fractions of *X. laevis* oocyte nuclei (total nuclei, LSP, HSP, and HSS) and in *X. laevis* cells of line A6 cells was analyzed by immu-
nblotting. Antibody NO145-H recognized its antigen in total oocyte nuclei as well as in the LSP and HSP fractions, indicating that NO145 is a nuclear protein associated with relatively large structures. The protein was detectable neither in the HSS fraction containing soluble nuclear proteins nor in cultured cells (Figure 4, A and A’). Moreover, we did not detect NO145 in immunoblots of total proteins from cultured cells of different species (human, bovine, rat, mouse, rat kangaroo) or in different tissues of X. laevis (heart, muscle, testis, kidney). This indicated that protein NO145 was either exclusively synthesized in Xenopus oocytes or that the antibodies used were very restricted in their cross-reactivities. Antibodies against other peptides of NO145 as well as the different mAbs gave essentially the same results.

The appearance of protein NO145 exclusively in the nuclear fraction of Xenopus oocytes was confirmed in analyses of manually dissected oocytes (Figure 4B). Moreover, on further fractionation of GV’s into nuclear contents and nuclear envelopes, protein NO145 was recovered only in the nuclear interior and was not detected in the nuclear envelope fraction (Figure 4, C and C’). The identity and integrity of the fractions was ascertained by reprobing the nitrocellu-
lose filters containing the blotted proteins with mAb Nuc-195 directed against lamin LIII.

All our attempts to solubilize protein NO145 from nuclear fractions such as LSP and HSP failed. On treatments of these fractions with buffers containing high salt (up to 1.5 M), nonionic detergent (1% Triton X-100), and Benzonase to digest nucleic acids, protein NO145 always remained in the residual pellet, classifying this protein as a bona fide karyoskeletal protein.

Because protein NO145 had been detected in the HSP fraction of oocyte nuclei known to be highly enriched in preribosomal particles, HSP components were further separated by centrifugation in 10–40% sucrose gradients, and the resulting fractions were analyzed by immunoblotting. Interestingly, NO145 was enriched in fractions containing the precursors for the large ribosomal subunit, recognized as 65S particle. In contrast, the protein synthesized in vitro sedimented with ~5.5S, indicative of a monomer (our unpublished data). However, characterization of the native state of protein NO145 and its possible association with other nucleolar molecules requires further experiments.

Figure 6. Double-label immunolocalization of protein NO145 on frozen sections through a X. laevis ovary. (A and A’) Double-label immunofluorescence staining with NO145-specific antibodies NO145-M (A) and mAb No-114 antibody (A’) directed against the nucleolar protein xNopp180 (Schmidt-Zachmann et al., 1984). Both antibodies show a bright immunostaining of the amplified nucleoli. In addition, mAb No-114 specifically decorates the smaller nucleoli of the surrounding follicle cells (some are denoted by arrows in A’). (B–B”) Laser scanning confocal microscopy. The intracellular distribution of protein NO145 (B) is compared with that of protein xNopp180 (B’). The corresponding merged picture is shown in (B”). Bars, 40 μm (A and A’) and 20 μm (B–B”).
Protein NO145 and Its mRNA during Oogenesis and Maturation

We had to recognize that NO145 is an oocyte-specific protein. When protein NO145 and its mRNA was studied during oogenesis and oocyte maturation, the protein was found only in trace amounts in stage I and II oocytes, in which it became detectable only upon loading of large amounts (our unpublished data). Its concentration per oocyte markedly increased in stages III, IV, and V and then appeared to decrease slightly in stage VI (Figure 5A). After maturation in vitro, i.e., at the time of GV breakdown, protein NO145 decreased drastically in the egg at the “white spot stage” and was no longer detectable by the antibodies. This rapid decrease in the level of NO145 was also apparent when NO145 was compared with the major nucleolar protein NO38/B23, which remained at a high concentration level throughout oogenesis and on maturation where it appeared in a hyperphosphorylated form (Figure 5A; cf. Schmidt-Zachmann et al., 1987, 1998).

We also determined the synthesis of protein NO145 at the mRNA level (Figure 5B). NO145 mRNA accumulated during oogenesis and even appeared to increase on oocyte maturation and in eggs. In comparison, the mRNA level of NO38/B23 accumulated during early oogenesis until stage IV then decreased somewhat in stages V and VI but remained high in the egg. These results indicate that protein NO145 is stockpiled during oogenesis, with an increased protein synthetic activity in stages III and VI, and that the rapid disappearance of protein NO145 on oocyte maturation does not correlate with, and hence is not due to, mRNA instability.
Immunolocalization Studies at Light Microscopic Level

Tissue Sections Immunofluorescence microscopy with the use of protein NO145-specific antibodies on cryostat sections through ovaries of X. laevis (Figure 6) and X. borealis (our unpublished data) showed a bright staining of the large nucleoli most of which were located in the GV periphery (Figure 6A). The exclusively nucleolar localization of protein NO145 was confirmed by double staining with mAb No-114 against the 180-kDa nucleolar protein (Schmidt-Zachmann et al., 1984), also termed xNopp180 (Cairns and McStay, 1995). Like protein NO145, xNopp180, a marker for the DFC, was found in the large amplified nucleoli, but in contrast to NO145, it was also present in the much smaller nucleoli of the surrounding follicle epithelial cells (Figure 6A′). Comparison of NO145 with xNopp180 disclosed that both proteins occurred in the nucleolus, but did not colocalize: although protein NO145 was enriched in the nucleolar cortex (Figure 6B), xNopp180 appeared mostly in the nucleolar interior (Figure 6B′). Moreover, closer inspection suggested that NO145 might also occur in some internucleolar filamentous structures (Figure 6B′).

We also compared the localization of protein NO145 with those of other nucleolar proteins known to be enriched in the GC of the nucleolus. Neither protein NO38/B23 (Schmidt-Zachmann et al., 1987) nor protein NOH61 (Zirwes et al., 2000) showed significant colocalization (our unpublished data). In summary, these results indicated that NO145 was a nucleolar protein that did not colocalize with markers for the DFC and GC but was strictly cortical, thus defining a novel nucleolar subcompartment.

Sprays of Nuclear Contents To analyze in more detail the topological relationship between protein NO145 and other nucleolar proteins in the amplified nucleoli of stage VI oocytes, we performed double-label immunolocalization experiments on spread nuclear structures. When GV contents were spread on a microscope slide, the lambrush chromosomes and other nuclear organelles such as nucleoli, Cajal bodies, and fibrillar elements were well separated from each other, thus allowing good resolution and accessibility of nuclear elements (Gall et al., 1999; Narayanan et al., 1999; Lange and Gerbi, 2000; Morgan et al., 2000). Immunofluorescence colocalization studies performed with this technique and analyzed by confocal laser scanning microscopy are presented in Figure 7, showing the intranucleolar distribution of proteins NO145 and xNopp180. The extrachromosomal nucleoli, varying in size from 1 to 15 μm, were brightly decorated by both antibodies. However, although protein NO145 was localized exclusively to the nucleolar periphery, xNopp180 was restricted to the nucleolar interior, specifically to the nucleolar cores representing mainly DFCs (Figure 7, A–D). Depending on the specific confocal plane, one could get the impression that protein NO145 formed a shell- or cage-like structure around the nucleolus (Figure 7, E and F). When focused on the nucleolar surface, however, protein NO145 appeared in a patchy arrangement, suggestive of a cortical net surrounding the entire nucleolus (Figure 7G). The exclusively nucleolar localization of protein NO145 is also demonstrated in Figure 7H in which xNopp180 is found in the DFC of the nucleoli as well as in the matrix of the Cajal bodies, whereas protein NO145 was exclusive for the cortex of the nucleoli.
When the spreading was performed in the absence of Mg$^{2+}$ ions the cortical structure of the nucleolus was disrupted and dispersed to varying degrees, and protein NO145 localized to loose fibrillar tangles surrounding the core structures (Figure 7, I–L). This core material also expanded, often appearing as chains of beads positive for xNopp180 (Wu and Gall, 1997).

The specific arrangement of protein NO145 in the cortical layer of the nucleoli was also shown in GV spreads in which it was compared with another major nucleolar protein, NO38/B23, a GC marker (Figure 8). This latter staining pattern was clearly different from that observed for the DFC protein xNopp180 (Figure 8, A–D; cf. Figure 7). Again, no significant colocalization of proteins NO38/B23 and NO145 was seen, indicating that protein NO145 defines a truly novel nucleolar substructure, a cortical meshwork representing the outermost part of the GC. GV spreads performed in the absence of Mg$^{2+}$ again resulted in a far-reaching dispersal of the GC, which then seemed to overlap partly with the filamentous meshwork stained by NO145 antibodies (Figure 8, E–H).

In cell cultures, the inactivation of nucleoli, whether naturally occurring or experimentally induced, such as by actinomycin D treatment, leads to a typical segregation of the fibrillar components (FC and DFC) from the GC material, usually resulting in the formation of distinct nucleolar hemispheres (Simard et al., 1974). Recently, this phenomenon has also been described for the amplified nucleoli of X. laevis oocytes (Mais and Scheer, 2001). Colocalization studies with the use of antibodies against proteins NO145, xNopp180, or NO38/B23 on nucleoli of actinomycin D-treated oocytes has led to an unexpected observation. Under these conditions, proteins NO145 and xNopp180 display an extensive, although not complete colocalization (Figure 9, A–A'), whereas proteins NO145 and NO38/B23 accumulate at opposite poles (Figure 9, B–B'). Obviously, during this inhibitory treatment and the extensive rearrangements, protein NO145 becomes, at least in part, associated with the DFC material.

**Electron Microscopic Immunolocalization of Protein NO145**

In the electron microscope, we examined the distribution of protein NO145 with the use of secondary antibodies coupled to colloidal gold particles on cryostat sections of frozen *Xenopus* ovaries. An intense and specific labeling of the outermost cortical layer, up to 0.1 $\mu$m in thickness, of the nucleolus was observed, whereas the nucleolar interior was practically devoid of gold particles (Figure 10, A and A'). Essentially the same result was obtained by immunoelectron localization of protein NO145 on GV spreads (our unpublished data). These analyses confirmed our immunolocalizations at the light microscopic level and allowed a better resolution of the reactive structure, demonstrating directly that protein NO145 was a specific marker for a cortical entity, different from the three major nucleolar subcompartments, i.e., FC, DFC, and GC. Occasionally we also noted, in addition, a specific labeling of certain filaments extending from the nucleolus into the nucleoplasm (Figure 10B).

**DISCUSSION**

Our molecular characterization of nucleolar protein NO145 has identified a novel type of karyoskeletal protein and also a molecular marker for a specific nucleolar substructure. Besides the nuclear envelope-associated lamin LIII, protein NO145 represents a major component of the residual fraction from *X. laevis* oocyte nuclei obtained after extraction in high salt buffers, nonionic detergents, and nucleases, and it is by far the predominant protein of the high salt- and nuclease-resistant nucleolar material (cf. Franke et al., 1981). Moreover, our immunolocalization studies at the light and electron microscopic level have revealed a very specific nucleolar location of the protein NO145-containing structures: Whereas all other nucleolar proteins known so far have been localized to one of the subnucleolar compartments, i.e., FC, DFC, or GC, protein NO145 is exclusive for a relatively thin cortical structure, forming a cage-like perinucleolar structure. The constitutive cortical cage containing the NO145 protein is a relatively stable, truly karyoskeletal structure, but is depending on a critical concentration of divalent cations, notably Mg$^{2+}$. Thus, the combination of our biochemical and structural (see also Franke et al., 1981; Moreno Díaz de la Espina et al., 1982) observations has led us to the conclusion that the extrachromosomal nucleoli of *Xenopus*...
Figure 10. Immunoelectron microscopic localization of protein NO145 on frozen sections through *X. laevis* ovary. Antibody NO145-E directed against protein NO145 was detected by secondary antibodies coupled to nanogold particles. The periphery of the nucleolus is specifically labeled (A), whereas the main body of the nucleolus, i.e., all other nucleolar subcompartments, is practically free of gold particles. The box indicated in A demarcates the nucleolar region that is presented at higher magnification in B. Occasionally, strongly labeled nucleolar filaments extending from the nucleolar cortex into the nucleoplasm can be observed (C). Bars, 0.5 μm.
contain an exoskeletal meshwork formed by patches of dense nodular aggregates interconnected by tangles of filaments with diameters varying between ~4 and 12 nm, sometimes displaying a beads-on-a-string appearance (cf. Benavente et al., 1984). We have begun to examine the self-assembly potential of protein NO145 in vitro.

Analysis of the amino acid sequence of protein NO145 has disclosed a remarkable homology to the rat SC protein 2 (SCP2), which is particularly striking (43%) in a domain of ~200 aa located in the N-terminal part of the two molecules. Whereas the functional significance of the homology between these two architectonic proteins and their karyoskeletal roles remains to be elucidated, it seems worth mentioning that some relationships between certain nucleolar and SC proteins have been reported previously. For example, nucleolar protein No55 has been reported to be almost identical to the rat SC protein SC65 (Ochs et al., 1996). Moreover, it has been described that some antibodies to SC proteins also label nucleoli and vice versa (Dresser, 1987; Moens et al., 1987), and the nucleolar protein Pch2 identified in yeast has also been detected in a punctate pattern along synapsed chromosomes (San-Segundo and Roeder, 1999). These observations may be taken as an indication that certain, although yet unknown amino acid sequence motifs have been conserved in proteins of both nuclear structures, SC and nucleoli, serving similar structure-defining functions.

Although our immunolocalization results in Xenopus oocytes are in line with those previously reported, they are at variance with some observations of a few tiny, nucleoli-associated fluorescent “dots” made with the same murine antisera on some somatic Xenopus cells such as hepatocytes, Sertoli cells, spermatagonia, and A6 cells (Krohne et al., 1982; Benavente et al., 1984). In contrast, the various well-characterized antibodies to protein NO145 used in the present study have shown an exclusive occurrence in amplified oocyte nucleoli, and we have failed to detect NO145 immunostaining and immunoblotting reactions in any other cell type than oocytes. Although we have no definitive explanation for this difference of reaction we cannot exclude that these tiny dots detected in the previous studies might be due to a component cross-reactive with an as yet unknown, apparently minor nucleolar protein present in somatic cells. In this context it is perhaps also worth stating that the Xenopus protein NO145 has no relationship to the mammalian nucleolar 120–145-kDa proteins reported in the literature (Freeman et al., 1986; Busch, 1997).

However, we have to mention that we have isolated a partial cDNA clone coding for NO145 from a X. laevis kidney expression library by DNA screening (our unpublished data). Possibly, this cDNA clone identified in the kidney cDNA library results from very low levels of transcription of the NO145 gene in these somatic cells. We are currently performing reverse transcription-PCR experiments to identify possible mRNAs encoding NO145-related proteins in somatic cells of Xenopus and other species. At present, we cannot decide whether similarly looking cortical nucleolar structures in other cells and outside the genus Xenopus are formed by more distantly related homologous proteins or represent analog structures.

A most remarkable phenomenon is the rapid and complete degradation of nucleolar protein NO145 during meiotic maturation and egg formation, which experimentally can be followed in detail upon addition of progesterone to oocytes (Wasserman and Smith, 1978). Closely correlated with nuclear envelope breakdown and disassembly of the nuclear lamina, the multiple amplified nucleoli also disassemble rapidly, all processes being accompanied by extensive phosphorylation events (Belenguer et al., 1990; Heald and McKeon, 1990; Peter et al., 1990a,b). Already minutes upon the appearance of the “white spot” at the pigmented animal pole, most nucleolar proteins are dispersed throughout the cytoplasm (Bell et al., 1992; Messmer and Dreyer, 1993; Bauer et al. 1994; some residual rDNA units detectable by DNA staining might correspond to FCS; cf. Shah et al., 1996). Interestingly, the disappearance of protein NO145 correlates perfectly with this rapid dispersion of nucleolar material, a behavior that distinguishes NO145 from all other nucleolar proteins so far studied in this system. Moreover, we have shown that the rapid decrease in NO145 protein concentration does not correspond to lowered levels of NO145 mRNA, which remains stable through oocyte maturation and in unfertilized eggs. We conclude that a special mechanism for the selective degradation of protein NO145 must exist. It would be interesting to know whether this degradation occurs via the proteasome pathway, with ubiquitin-conjugated intermediates, as recently described for the cytoplasmic polyadenylation element-binding protein (CPEB; Reverte et al., 2001), or whether it is regulated by rapid changes in the phosphorylation state of protein NO145 (for a high density of potential phosphorylation sites and a high degree of phosphorylation, see this study; Benavente et al., 1984).

Regardless, the nucleolar cortex protein NO145 presents a remarkable dual character: Although it is a major component of a rather stable structure in the nucleolar periphery, where it may be associated with other, yet unknown minor components, it is obviously also very sensitive to regulated proteolysis. Future studies will have to elucidate the specific mechanisms involved in both the formation and maintenance of the stable cage structure and in the rapid disassembly and degradation.

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