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NO66, a Highly Conserved Dual Location Protein in the Nucleolus and in a Special Type of Synchronously Replicating Chromatin[D]

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It has recently become clear that the nucleolus, the most prominent nuclear subcompartment, harbors diverse functions beyond its classic role in ribosome biogenesis. To gain insight into nucleolar functions, we have purified amplified nucleoli from *Xenopus laevis* oocytes using a novel approach involving fluorescence-activated cell sorting techniques. The resulting protein fraction was analyzed by mass spectrometry and used for the generation of monoclonal antibodies directed against nucleolar components. Here, we report the identification and molecular characterization of a novel, ubiquitous protein, which in most cell types appears to be a constitutive nucleolar component. Immunolocalization studies have revealed that this protein, termed NO66, is highly conserved during evolution and shows in most cells analyzed a dual localization pattern, i.e., a strong enrichment in the granular part of nucleoli and in distinct nucleoplasmic entities. Colocalizations with proteins Ki-67, HP1α, and PCNA, respectively, have further shown that the staining pattern of NO66 overlaps with certain clusters of late replicating chromatin. Biochemical experiments have revealed that protein NO66 cofractionates with large preribosomal particles but is absent from cytoplasmic ribosomes. We propose that in addition to its role in ribosome biogenesis protein NO66 has functions in the replication or remodeling of certain heterochromatic regions.

INTRODUCTION

The nucleolus is the most prominent nuclear structure, representing the main site of ribosome biogenesis, a complicated process that includes the transcription of rRNA genes, the processing and modification of these transcripts, and their assembly with both ribosomal as well as nonribosomal proteins to guide the formation of preribosomal particles (reviewed by Scheer and Hock, 1999; Grummt, 2003). More recent evidence, however, has shown that the nucleolus is also involved in the assembly of various other kinds of ribonucleoprotein particles, the modification of small RNAs, the control of the cell cycle, the sequestration of regulatory molecules, and nuclear export processes (reviewed by Pederson, 1998; Olson *et al.,* 2002; Gerbi *et al.,* 2003). The discovery of novel functional importance of the nucleolus was paralleled by two recent proteomic analyses of human nucleoli (Andersen *et al.,* 2002; Scherl *et al.,* 2002), in which a total of ~350 different proteins have been identified, adding further support to the concept of the plurifunctional nature of nucleoli.

Morphologically, the nucleolus is characterized by the presence of three major structural components defined by electron microscopy: The internal fibrillar center (FC) is surrounded by the dense fibrillar component (DFC) and the granular component (GC), constituting the bulk of an active nucleolus. Localization studies using specific antibodies as well as hybridization probes have disclosed that the vectorial process of ribosome synthesis can be correlated with distinct nucleolar substructures, i.e., nascent preribosomes move from the DFC region to the peripherally located GC (e.g., Thiry *et al.,* 2000; Huang, 2002). In addition, a nucleolus-specific karyoskeletal element has been demonstrated in the nucleolar cortex of amphibian oocytes (Franke *et al.,* 1981; Kneissel *et al.,* 2001).

Extended immunolocalization studies of nuclear proteins and, in particular, live-cell imaging have disclosed that nuclear processes rely on a constant flow of molecules between nuclear subcompartments (reviewed by Carmo-Fonseca, 2002; Leung and Lamond, 2003). Consequently, certain nuclear proteins may not be restricted to one nuclear substructure alone, but may also occur—at least transiently or in special phases—in other nuclear substructures. Indeed, a number of nucleolar proteins, such as fibrillarin, Nopp140, and NAP57, have been also found in Cajal bodies (Ochs *et*

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Abbreviations used: aa, amino acid(s); AMD, actinomycin D; DFC, dense fibrillar component; FACS, fluorescence-activated cell sorting; FC, fibrillar center; FITC, fluorescein isothiocyanate; GC, granular component; HSP, high-speed pellet; HSS, high-speed supernatant; LSP, low-speed pellet; MALDI, matrix-assisted laser desorption ionization; SMN, survival of motor neuron; PCNA, proliferating cell nuclear antigen; PML, promyelotic leukemia.

al., 1985; Meier and Blobel, 1990, 1994; Raska *et al.*, 1991), and the survival of motor neuron (SMN) protein as well as its interacting proteins have been localized to "gems" and nucleoli (Charroux *et al.*, 2000; Wehner *et al.*, 2002). Under certain conditions proteins normally found in promyelotic leukemia (PML) bodies or "paraspeckles" can move to the nucleolus (Lin and Shih, 2002; Fox *et al.*, 2002), and protein Ki-67, a widely used tumor marker, localizes to both nucleoli and heterochromatic regions (Starborg *et al.*, 1996; Bridger *et al.*, 1998).

The number of nucleoli per nucleus can vary greatly, from one or a few located at chromosomal nucleolar organizers, to more than thousand extrachromosomal nucleoli in certain amphibian oocytes (Hadjiolov, 1985). The presence of a high copy number of rRNA genes and the absence of nonribosomal DNA make the *Xenopus* oocyte nucleoli a particularly valuable model to analyze nucleolar proteins and their functions. This prompted us to improve the purification of amplified nucleoli from *Xenopus* oocyte nuclei by fluorescence-activated particle sorting, originally described by Franke *et al.* (1981). The resulting "pure" nucleolar fraction was subsequently analyzed with protein chemical and immunolocalization methods.

Here, we report the identification and molecular characterization of a so far unknown nucleolar protein of ~66 kDa from *Xenopus laevis*, hence termed protein NO66. This protein, highly conserved from *Caenorhabditis elegans* to man, displays an unusual distribution: Besides a strong accumulation in nucleoli, it is also localized in distinct intranuclear bodies that represent clusters of late replicating chromatin. In biochemical studies we further show that in the nucleolus NO66 is enriched in large, most likely preribosomal particles, and on immunoprecipitation it copurifies with a number of well-characterized nucleolar constituents. We conclude that we have identified a dual location intranuclear protein that on the one hand plays a role in ribosome biogenesis and on the other participates in the replication or silencing of certain heterochromatic regions.

MATERIALS AND METHODS

Biological Material

Clawed toads (*X. laevis*) were purchased from the South African Snake Farm (Krysna, Republic of South Africa).

Cell culture lines used in this study included vulvar squamous carcinoma line A-431, astrocytoma derived glioma line U333 CG/343 MG, spontaneously transformed keratinocyte line HaCaT, cervical adenocarcinoma line HeLa, breast adenocarcinoma line MCF-7, primary liver carcinoma line PLC, SV40-transformed fibroblast line SV80 (all of human origin), mouse embryonic fibroblast line 3T3, rat smooth muscle cell line RV, bovine mammary gland–derived cell line BMGE+H, canine kidney cell line MDCK, potoroo kidney cell line PtK2, chicken embryonic fibroblast line CEF, rainbow trout gonadal cell line RTG-2, and *X. laevis* kidney epithelium line XLKE-A6. For sources of all cell lines see American Type Culture Collection (ATCC, Manassas, VA) and previous reports from this laboratory (Franke *et al.*, 1979; Schmid *et al.*, 1983; Cordes *et al.*, 1996). Primary human umbilical vein endothelial cells (HUVEC) were prepared as described by Peitsch *et al.* (1999).

HeLa cells were synchronized at the G1/S-phase border by a double-thymidine block according to standard protocols. Cells were released by adding fresh culture medium and their progression toward the cell cycle was analyzed at different time points by flow cytometry and immunofluorescence microscopy.

Large-scale Isolation and Fractionation of X. laevis Oocyte Nuclei and Preparation of Egg Extracts

Nuclei from mature *X. laevis* oocytes (stages IV–VI; Dumont, 1972) were obtained by mass isolation using the procedure of Scalenghe *et al.* (1978), with modifications of Kleinschmidt and Franke (1982). Subsequent fractionation of nuclear contents by differential centrifugation was performed as described (Hügle *et al.*, 1985; Kneissel *et al.*, 2001). Fractions obtained were termed low-speed pellet (LSP), high-speed pellet (HSP), and high-speed supernatant (HSS). LSP fractions were cleared from yolk by extraction with freon (Evans and Kay, 1991). Unfertilized eggs from *X. laevis* were extracted as described (Cordes *et al.*, 1993).

Isolation of Amplified Nucleoli by Fluorescence-Activated Particle Sorting

Mass isolated *X. laevis* nuclei densely packed in 5:1 medium (83 mM KCl, 17 mM NaCl, 2 mM $MgCl_2$, 1 mM dithiothreitol, 100 μM Pefabloc, 10 mM Tris-Cl, pH 7.4) containing 100 mM sucrose were gently homogenized by sucking through a 200-μl pipette tip and labeled with fluorescein isothiocyanate (FITC)-conjugated mAb No-185 (see below, final concentration 50 μg/ml homogenate). Subsequently, the homogenate was analyzed with a fluorescence-activated cell sorting (FACS) flow cytometry system (FACS II; Becton Dickinson, Heidelberg, Germany) using the 488-nm line of an argon ion laser and 5:1 medium as sheath fluid. The relative FITC fluorescence of passing particles was measured with a 520–620-nm bandpass filter. Information about the relative particle size was obtained with a light scatter sensor. Large, strongly fluorescing particles (i.e., nucleoli) were sorted by appropriate threshold settings in the two-dimensional distribution of FITC fluorescence and light scatter (cf. Figure 1A). The collected nucleoli were sedimented (10 min, 3200 × *g*) and resuspended in PBS or SDS-PAGE sample buffer.

Protein NO66-specific Antibodies

Monoclonal antibodies against proteins from purified *X. laevis* oocyte nucleoli were generated essentially as described by Köhler and Milstein (1975). One of the resulting antibodies, mAb No-5-1-1 (IgG1) showed a strong nucleolar immunostaining on *Xenopus* A6 cells and reacted specifically with a 66-kDa polypeptide (NO66; cf. Figure 2, A and B).

Guinea pig antisera against protein NO66 were obtained by immunization with synthetic peptides (PSL, Heidelberg, Germany). Antisera NO66-1 and NO66-3 were raised against peptides NSSEFLPQFSSVNFR and HNHGYYDGLFSTK, respectively, representing parts of *X. laevis* NO66 as determined by peptide sequencing. NO66-2 was directed against the peptide TPPGTQGFAPHYDDIEAFVLQLEGKKHWRV derived from an expressed sequence tag (EST) from chicken (acc. no. AL587154). All antisera showed the same specificity and were used after affinity purification on corresponding peptide-conjugated matrices (Mertens *et al.*, 1996).

Other Antibodies

The following antibodies were used for colocalization studies: Rabbit antibodies to coilin (Bohmann *et al.*, 1995), PSP1 (Fox *et al.*, 2002), fibrillarin (Azum-Gélade *et al.*, 1994), CENP-B (Goldberg *et al.*, 1996), XOrc1, XOrc2, XMcm3, XMcm7, and XCdc6 (Madine *et al.*, 2000). Rabbit antiserum against human $SF3b^{155}$ was obtained by immunization with baculovirus expressed recombinant protein. mAbs P7–1A4 against nucleolin and SH54 against PTB (Meßmer and Dreyer, 1993; Huang *et al.*, 1997). mAb MB67 recognizing the Ki-67 protein and mAb PC10 against the proliferating cell nuclear antigen (PCNA) was from Dianova (Hamburg, Germany) and Dako (Hamburg, Germany), respectively. SMN, an mAb directed against the homonymous SMN protein was purchased from Becton Dickinson, mAb PML (PG-M3) against the PML protein and mAb Sam68 (7-1) against protein Sam68 were from Santa Cruz (Heidelberg, Germany), and mAb 2HP 1H5 against HP1α was from Euromedex (Mundolsheim, France). Lamin-mAb $L_0$46F7 against *Xenopus* oocyte-specific lamin L_{III}, mAb 9E10 recognizing the human c-myc protein and the guinea pig serum B2.4–1 against $SF3b^{155}$ have been described (Benavente *et al.*, 1985; Evan *et al.*, 1985; Schmidt-Zachmann *et al.*, 1998). mAb No-440, which recognized the nucleolar RNA helicase NOH61 (Zirwes *et al.*, 2000), was raised against recombinant His_6-tagged NOH61. mAb No-114 against xNopp180 and mAb No-185 against protein NO38/B23 were described (Schmidt-Zachmann *et al.*, 1984, 1987).

For fluorescence-activated particle sorting, mAb No-185 was purified from serum free cell culture supernatant by affinity chromatography using HiTrap protein G-Sepharose columns (Amersham, Freiburg i. Br., Germany). The purified antibodies were covalently coupled to FITC as described by Harlow and Lane (1998).

Secondary antibodies used for immunofluorescence microscopy were Cy3- or Alexa 488–conjugated antibodies against immunoglobulin G (IgG) of mouse, guinea pig, or rabbit (Dianova). Western blot experiments were performed with horseradish peroxidase–conjugated secondary antibodies (Dianova). For immunoelectron microscopy, anti-guinea pig– or anti-mouse IgG-conjugated Nanogold (Nanoprobes, Stony Brook, NY) was used.

cDNA Clones

IMAGE Consortium (LLNL) cDNA clones (Lennon *et al.*, 1996) with CloneIDs 4340013 (GenBank acc. no. BC011350) and 3916148 (acc. no. BC014928) were obtained from the German Resource Center for Genome Research (RZPD, Berlin). These clones contained open reading frames encoding full-length hsNO66 and hsNO52, respectively. The hsNO66-cDNA was subcloned into expression vector pcDNA3.1(+) by *Eco*RI/*Not*I, resulting in clone pcDNA3.1-NO66. pcDNA3.1-mycNO66 was constructed by digestion of pcDNA3.1-NO66 with *Eco*RV/*Not*I. A 2477-base pair cDNA fragment encoding hsNO66 was isolated and cloned in-frame with the myc-tag into the *Sma*I/*Not*I-

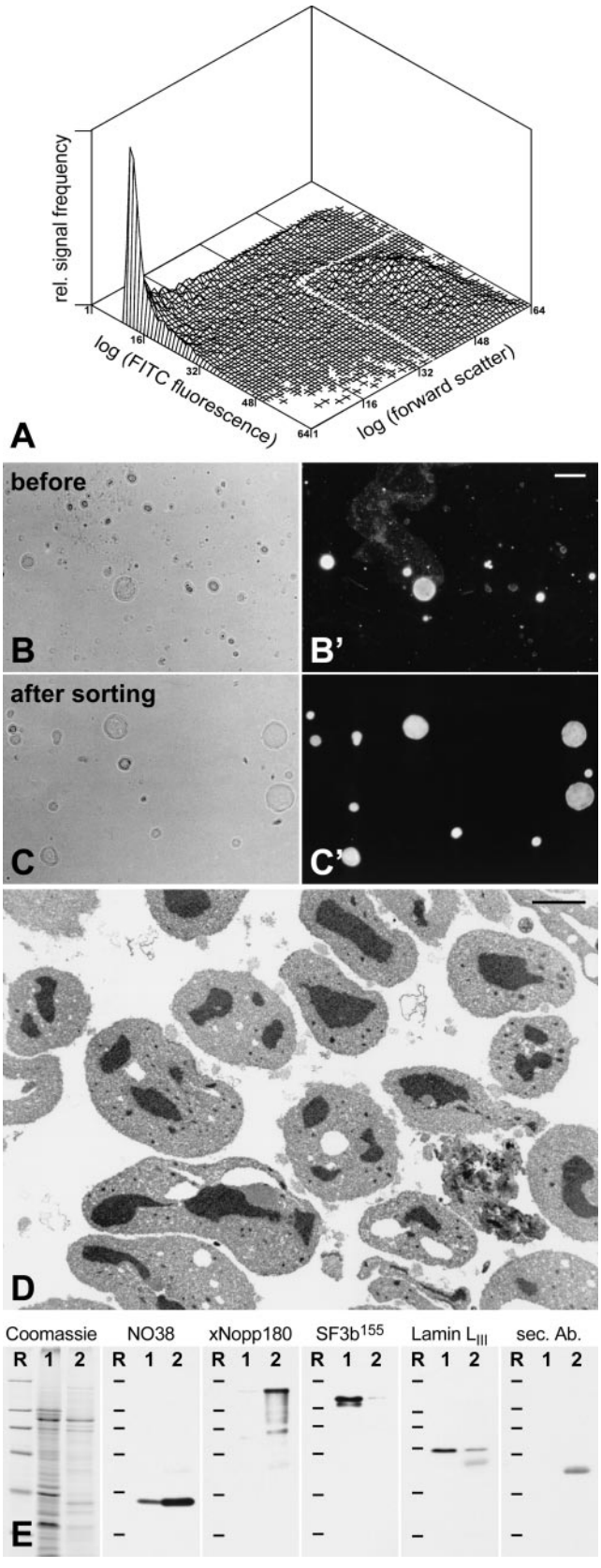

Figure 1. Purification of nucleoli from mass-isolated *Xenopus laevis* oocyte nuclei by fluorescence labeling and particle sorting. Amplified nucleoli were labeled by incubating nuclear homogenates with FITC-coupled mAb No-185 (No-185-FITC) directed against the major nucleolar protein NO38. (A) Two-parameter frequency profile obtained by flow cytometry. Besides FITC fluorescence, light scattering depending on the particle size was measured. Strongly fluorescent large particles, i.e., nucleoli, appearing in the indicated rectangle were separated from other particles, including yolk platelets and debris of nuclear membranes. (B and C) Light microscopy of ruptured oocyte nuclei labeled with No-185-FITC before (B and B′) and after (C and C′) particle sorting. (B and C) Phase contrast. (B′ and C′) FITC fluorescence. Bar, 20 μm. (D) Electron microscopy of amplified nucleoli of *Xenopu*s enriched by particle sorting. Bar, 5 μm. (E) Mass-isolated total nuclei (lane 1) and purified nucleoli (lane 2) were analyzed by immunoblotting using anti-NO38, anti-xNopp180, anti-SF3b^{155}, and antilamin L_{III}. The 50-kDa signal appearing in the nucleoli fraction after long exposure time results from the secondary antibodies to mouse-IgG detecting the heavy chain of the mAb No-185-FITC antibody originally used as fluorescence marker for particle sorting. Reference proteins (lane R) represent, from top to bottom: 205, 116, 97, 66, 45, and 29 kDa.

digested construct BTmyc (Schmidt-Zachmann and Nigg, 1993). The resulting fusion construct was transferred by *Hin*dIII/*Not*I into pcDNA3.1(+).

RNA Isolation, Northern Blot Hybridization, and Coupled In Vitro Transcription-Translation

Total RNA from MCF-7 cells was prepared as described (Chirgwin *et al.*, 1979). Poly(A)$^+$ RNA was obtained using a mRNA purification kit (Amersham). RNAs (5 μg per lane) were separated on 1% agarose gels containing 0.6% formaldehyde, transferred to Biodyne B nylon filters (Pall, Dreieich, Germany) and hybridized as described (Kneissel *et al.*, 2001) with a random-primed, ^{32}P-labeled 554-base pair *Fsp*I/*Xcm*I fragment of the hsNO66 cDNA.

In vitro synthesis of ^{35}S-methionine–labeled proteins by coupled transcription/translation was carried out with the TNT reticulocyte lysate system (Promega, Mannheim, Germany).

Preparation of Total Cellular Lysates, Cell Extracts, and Isolation of Nuclei and Nucleoli from MCF-7 Cells

Total cellular lysates were prepared as described by Schmidt-Zachmann *et al.* (1998). Extracts from human A-431 and *X. laevis* A6 cells were obtained according to Zirwes *et al.* (2000) with lysis buffer containing 50 mM Tris-Cl, pH 7.4, 250 mM NaCl, 0.5% Triton X-100, 1 mM β-mercaptoethanol, and 1 mM Pefabloc. The preparation of nuclei and nucleoli from MCF-7 cells was performed essentially as described by Zirwes *et al.* (2000).

Immunoprecipitation Experiments, Mass Spectrometry, and Peptide Sequencing

Immunoprecipitation from *X. laevis* A6 cell extract with mAbs was done according to Zirwes *et al.* (2000). Excision of immunoprecipitated protein bands from gels, digestion with trypsin, matrix-assisted laser desorption/ionization (MALDI) mass spectrometry, amino acid sequence analysis, and database searches were performed as described (Kuhn *et al.*, 2001).

Sucrose Density Gradient Centrifugation and Gel Filtration

A-431 cell extracts and HSP fractions were separated as described (Zirwes *et al.*, 2000) in density gradients containing 5–30% and 10–40% sucrose, respectively. In parallel experiments protein samples were preincubated with 50 μg of DNase-free RNase A for 30 min on ice before loading onto gradients.

For gel filtration, 200 μl of A-431 cellular extract corresponding to $\sim 10^7$ cells were loaded onto a Superdex 200 HR10/30 column (Amersham) and eluted with lysis buffer (without detergent). For calibration, mixtures of dextran blue and reference proteins (thyroglobulin, ferritin, catalase, aldolase, BSA, and ovalbumin; all from Amersham) were separated under the same conditions.

Gel Electrophoresis and Immunoblotting

Protein fractions were separated by SDS-PAGE according to Thomas and Kornberg (1975), and immunoblot analysis was performed as described by Zirwes *et al.* (2000).

DNA Transfection and Microinjection into Cultured Cells

Transient transfection of MCF-7 cells was achieved with Fugene 6 transfection reagent (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol. Immunofluorescence microscopy analysis was performed 24 h after transfection. For replication foci labeling, 25 mM Cy3-dCTP in PBS was microinjected into the nucleoplasm of MCF-7 cells as described by

Görisch *et al.* (2003). Cells were fixed and processed for immunofluorescence microscopy 45 min after injection.

Immunofluorescence Microscopy

For immunofluorescence microscopy, cells were fixed with methanol (5 min, −20°C) and acetone (30 s, −20°C). Nuclease digestion was performed according to Spector *et al.* (1991). In some experiments, cells were extracted with Triton X-100 before fixation as described by Fujita *et al.* (2002). Incubation with antibodies and DAPI staining was performed essentially as described (Zirwes *et al.*, 2000). Dilutions in PBS of the primary antibodies raised during this study were 1:25 for NO66-2 and 1:10 for NO66-3. Concentrated hybridoma cell culture supernatant containing mAb No-440 was diluted 1:500, mAb No-5-1-1 cell culture supernatant was used undiluted.

Electron Microscopy

For conventional thin-section studies, purified *X. laevis* oocyte nucleoli were processed as described (Rose *et al.*, 1995). For immunoelectron microscopy, cells grown on coverslips were fixed in 2% formaldehyde in PBS (see Figure 5I) as specified by Brandner *et al.* (1998) or in cold methanol and acetone (see Figure 9, F and G) as described for immunofluorescence microscopy, followed by immunogold labeling according to Zirwes *et al.* (2000). Antibody dilutions were the same as for immunofluorescence microscopy. For some experiments, cells were incubated with 5 μg/ml actinomycin D (AMD) for 4 h before fixation.

RESULTS

Identification of a Novel, Ubiquitous Nucleolar Protein (NO66)

A particularly advantageous system for studies of the biochemical composition of the nucleolus are the numerous extrachromosomal amplified nucleoli of amphibian oocyte nuclei. Because of their enormous size, high number of rRNA genes, and their high transcriptional activity, the amounts of most nucleolar proteins are significantly higher in oocyte nuclei than in the nuclei of somatic cells. Because the extrachromosomal nucleoli are not associated with heterochromatin or any other chromosomal material, they can be isolated in a much purer form than somatic nucleoli. We therefore have improved the isolation of nucleoli by FACS originally described by Franke *et al.* (1981) and Schmidt-Zachmann *et al.* (1984). Here, nuclear homogenates obtained from mass-isolated oocyte nuclei were incubated with FITC-coupled mAb No-185 directed against the well-characterized nucleolar protein NO38/B23 (Schmidt-Zachmann *et al.*, 1987; Zirwes *et al.*, 1997a). This treatment led to a strong and specific labeling of the amplified nucleoli and thus allowed their isolation by FACS (Figure 1A). The purity and integrity of the resulting fraction was analyzed by light and electron microscopy (Figure 1, B–D) as well as by immunoblot experiments (Figure 1E).

The purified amplified nucleoli, varying in size from 1 to 20 μm were highly enriched, exhibiting the typical intranucleolar morphology (Figure 1D; see also Mais and Scheer, 2001). To verify the purity of the isolated nucleoli, protein fractions from total oocyte nuclei in comparison to purified nucleoli were analyzed by SDS-PAGE, transferred to nitrocellulose filters, and probed with antibodies specific for other nucleolar proteins such as NO38, xNopp180, or nuclear proteins SF3b^{155} (a component of the splicing factor SF3b that associates with the U2 snRNP) and the oocyte-specific lamin L_{III} (Figure 1E). Both nucleolar proteins xNopp180 and NO38 (Schmidt-Zachmann *et al.*, 1984, 1987) were highly enriched in the nucleolar protein fraction, whereas the relative amounts of two other nuclear proteins, SF3b^{155} known to be highly enriched in nuclear speckles (Schmidt-Zachmann *et al.*, 1998) as well as the lamina protein lamin L_{III} (Krohne *et al.*, 1984) were greatly reduced in the nucleolar fraction.

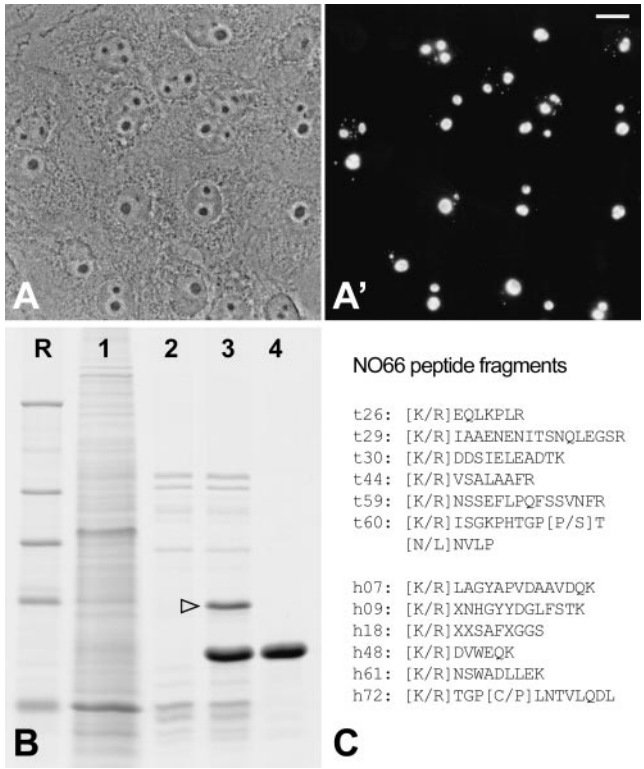

Figure 2. Immunological characterization of mAb No-5-1-1 and identification of protein NO66. (A and A′) Immunolocalization of the protein recognized by mAb No-5-1-1 in cultured *Xenopus laevis* kidney epithelial cells, line A6 (XLKE-A6). (A) Phase contrast. (A′) Corresponding immunofluorescence micrograph. Bar, 10 μm. (B) Immunoprecipitation experiments using mAb No-5-1-1. Proteins of the following fractions were separated by SDS-PAGE and stained with Coomassie blue: XLKE-A6 cell extract before immunoprecipitation (lane 1); cell lysate proteins nonspecifically bound to protein G-Sepharose in the absence of antibody (lane 2); immunoprecipitate obtained from XLKE-A6 cell lysate (lane 3); and immunoprecipitate after incubation with extraction buffer as a negative control (lane 4). Reference proteins (lane R) are the same as in Figure 1. A protein of ~66 kDa (termed NO66, indicated by an arrowhead in lane 3) is specifically immunoprecipitated by mAb No-5-1-1. (C) Peptide sequences of *Xenopus laevis* protein NO66 obtained after excision of the specifically immunoprecipitated protein band from gels as shown in B, lane 3 and "in-gel" digestion with trypsin, followed by chromatographic separation and Edman sequencing of the tryptic fragments.

The protein fraction containing amplified nucleoli in FACS-purified form was separated by two-dimensional gel electrophoresis and analyzed by mass spectrometry (MS) and microsequencing (our unpublished results). A large-scale MS-based protein identification was hampered by the lack of comprehensive *Xenopus*-specific protein and cDNA databases. Because the identification of the individual protein spots progressed more slowly than expected, a second approach was taken in parallel. The same protein fraction was used for the immunization of mice to generate mAbs directed against nucleolar components. Besides a number of mAbs recognizing well-known nucleolar proteins, such as NO38, xNopp180, and nucleolin, a mAb termed No-5-1-1 was obtained that showed a strong and specific labeling of

nucleoli in somatic cells (Figure 2A) as well as in oocytes from *X. laevis* (our unpublished results). Immunoprecipitations from protein extracts prepared from XLKE-A6 cells using mAb No-5-1-1 revealed the enrichment of a ca. 66-kDa polypeptide (Figure 2B, lane 3). To identify this polypeptide, hereafter referred as to NO66, the immunoprecipitated band was excised from the gel and digested with trypsin. The resulting peptides were analyzed by MALDI-TOF mass spectrometry, and the peptide mass fingerprints were compared with predicted peptides of the NCBInr database by the ProFound search aligorithm. As the mass information obtained for the 66-kDa protein could not be assigned to any known protein in the database, we prepared material sufficient for microsequencing analysis of the polypeptide.

Twelve amino acid sequences were obtained (Figure 2C) and subsequently used for detailed database searches. We identified a human cDNA clone encoding the hypothetical protein FLJ21802 displaying significant homology to various peptides derived from the *Xenopus* protein NO66 (thereafter termed hsNO66; Figure 3A). The human cDNA clone was received from the RZPD and used for further studies of this novel nucleolar protein (acc. no. AY390535). The cDNA clone coding for hsNO66 (2451 base pairs) contained an initiation codon at position 52, an open reading frame of 1926 base pairs and a 3′-untranslated region of 474 base pairs. The open reading frame encoded a polypeptide of 641 aa with a calculated molecular mass of 71.1 kDa and an isoelectric point of 6.4. Inspection of the primary sequence of hsNO66 disclosed the presence of two putative nuclear localization signals, one of the monopartite type (aa 17–21; RRRRK) and the other of the bipartite type (aa186–201; RR $(x)_{11}$ RRR; cf. Dingwall and Laskey, 1991). A high number of potential phosphorylation sites for cAMP-dependent kinase, protein kinase C, casein kinase II, and thyrosine kinase were also noticed. Presently, we do not know the actual degree of phosphorylation of NO66, but posttranslational modifications could well account for the observed difference in molecular mass calculated for the polypeptide from the cDNA-derived sequence (71 kDa) or from its SDS-PAGE mobility (79 kDa, see below). A closer analysis of the primary sequence of the protein revealed the presence of a putative JmjC domain (aa 290–429). This kind of domain has been found in numerous eukaryotic proteins and has been postulated to be involved in chromatin remodeling processes (Clissold and Ponting, 2001).

Searches of current databases have disclosed a striking homology of hsNO66 with hypothetical proteins from *Anopheles gambiae*, *C. elegans*, *Drosophila melanogaster*, *Rattus norvegicus*, and *Mus musculus* (acc. nos. EAA01582, T15138, NP_572160, Xp_234396, and BAA95038, respectively) as well as with expressed sequence tags of *Gallus gallus* (Figure 3B; acc. no. AL587154), and *Xenopus tropicalis* (Figure 3C; acc. nos. AL650484 and AL786378). These proteins of unknown functions comprise of 693, 817, 653, 597, and 603 aa residues, and differ markedly in size from the human protein, because of different lengths of the amino-terminal portions ranging from 147 aa (*M. musculus*) to 361 aa (*C. elegans*). Notably, all proteins show a significantly high sequence homology upstream of the less conserved amino-terminal third, corresponding to aa 186–641 of hsNO66. Here, the sequence identity between hsNO66 and the homologues in *A. gambiae*, *C. elegans*, *D. melanogaster*, and *M. musculus* are 44, 39, 40, and 92%, respectively (our unpublished results). This remarkable conservation indicated that these proteins were indeed homologous and most likely participate in a fundamental cellular process.

Moreover, database searches revealed the existence of another related but distinct hypothetical human protein (FLJ14393) with a molecular mass of ~52 kDa (termed NO52; acc. no. AY390536), which might be correlated to the recently described Mina53 protein proposed to be involved in cell proliferation (Tsuneoka *et al.*, 2002). The overall aa sequence identity between the two human proteins was 36% but even higher in a central domain of 140-aa residues (57%). Like protein NO66, protein NO52 was also highly conserved during evolution and was found enriched in nucleoli. Further studies, however, revealed that the two nucleolar proteins differed in their biochemical properties as well as in the ways their precise intracellular localization is affected by certain treatments such as serum starvation and transcription inhibition by AMD (our unpublished results; a detailed characterization of protein NO52 will be presented elsewhere).

Because for unknown reasons all attempts to clone the *X. laevis* ortholog failed, we used the cDNA clone coding for the human protein (hsNO66) for the molecular and biochemical characterization of this novel nucleolar protein.

Molecular Characterization of Protein NO66

Total RNA as well as poly(A^+) RNA from human MCF-7 cells were probed in Northern blot experiments, using a random prime-labeled fragment of the hsNO66-cDNA. Although only a faint signal could be observed in total cellular RNA after long exposure, a specific signal corresponding to a mRNA of ~2.3 kb was detected in the poly(A^+) fraction, indicating that the analyzed cDNA clone encoding protein NO66 was of full length and that the amount of the endogenous mRNA encoding this nucleolar protein was rather low in the cells used (Figure 3D). When the expression of the gene encoding protein NO66 was examined, the 2.3-kb mRNA was detected in all eight tissues (heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas), although the intensity of the radioactive signal varied somewhat (our unpublished results). To analyze the localization of hsNO66, a chimeric cDNA construct encoding the full-length protein carrying a myc tag at its amino-terminus was transfected into MCF-7 cells. Immunofluorescence analysis with the myc antibody revealed that the protein was highly enriched in nucleoli. However, additional labeling of small nucleoplasmic granules was also observed (Figure 3, E and E′).

Because mAb No-5-1-1 recognizing *X. laevis* NO66 did not cross-react with other species, except for *X. borealis*, as indicated from the immunofluorescence studies, and was negative in immunoblots, we generated further antibodies to study the intracellular distribution and localization of the endogenous protein hsNO66. Three polyclonal antibodies (NO66–1, -2 and -3) raised against peptides representing different sequence elements of protein NO66 were obtained, which varied in their cross-species reactivities but showed the same specificity. To prove that the antisera indeed recognized the same protein originally detected by mAb No-5-1-1 in cellular extracts from XLKE-A6 cells, we repeated the immunoprecipitation shown in Figure 2B and analyzed the resulting protein fractions by immunoblotting using these polyclonal NO66 antibodies (Figure 4). The antibodies showed a strong reaction with the *Xenopus* protein present in the total cellular extract before and after incubation with protein G-Sepharose in the absence of antibody No-5-1-1 (Figure 4B, lanes 1 and 2), which was no longer detectable in the extract upon incubation with protein G-Sepharose in the presence of the antibody. Moreover, the NO66 antibodies specifically reacted with the polypeptide immunoprecipitated in Coomassie blue-visible amounts by the mAb (Figure

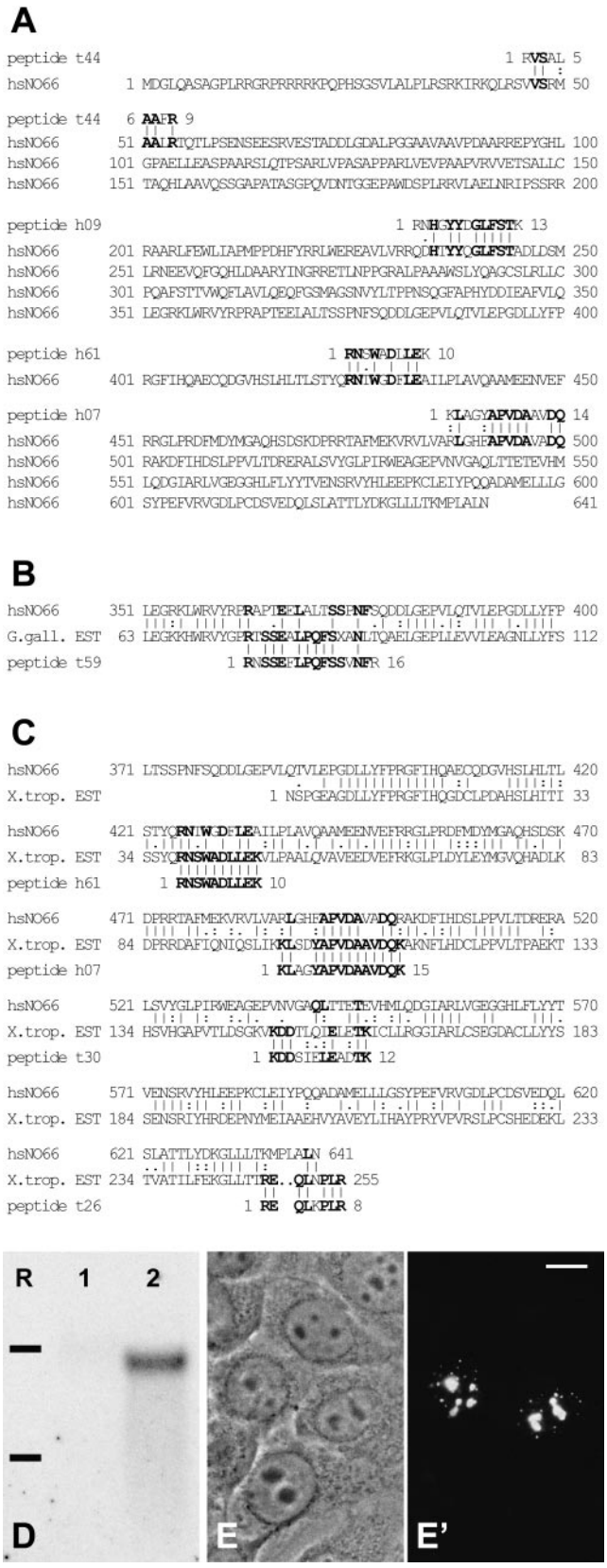

Figure 3. Identification of proteins homologous to *Xenopus laevis* protein NO66. (A) Database searches with NO66 peptides listed in Figure 2 revealed the hypothetical human protein "hsNO66" as a putative homologue of protein NO66. An alignment of hsNO66 with matching NO66 peptides obtained from *Xenopus laevis* is shown (identical aa residues are indicated in bold letters). (B) The homology of the human hypothetical protein with *Xenopus laevis* protein NO66 was confirmed by analyses of homologues found in other species. A fragment of the corresponding chicken NO66 homolog encoded by an EST shows high similarity to *X. laevis* peptide t59, which fits only weakly to the human sequence. (C) Two EST clones were identified that together encoded the carboxy-terminal part of the protein NO66 homolog of *Xenopus tropicalis*. Peptides t30 and t26 of NO66 could not be directly allocated to the human sequence but showed significant homology to the *Xenopus tropicalis* sequence. (D) Northern blot analysis of total RNA (lane 1) and poly(A)$^+$ RNA (lane 2) from human MCF-7 cells with a random prime-labeled probe obtained from a cDNA clone encoding hsNO66. RNA size markers (R): 2.4 (top) and 1.4 kb (bottom). (E) Phase-contrast microscopy of human MCF-7 cells used to determine the subcellular localization of the hsNO66 protein carrying an amino-terminal myc-tag in transient transfection experiments. (E′) Corresponding immunofluorescence using mAb 9E10 recognizing the myc-tag. Bar, 10 μm.

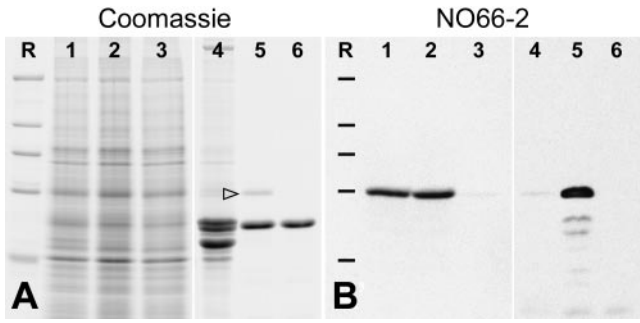

Figure 4. Characterization of antibodies to protein NO66. (A) Immunoprecipitation of the *X. laevis* protein NO66 with mAb No-5-1-1. Proteins of the following fractions were separated by SDS-PAGE and stained with Coomassie blue: XLKE-A6 cell extract before immunoprecipitation (lane 1), supernatant after incubation of cell extract with protein G-Sepharose in the absence of antibody (lane 2), supernatant after incubation of cell extract with protein G-Sepharose in the presence of mAb No-5-1-1 (lane 3), extract nonspecifically bound to protein G-Sepharose (lane 4), immunoprecipitate obtained from XLKE-A6 cell lysates (lane 5), and immunoprecipitate after incubation with lysis buffer, as a negative control (lane 6). The arrowhead (lane 5) points to the immunoprecipitated *Xenopus* NO66. (B) Corresponding immunoblot probed with the guinea pig antibodies NO66–2 to protein NO66 reacting specifically with the 66-kDa protein present in the cell extract (lanes 1 and 2) and in the immunoprecipitate (lane 5). Reference proteins (lanes R) are the same as in Figure 1.

4B, lane 5). From these results, we concluded that the hsNO66 cDNA clone coded for the human homologue of the *Xenopus* protein NO66, originally identified with the mAb No-5-1-1. We also verified that the NO66 antisera did not cross-react with the sequence-related 52 kDa polypeptide mentioned before (our unpublished results).

In immunoblots of total proteins isolated from different cultured human cells antibody NO66–2 reacted exclusively with a polypeptide of ~79 kDa (Figure 5, A and A′), in good agreement with the size of the product of in vitro translation of the hsNO66 cDNA (our unpublished results). Although the antigenic polypeptide was present in similar amounts in most cell lines analyzed, it was clearly underrepresented in the cells of the glioma line U333 (Figure 5A′, lane 2). Similar analyses of protein fractions

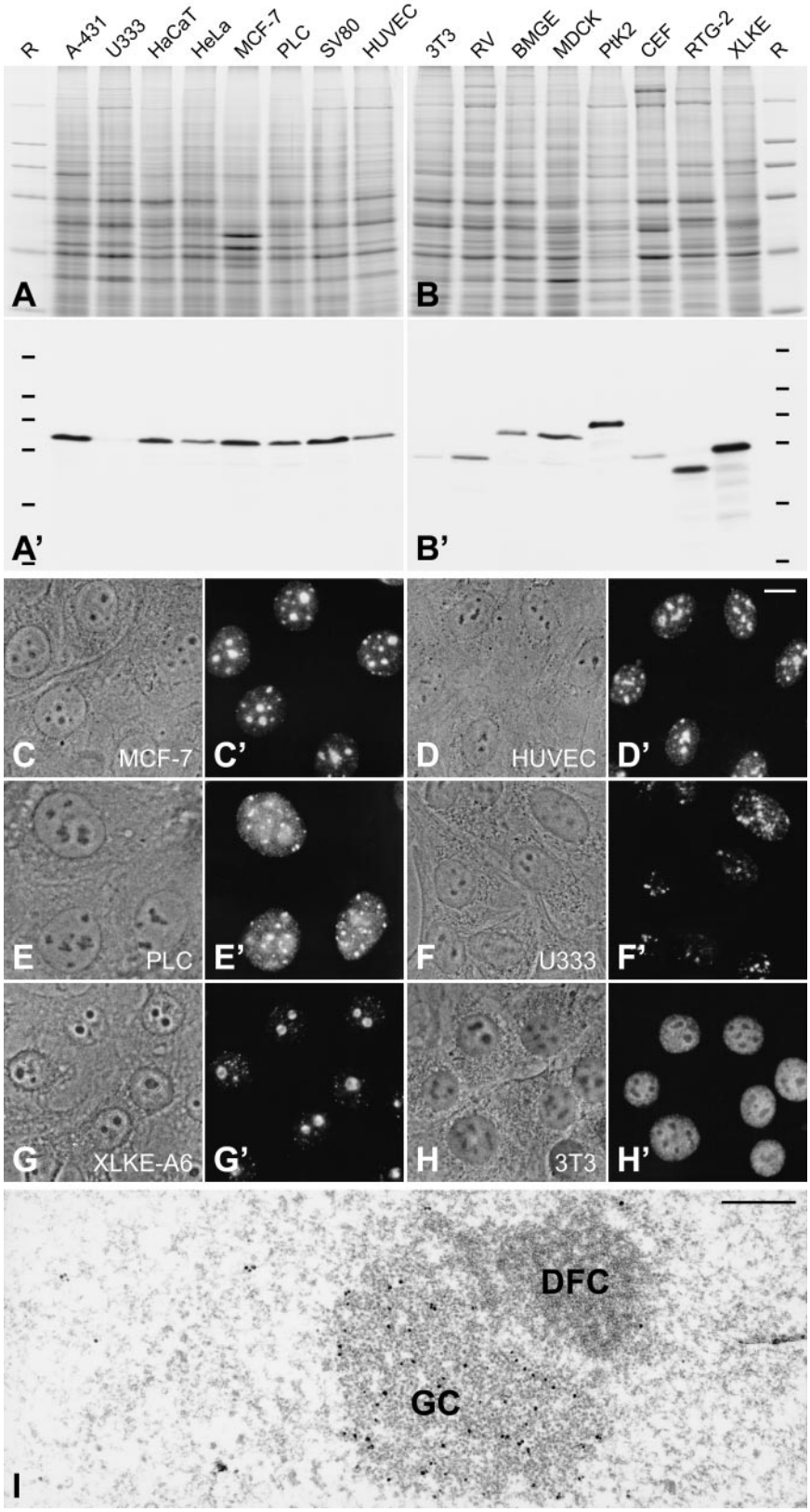

Figure 5. Identification and immunolocalization of protein NO66 and its homologues in different cultured cell lines from diverse species. (A) Coomassie blue–stained total cellular proteins of different human cell lines. Reference proteins (lane R) are the same as in Figure 1. (A′) Corresponding autoradiogram showing ECL detection of the antigenic polypeptide detected by antibody NO66-2. (B) Separation of total cellular proteins from cultured cells of different species by SDS-PAGE and Coomassie blue staining. (B′) Corresponding immunoblot, using antibody NO66-2, reveals the presence of homologous proteins in all the species analyzed. Note that only one polypeptide is recognized in A′ and B′, but that the M_r of the protein exhibits some variation in the diverse species. (C and C′–H and H′) The intracellular distribution of protein NO66 was analyzed by immunofluorescence microscopy in cultured cells from different species (as indicated). Shown here are examples of human (C–F), *Xenopus* (G), and murine (H) origin. Phase-contrast micrographs are shown in C–H, and the corresponding immunofluorescence micrographs are shown in C′–H′. Bar, 10 μm. (I) Immunoelectron microscopy of protein NO66 within the nucleolus of XLKE-A6 cells showing segregation of nucleolar components upon AMD-treatment. Antibody NO66-2 was detected by secondary antibodies coupled to nanogold particles. The granular component (GC) of the segregated nucleolus is specifically labeled, whereas the dense fibrillar component (DFC) is almost devoid of silver-enhanced gold particles. Bar, 0.5 μm.

derived from diverse species (mouse, rat, bovine, canine, rat kangaroo, chicken, trout, and *Xenopus*) disclosed the presence of the antigen with molecular weights ranging from ~55 kDa (trout) to ~80 kDa (potoroo), reflecting variations of the length of the amino-termini (Figure 5, B and B′). The broad cross-reaction observed again empha-

Table 1. Patterns of intracellular distribution of protein NO66 as determined by immunofluorescence microscopy

Pattern	Description	Cell lines
I	Strong nucleolar staining; nucleoplasmic foci, some of them associated with nucleoli	A-431, HaCaT, HeLa, HUVEC, MCF-7, SV80 (all human), MDCK (canine), CEF (chick), XLKE (*Xenopus*)
II	Weak nucleolar fluorescence; prominent nucleoplasmic foci, often associated with nucleoli	PLC (human), BMGE (bovine)
III	Diffuse labeling of the whole nucleus including the nucleoli	RV (rat), PtK2 (potoroo), RTG-2 (trout)
IV	Rather diffuse labeling of the nucleoplasm excluding the nucleoli	3T3 (murine)
V	Nucleoplasmic dots, no nucleolar staining	U333 (human)

sized that protein NO66 has been highly conserved during evolution.

Intracellular Distribution of Protein NO66

We next examined the intracellular localization of native protein NO66 by immunofluorescence microscopy on the same monolayer cell cultures, which were all analyzed at approximately the same cell density (80% confluence). Surprisingly, significant differences in the intracellular staining pattern could be observed that conformed to one of five patterns of distribution as summarized in Table 1. In most cell lines studied, NO66 antibodies brightly stained the nucleoli, the nucleoplasm in a finely dispersed form and, additionally, some distinct nuclear "dots," some of which were often seen in association with nucleoli (pattern I in Table 1). A weak nucleolar fluorescence, but prominent nucleoplasmic dots were observed in other cells such as those of lines PLC and BMGE. Poorly stained nucleoli and diffuse labeling of the nuclear interior were seen in other cell types (RV, PtK2, and RTG-2). The next class of intracellular distribution pattern was defined by a rather diffuse nucleoplasmic staining that, however, excluded the nucleoli (3T3), and the glioma line U333 showed strongly labeled nuclear dots, but again no nucleolar fluorescence (Figure 5, C and C′–H and H′; and our unpublished results).

Because the majority of cells showed a strong nucleolar accumulation of protein NO66, we investigated the intranucleolar distribution of the protein in greater detail by performing immunoelectron microscopy on AMD-treated XLKE-A6 cells. Under these conditions the nucleolar subcompartments became separated from each other, thereby facilitating intranucleolar localization. As shown in Figure 5I, protein NO66 was significantly enriched in the granular component, whereas the dense fibrillar component was practically devoid of gold particles. The same intranucleolar distribution was observed in untreated cells (our unpublished results), indicating that protein NO66 may have a role in later stages of the processing of preribosomal particles, at least in some cell types.

Many nuclear complexes disperse in mitotic cells, but some remain in association with the segregating chromosomes. Interestingly, the intracellular distribution of protein NO66 changes drastically during mitosis. During meta- and early anaphase the protein is diffusely distributed over the perichromosomal cytoplasm, whereas during late anaphase a significant fluorescence is seen on the surfaces of the chromosomes. Later in telophase, the protein appears to be enriched in prenucleolar bodies (our unpublished results). This pattern of distribution during mitosis is similar, but not identical to the mitotic behavior described for other nucleolar proteins such as NO38, NO29, and NOH61, which associate with the chromosomes already during metaphase (Schmidt-Zachmann *et al.*, 1987; Zirwes *et al.*, 1997b, 2000).

Notably, the immunolocalization pattern of protein NO66 was unaffected after incubation the cells with DNase but was drastically altered by treatment with RNase, resulting in a complete release from the nucleoli as well as from the nucleoplasmic structures (our unpublished results). On treatment of cells with AMD protein NO66 was to some extent released from the nucleoli and translocated to the nucleoplasm. These experiments indicated that protein NO66 binds to RNA and that its nucleolar accumulation depends on ongoing transcription.

Biochemical Characterization and Immunoprecipitation Experiments

Our transfection experiments (Figure 3, E and E′) and immunolocalization studies (Figure 5) had demonstrated a mostly nucleolar localization of protein NO66. To examine this on the biochemical level, we analyzed the distribution of protein NO66 in cellular fractions from cultured human cells by gel filtration and sucrose gradient centrifugation.

On cell extractions with different detergent and salt concentrations, the protein was largely solubilized in buffers containing 250 mM NaCl and 0.1% Triton X-100 (our unpublished results). On gel filtration the solubilized protein appeared with an apparent molecular mass (M_{app}) of 600,000 (Figure 6, A and A′) and was identified in a complex with a sedimentation coefficient of ~12S (Figure 6, B and B′) when analyzed by sucrose gradient centrifugation. According to Siegel and Monty (1966), this allowed to estimate a molecular mass of ~350,000 Da for the solubilized form of protein NO66. RNase treatment of the cellular lysates before centrifugation led to a significant degradation of the protein as indicated by the much weaker signal obtained by immunoblotting. The remaining protein seemed to form large aggregates, which sedimented at the bottom of the gradient (Figure 6C). These results suggested that native protein NO66 is a constituent of a larger particle containing an RNA component of so far unknown identity.

On biochemical fractionation of MCF-7 cells protein NO66 was absent from the cytoplasmic fraction but significantly enriched in the nuclear and nucleolar fraction (Figure 6, D and D′). The fractionation procedure was verified by immunoblot analyses with antibodies B2.4-1 directed against the nuclear protein SF3b^{155} (Figure 6D″) as well as with antitubulin, anti-NOH61, and antilamin B_2 (our unpublished results).

The distribution of protein NO66 in different fractions from *Xenopus* oocyte nuclei, egg extracts, and somatic cells was analyzed by immunoblotting with NO66-specific antibodies. Here, the protein was detected in total nuclei as well

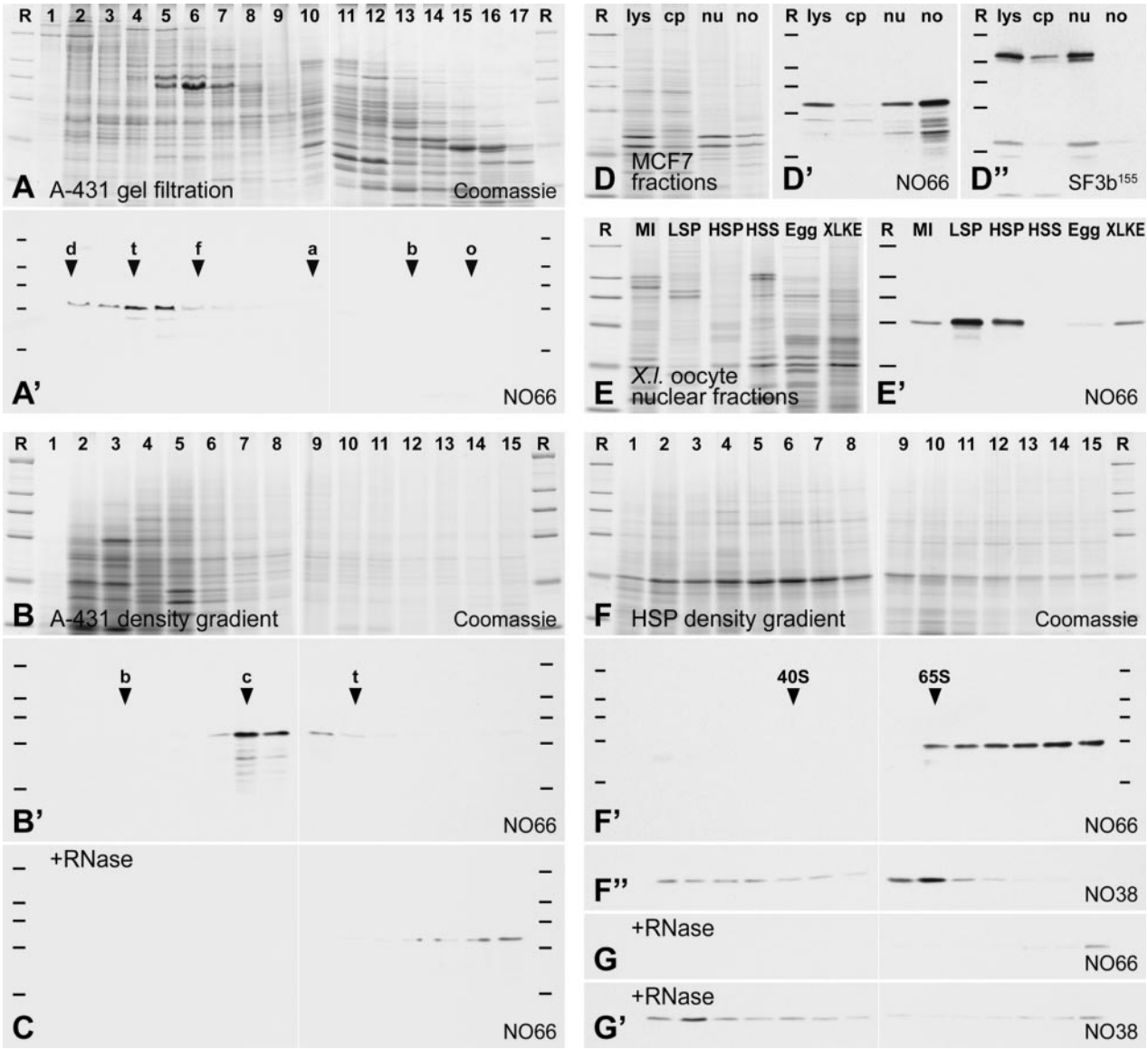

Figure 6. Identification of protein NO66 in cellular fractions from *Xenopus laevis* and human cell cultures and biochemical analysis by sucrose gradient centrifugation and gel filtration. (A) Proteins of cell extracts from A-431 cells were fractionated by gel filtration, separated by SDS-PAGE, and stained with Coomassie blue. (A′) Corresponding immunoblot probed with the antiserum NO66-2. Peak positions of reference macromolecules are indicated by arrowheads: dextran blue (d; M_{app} 2000,000; fraction 2), thyroglobulin (t; M_{app} 669,000; fraction 4), ferritin (f; M_{app} 440,000; fraction 6), aldolase (a; M_{app} 158,000; fraction 10), bovine serum albumin (b; M_{app} 66,000; fraction 13), and ovalbumin (o; M_{app} 43,000; fraction 15). Protein NO66 is mainly detected in fractions 4 and 5, corresponding to a mean M_{app} 600,000. (B) Cell lysates of human A-431 cells were fractionated after sucrose density centrifugation. The resulting fractions (lanes 1–15 from top to bottom of the gradient) were separated by SDS-PAGE and subjected to Coomassie blue staining. (B′) Corresponding immunoblot analysis using the NO66–2 antiserum. Arrowheads indicate the peak positions of the reference proteins fractionated in parallel: bovine serum albumin (b; 4.3 S, fraction 3), catalase (c; 11.3 S, fraction 7), and thyroglobulin (t; 16.5 S, fraction 10). Protein NO66 is recovered in fractions 7 and 8, with a sedimentation coefficient of ~12S. (C) Same experiment as presented in B and B′, but after treatment of the cell lysates with RNase A. Under these conditions, the antigenic polypeptide was only weakly detectable by the NO66-specific antibodies and sedimented to the bottom of the gradient (fractions 13–15). (D) Coomassie blue staining of various fractions from MCF-7 cells separated by SDS-PAGE (lys, total cell lysate; cp, cytoplasmic fraction; nu, fraction enriched in nuclei; no, fraction enriched in nucleoli). (D′) Corresponding immunoblot using antibody NO66-2, which reacts specifically with a ~79 kDa protein (hsNO66) highly enriched in the nuclear and nucleolar fractions. (D″) To ascertain the fractionation procedure, a parallel immunoblot was probed with antibody B2.4-1 directed against the 146-kDa splicing factor $SF3b^{155}$. (E and E′) Total proteins of mass isolated oocyte nuclei (MI), of the LSP, HSP, and HSS fractions from oocyte nuclei, of a *Xenopus* egg extract (Egg) and from total *Xenopus* XLKE-A6 cells (XLKE) were separated by SDS-PAGE, stained with Coomassie blue (E) and analyzed by immunoblotting using antibody NO66–2 (E′). (F–F″) Fractionation of HSP fractions from *Xenopus* oocyte nuclei by sucrose gradient centrifugation. The resulting protein fractions were analyzed by SDS-PAGE and Coomassie blue staining (F) and further examined by immunoblotting using antibody NO66–2 (F′) or mAb No-185 against the nucleolar protein NO38 (F″). The peak fractions (indicated by arrowheads) of the preribosomal precursor molecules of 40S (fraction 6) and 65S (fraction 10) were determined by their extinction at 260 nm (marked in F′). Although protein NO38 was associated with both the 40S and the 65S preribosomal particles, protein NO66 was detected in fractions 10–15, suggesting an association with the 65S preribosomes and larger particles. (G and G′) RNase treatment of the HSP fractions before centrifugation resulted in the expected shift of the protein NO38 position to lower density fractions, where it peaked in fraction 3 (G′). By contrast, only trace amounts of protein NO66, sedimenting to the bottom of the gradient, were detected (G). Reference proteins (lanes R) are the same as in Figure 1.

as in the LSP and HSP fraction, indicating that NO66 was a nuclear protein bound to relatively large structures. By contrast, the protein was not detected in the HSS fraction and present only in trace amounts in egg extracts (Figure 6, E and E′). Protein NO66 was also highly enriched in the fraction containing amplified nucleoli obtained by particle sorting (our unpublished results). As the NO66-containing HSP fraction was enriched in preribosomes (Hügle *et al.,* 1985), we fractionated it further on sucrose gradients and analyzed the resulting fractions by SDS-PAGE and immunoblotting (Figure 6F). Protein NO66 was specifically enriched in fractions containing the precursors for the large ribosomal subunit and even larger particles, probably representing intermediates of the 90 S preribosomal particle (Figure 6F′). By contrast, the major nucleolar protein NO38 was specifically enriched in 40S and 65S particles representing precursors for the small and large ribosomal subunits (Figure 6F″; cf. Schmidt-Zachmann *et al.,* 1987). Again, RNase treatment led to an almost complete degradation of protein NO66 with the remaining protein sedimenting at the bottom of the gradient (Figure 6G), whereas protein NO38 was released from the ribonucleoprotein particles and shifted to the top of the gradient (Figure 6G′).

In conclusion, the biochemical characterization of NO66 indicates that this protein represents a factor associated with preribosomal complexes and may accompany preribosomes through several stages of maturation.

A number of nucleolar proteins have been implicated directly or indirectly in pre-rRNA packaging and metabolism in higher eukaryotic cells such as protein NO38/B23, the box C/D snoRNA-associated protein fibrillarin, protein Nopp140 (xNopp180 in *Xenopus*) transiently interacting with both major classes of snoRNPs, box H/ACA and box C/D, the nucleolar helicase NOH61, and the very acidic protein NO29 (reviewed by Olson *et al.,* 2002). Therefore, we determined whether these proteins are among those that copurify with protein NO66 by immunoblot analysis (Figure 7). Indeed, nucleolin (Figure 7A′) and NO38/B23 (Figure 7A″) were detected in the complexes isolated with mAb No-5-1-1. An interaction between protein NO66 and nucleolin as well as protein NO38 was confirmed in reciprocal immunoprecipitations using nucleolin- and NO38-specific antibodies, respectively (Figure 7, B and C). In both cases, protein NO66 was found exclusively in the fractions containing the purified immunocomplexes (Figure 7, B′ and C′). In contrast, none of the other aforementioned nucleolar proteins (xNopp180, NO29, NOH61, fibrillarin) was detectable in complexes with protein NO66 (our unpublished results). Therefore, we concluded that only a subset of nucleolar proteins are found in association with protein NO66, which is in agreement with recent reports of a large number of preribosomal particles of different molecular composition (reviewed by Tschochner and Hurt, 2003).

Characterization of the Protein NO66-Containing Complexes by Colocalization with Nuclear Marker Proteins

As mentioned earlier, protein NO66 displayed cell type-dependent differences of intranuclear localization (Table 1), raising the question whether its location is unique or overlaps with other nuclear entities (for recent reviews of distinct nuclear domains see Lamond and Earnshaw, 1998; Misteli, 2000). By confocal laser scanning microscopy, we directly compared the localization of protein NO66 with that of well-characterized marker proteins of different kinds of "nuclear bodies," in particular the locations of nucleoli (Figure 8, A–A‴), Cajal bodies (Figure 8, B–B‴), gems (Figure 8, C–C‴),

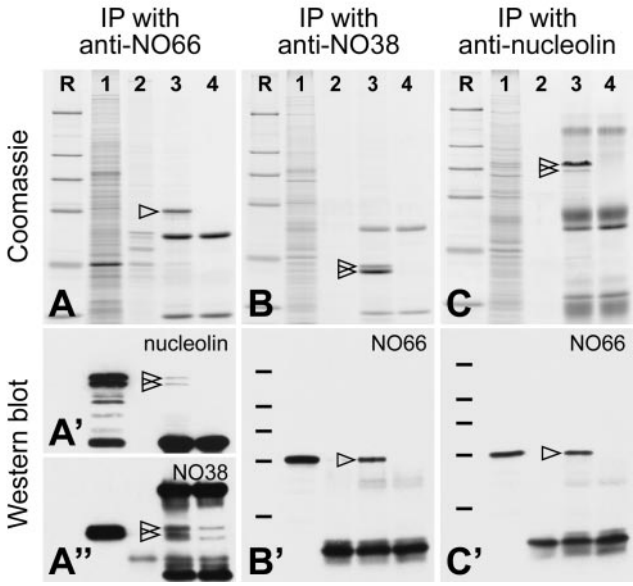

Figure 7. Identification of constitutive nucleolar proteins associated with protein NO66 in immunoprecipitation experiments. (A–A″) Immunoprecipitation of proteins from XLKE-A6 cell extract with mAb No-5-1-1 directed against NO66. The analyzed fractions are the same as shown in Figure 2B. The arrowhead (A) points to immunoprecipitated protein NO66 seen after Commassie blue staining. Corresponding immunoblot analysis with mAb P7-1A4 against nucleolin (A′) and mAb No-185 against protein NO38 (A″) shows that the 90- and 95-kDa homologues of nucleolin (arrowheads in A′) and the two isoforms of NO38 (arrowheads in A″) were coimmunoprecipitated by mAb No-5-1-1. Strong signals in the range of 25 and 50 kDa are due to binding of the secondary antibody used for immunodetection to the light and heavy chain of the NO66-specific antibody. (B and B′) In a reciprocal experiment, immunoprecipitation from extracts of XLKE-A6 cells was performed with mAb No-185. (B) SDS-PAGE-separated immunoprecipitation fractions after staining with Coomassie blue. Arrowheads mark the immunoprecipitated protein NO38 variants. (B′) Coimmunoprecipitated protein NO66 (arrowhead) was detected by immunoblotting with antiserum NO66-2. (C and C′) When immunoprecipitation from XLKE-A6 cell extracts was performed with mAb P7–1A4, which precipitated both forms of nucleolin (arrowheads in Coomassie blue-stained gel in C), significant amounts of protein NO66 were detected with NO66-2 antibodies (arrowhead in C′).

PML bodies (Figure 8, D–D‴), nuclear speckles (Figure 8, E–E‴), paraspeckles (Figure 8, F–F‴), or the perinucleolar compartment (PNC; Figure 8, G–G‴). Clearly, protein NO66 did not colocalizes with NOH61 in dots outside nucleoli. In addition, subnuclear structures containing coilin, SMN, PML, SF3b^{155,} PSP1, and PTB protein all showed no significant codistribution with protein NO66. Moreover, the NO66-positive nuclear dots were clearly different from Sam68 domains as well as from transcription sites (our unpublished results). In summary, these results demonstrated that protein NO66 can occur besides nucleoli in unique nucleoplasmic locations distinct from the major subnuclear domains involved in RNA processing and transcriptional regulation.

Because of similarities in the intranuclear distribution between NO66-positive nuclear dots and another type of nuclear bodies, the replication foci, we microinjected Cy3-dCTP into the nuclei of MCF-7 cells. After costaining with NO66-antibodies we observed a significant overlap between the protein NO66-containing structures and the sites of Cy3-

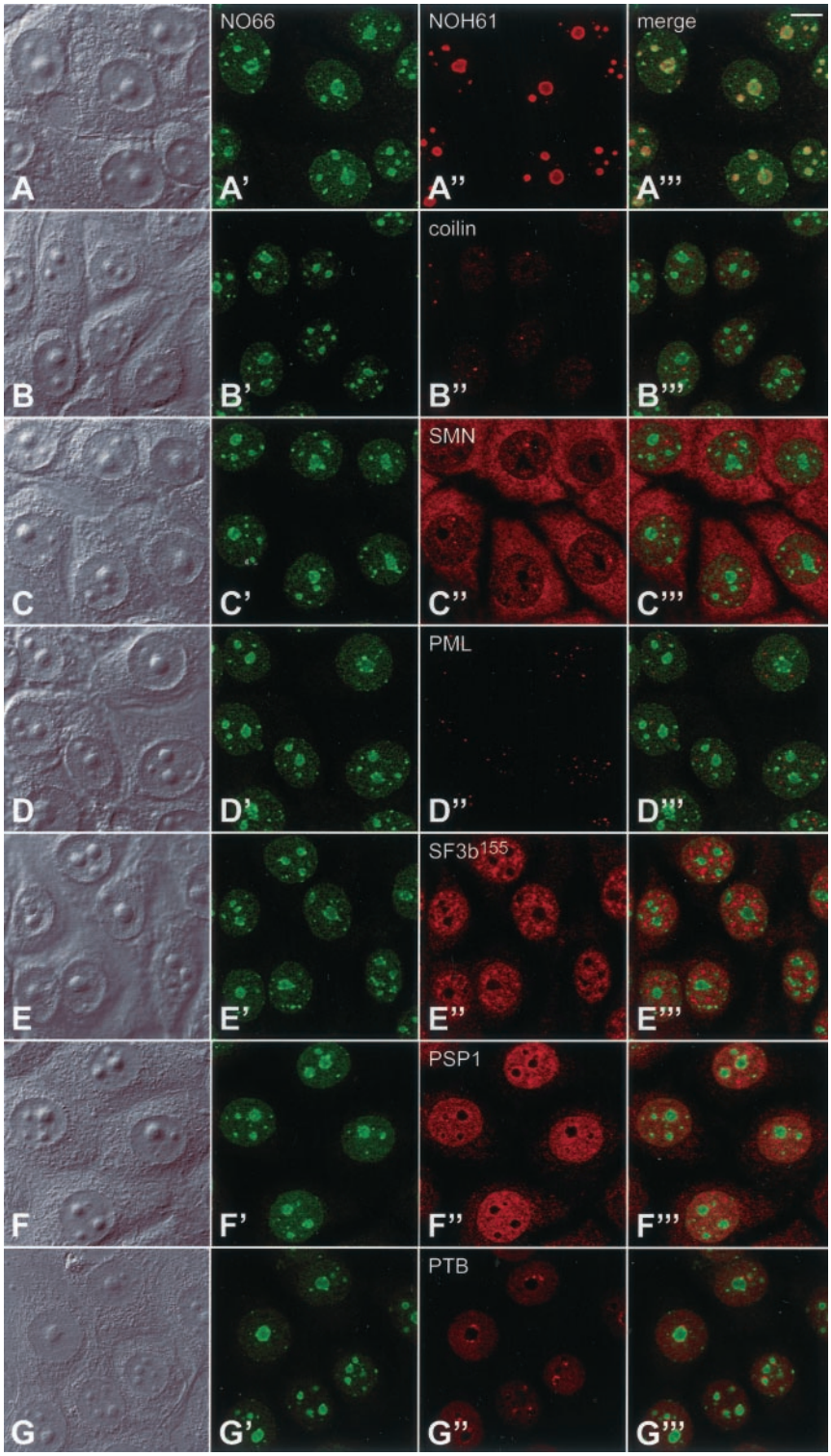

Figure 8. Double label immunolocalization studies of protein NO66 in comparison with various marker proteins characteristic for distinct nuclear subcompartments. Micrographs were taken with a confocal laser scanning microscope and single optical sections are shown. (A–G) Differential interference contrast (DIC) micrographs. (A′-G′) Immunolocalization of protein NO66 with antiserum NO66-2. (A″–G″) Immunolocalization of constituents of different nuclear domains: nucleolar RNA helicase NOH61 (A″), p80-coilin as a marker for Cajal bodies (B″), survival of motor neuron (SMN) protein present in the cytoplasm and in nucleoplasmic SMN bodies, also termed gems (C″), promyelotic leukemia (PML) protein as a marker for PML bodies (D″), splicing factor $SF3b^{155}$ accumulating in nuclear speckles (E″), protein PSP1 defining paraspeckles (F″), and PTB protein decorating the perinucleolar compartment (PNC; G″). (A‴–G‴) Corresponding overlay images. Note that the extranucleolar structures stained by the antibodies to protein NO66 do not show significant colocalization with any of the other defined nuclear domains. Bars, 10 μm.

dCTP incorporation (Figure 9, A–A‴). This positive correlation was further confirmed by colocalizing protein NO66 with the proliferating cell nuclear antigen (PCNA; Figure 9, B–B‴), a processivity factor of DNA polymerase δ (reviewed by Wyman and Botchan, 1995; Hingorani and O'Donell, 2000). In contrast to PCNA, which was removed from non–S-phase cells treated with Triton X-100 (Sasaki *et al.*, 1994; Lohr *et al.*, 1995), protein NO66 remained in non–S-phase cells and showed the same intranuclear distribution as in S-phase cells (Figure 9, C–C‴). The nuclear sites that had incorporated Cy3-dCTP and were positive for PCNA were rather large and often were located in the nuclear periphery or in association with nucleoli. This pattern closely resembled replication foci described for cells in mid-to-late S-phase, i.e., when switching from the replication of euchromatin to that of heterochromatin (Leonhardt *et al.*, 2000).

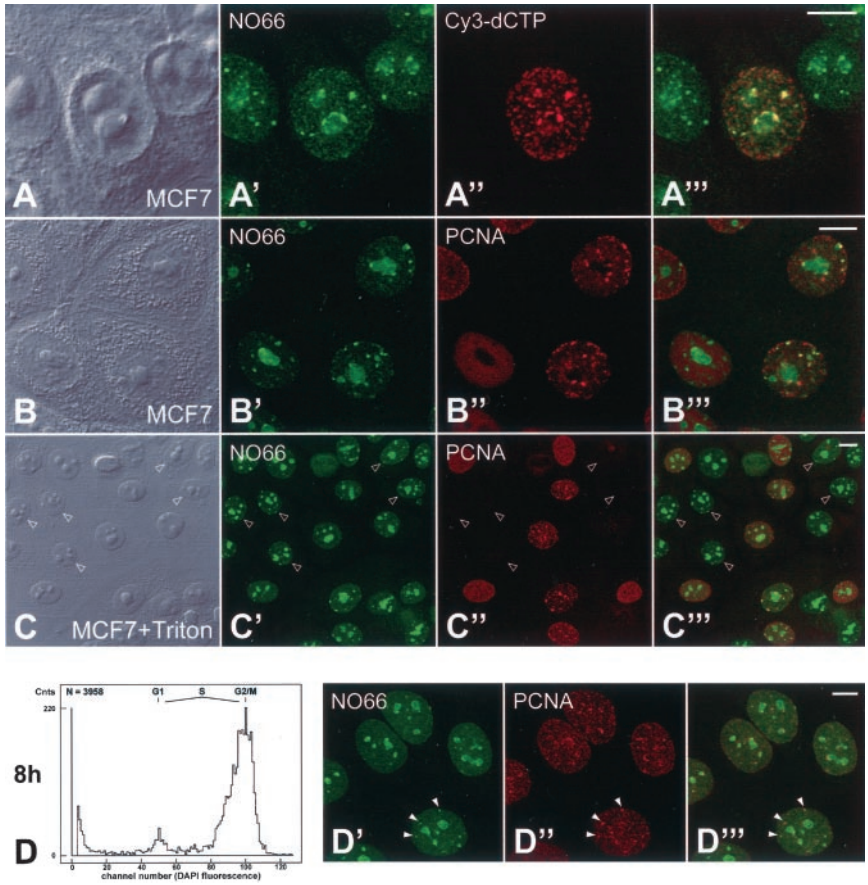

Figure 9. Relationship of nuclear bodies containing protein NO66 to replication foci. (A) Differential interference contrast (DIC) micrograph of MCF-7 cells microinjected with Cy3-dCTP. (A′) Immunolocalization of protein NO66 with antiserum NO66-2. (A″) Fluorescence of microinjected Cy3-dCTP, which was subsequently incorporated into newly synthesized DNA during replication. (A‴) Corresponding merged image. (B and C) DIC micrographs of MCF-7 cells. (B′ and C′) Immunolocalization of protein NO66 with antiserum NO66-2. (B″ and C″) Immunolocalization of PCNA using mAb PC10. (B‴ and C‴) Corresponding merged images. The cells presented in C and C‴ had been treated with Triton X-100 before fixation to remove PCNA from non–S-phase cells (indicated by arrowheads). (D) Flow cytometric analysis of the relative DNA content of synchronized HeLa cells during late S-phase, 8 h after release from a double-thymidine block. The DNA content according to the DAPI fluorescence intensity of the cells corresponds to G1-, S-, or G2/M-phase as indicated. (D′–D‴) Immunofluorescence analysis of the same HeLa cell population with antiserum NO66-2 against protein hsNO66 (D′) and with mAb PC10 against PCNA (D″). The corresponding merged image is given in D‴. NO66 and PCNA colocalize in replication foci during late S-phase (arrowheads) The micrographs in A–D‴ have been obtained with a confocal laser scanning microscope and show single optical sections, with exception of C′–C‴ where maximum intensity projections of multiple optical sections are shown. Bars, 10 μm.

Because PCNA-containing DNA replication foci undergo characteristic changes as replication progresses, we examined the temporal and spatial relationship between NO66 and PCNA foci during S-phase progression in synchronized cells. No colocalization could be observed at the G1/S border (time point 0 h) or in early to mid–S-phase (time points 2–6 h after release). In contrast, 8 h after the cells had been released, i.e., in late S-phase, the two proteins were substantially colocalized (Figure 9, D–D‴; for the complete analysis see Supplemental Figures, Figure 1s). Notably, the intracellular concentration of protein NO66 was not altered during the cell cycle stages analyzed, which argued against a direct functional role of the protein in cell cycle regulatory processes (Supplemental Figures, Figure 2s). Thus, colocalization of NO66 and replication foci were demonstrated exclusively in late S-phase cells, suggesting that this protein marks distinct heterochromatic regions.

Another nucleolar protein known to also occur in nuclear dots representing specific heterochromatin domains is the proliferation marker protein Ki-67 (Bridger *et al.*, 1998). In double localization studies using the appropriate antibodies, we have indeed observed a striking, although not complete colocalization of these two proteins (Figure 10, A–A‴). Well-known marker proteins for distinct heterochromatic regions are the proteins CENP-B and HP1α. whereas CENP-B is distributed throughout the centromeric heterochromatin by binding to a distinct DNA sequence located in the repeats of α-satellite DNA (Cooke *et al.*, 1990), the HP1α protein is a constituent of pericentromeric heterochromatin (reviewed by Eissenberg and Elgin, 2000). Comparison of the intranuclear distribution of NO66 with that of CENP-B has not disclosed a significant overlap of the respective foci, although NO66- and CENP-B–positive subnuclear structures are often found in close proximity (Figure 10, B–B‴). In contrast, when NO66 has been compared with HP1α, striking colocalization has been observed in all cell lines examined, which, however, has been incomplete because the number of HP1α foci is higher than the number of NO66 foci. Remarkably, almost every NO66-positive nucleoplasmic structure also presents local accumulation of HP1α (Figure 10, C–E).

In summary, protein NO66 displays a characteristic dual location. Besides its nucleolar accumulation, it is also enriched in nuclear foci that most probably correspond to a certain type of heterochromatin, which is replicated during late stages of S-phase.

DISCUSSION

Starting with an immunological approach to isolate and purify amplified nucleoli of *X. laevis* oocytes, we have identified and characterized a novel, ubiquitous nucleolar protein termed NO66 (nucleolar protein of ~66 kDa) with a hitherto unknown localization. Protein NO66 is present in various cell types of diverse species from amphibian to human, where it appears with different molecular weights due to variations in length of the amino-terminal region. Both, the high evolutionary conservation in aa sequence between the human protein and putative counterparts in *C.*

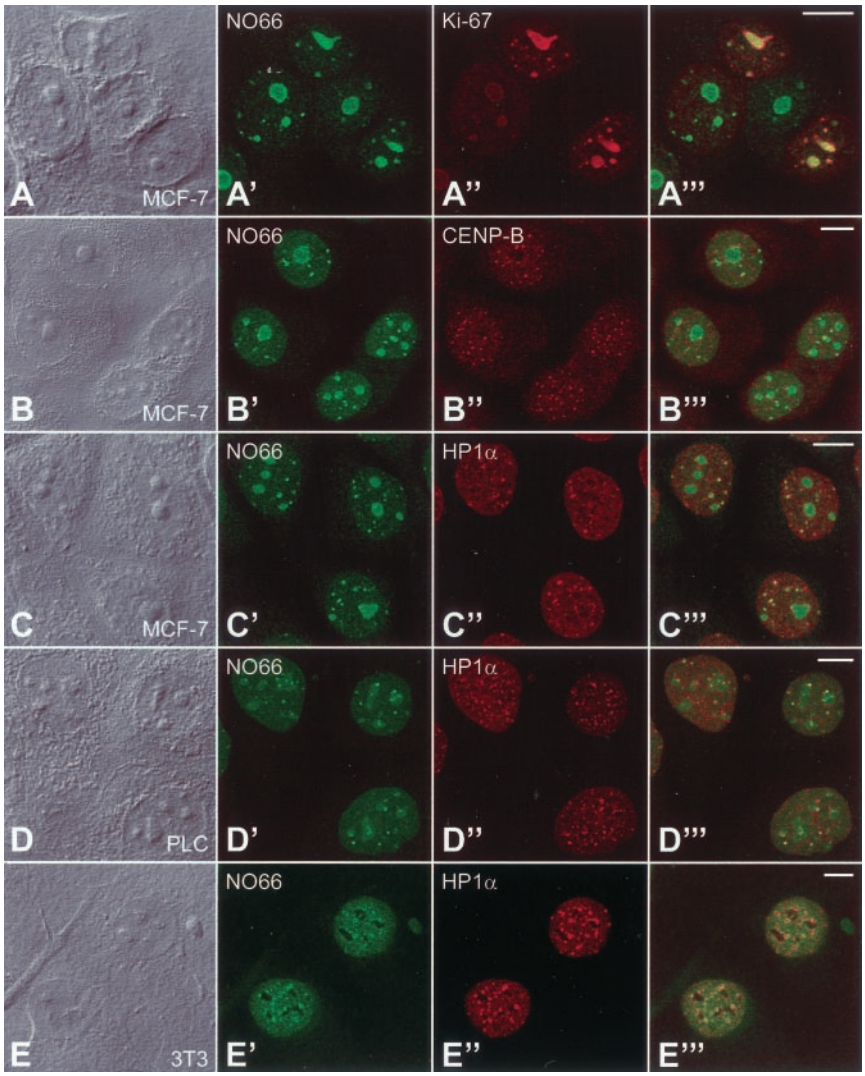

Figure 10. Double immunolocalization of protein NO66 in comparison with proteins Ki-67, CENP-B, and HP1α. Micrographs were taken with a confocal laser scanning microscope and show single optical sections. (A–E) Differential interference contrast (DIC) micrographs of cells of lines MCF-7, PLC and 3T3 as indicated. (A′–E′) Immunolocalization of protein NO66 with antiserum NO66-2. (A″) Immunolocalization of the proliferation marker protein Ki-67 with mAb MB67, (B″) of centromeric protein CENP-B with antiserum anti-CENP-B and (C″–E″) of heterochromatin protein HP1α with mAb 2HP 1H5. (A‴–E‴) Corresponding merged immunofluorescence images. Bars, 10 μm.

elegans and *X. tropicalis* as well as the wide cross-reactivity of NO66 antibodies suggest that this protein serves a fundamental cellular process. Remarkably, however, no homologue is found in yeast, and the protein is not included in the recently published lists of nucleolar proteins identified in the course of proteomic analyses of human nucleoli (Andersen *et al.*, 2002; Scherl *et al.*, 2002).

The primary sequence of protein NO66 did not disclose obvious functional roles, and no well-characterized motifs were detectable. However, NO66 contains a region showing striking similarity to the COG2850 (Cluster of Orthologous Groups of proteins; http://www.ncbi.nlm.nih.gov/BLAST) consensus sequence that defines a group of 49 otherwise uncharacterized conserved proteins. In addition to NO66 this group comprises several predictable bacterial proteins, hence underscoring the high evolutionary conservation of protein NO66. Interestingly, all these proteins also exhibit a JmjC-domain and are candidates for enzymes involved in chromatin remodeling, thereby regulating gene expression (Clissold and Ponting, 2001; Ayoub *et al.*, 2003).

The intranuclear topology of protein NO66 is as characteristic as perplexing and can differ in different kinds of cells. Typically, it occurs in more than one structural entity and thus can be counted among the "dual location–dual functional candidate" proteins (for review see Smalheiser, 1996). In light and electron microscopic immunolocalization studies five main patterns of intranuclear distribution have been distinguished, depending on the specific cell type (Table 1). However, in the majority of cell types studied, protein NO66 is strongly enriched in nucleoli but additionally occurs in distinct nucleoplasmic foci, often located in close proximity to the nucleoli. Such different distribution patterns may reflect either regulations by intracellular signals, including conformational changes induced by posttranslational modifications or differences in the expression level. Surprisingly, transient transfection experiments performed in various cell lines (MCF-7, PLC, 3T3) disclose the almost exclusive nucleolar localization of ectopically expressed protein NO66 without affecting the nucleoplasmic location of the endogenous protein. Moreover, photobleaching experiments have demonstrated that many nuclear proteins move constantly in and out of a given nuclear substructure such as the nucleolus (Chen and Huang, 2001), with transient accumulations in other structural entities (Leung and Lamond, 2003). Thus, protein NO66 might shuttle between the nucleolus and the distinct nuclear dots, and this equilibrium may be differently regulated in the various cell types.

Our biochemical data also justify to consider protein NO66 as a constitutive nucleolar component: The protein is detectable in purified extrachromosomal nucleoli of *Xenopus* oocytes as well as in chromosomal nucleoli of somatic cells. In addition, our finding that it is associated with large preribosomal particles (>60S) and coimmunoprecipitates with two nucleolar hallmark proteins, NO38 and nucleolin, suggests that NO66 plays a role in the assembly and processing of ribosomal subunits, notably of early 65S–90S preribosomal complexes. Such a role would also be in agreement with its localization to the granular component as the site of maturation of preribosomal particles and would also be compatible with the demonstrated RNase effects shown. Here, the unexpected observation that protein NO66, in contrast to other nucleolar proteins known to be associated with preribosomes, does not dissociate under the influence of RNase action but rather forms aggregates reminds one of earlier studies reviewed by Pederson (2000), showing that nuclear RNA-bound proteins can rearrange to large particles when dislodged from their usual RNA associations.

In most cell culture lines examined, protein NO66 did not exclusively localize to nucleoli but also accumulated in certain extranucleolar, nucleoplasmic bodies. To identify the nature of these intranuclear structures, a number of colocalization studies have been performed using antibodies against appropriate marker proteins for well-established nuclear entities such as Cajal bodies, speckles, PML bodies, paraspeckles, and the perinucleolar compartment. However, none of these proteins shows colocalization with protein NO66. On the other hand, the nucleoplasmic staining pattern of NO66, although persistent over the entire interphase, is somewhat reminiscent of DNA replication foci. It is well known that the pattern of DNA replication sites within the nucleus changes in many cultured cells from a finely punctate distribution in early S-phase cells, followed by the accumulations of larger granules at the nucleolar periphery and the nuclear envelope in mid-S-phase, to finally, the appearance of large foci in late S-phase (Leonhardt *et al.*, 2000). Moreover, no fundamental differences in the distribution of DNA replication sites and the spatiotemporal sequence of replication patterns have been found in normal, immortalized and transformed cells (Dimitrova and Berezny, 2002). Indeed, we have detected a significant overlap between the NO66-positive dots and the replication sites labeled by incorporated Cy3-dCTP and PCNA-staining, which is restricted to late S-phase cells. The enrichment of protein NO66 in extranucleolar foci persists throughout the interphase, whereas PCNA shows a diffuse nucleoplasmic distribution outside the S-phase. Moreover, in contrast to PCNA, which is extractable from nuclei of non–S-phase cells under certain conditions (Sasaki *et al.*, 1994; Lohr *et al.*, 1995), the staining pattern of NO66 essentially remains unchanged in all interphase cells. Because we have also noted DNA replication foci devoid of NO66, we conclude that protein NO66 is not a part of the DNA synthesizing machinery like PCNA, but might rather be involved in other heterochromatin-specific events. The latter assumption is strengthened by our observation that NO66 colocalizes with HP1α, but not with the centromeric protein CENP-B. In future experiments we will identify the distinct type of heterochromatin marked by protein NO66 during interphase. Recent work in yeast has uncovered for rDNA-associated heterochromatin a role in the maintenance of nucleolar structure (reviewed by Carmo-Fonseca *et al.*, 2000). On the other hand, the occurrence of inactive NORs surrounding the nucleolus has been described for mammalian cells (Wachtler *et al.*, 1986; Weipoltshammer *et al.*, 1996; Sullivan *et al.*, 2001). Our first attempt to characterize the NO66 foci at the DNA level by immuno-FISH using a 18S rDNA probe has not revealed evidence for their colocalization with extranucleolar rDNA repeats (Supplemental Figures, Figure 3s).

The most prominent nucleolar protein for which, in addition, accumulation in dot-like structures corresponding to specific heterochromatic regions has been reported, is the proliferation marker Ki-67 (for recent reviews see Endl and Gerdes, 2000; Brown and Gatter, 2002). However, the function of protein Ki-67 is still not clear. Our double-label studies comparing proteins NO66 and Ki-67 disclosed, in a subset of cells, probably in G1, a significant, although not complete colocalization (Figure 10, A–A‴). Like Ki-67, NO66 accumulates in regions outside the nucleoli, but in contrast to Ki-67 this characteristic pattern is not restricted to a specific cell cycle stage. Another remarkable similarity between the two proteins is their redistribution during mitosis. Protein NO66 associates with the surfaces of mitotic chromosomes, similarly to several other nucleolar proteins such as Ki-67, NO38, NO29, and NOH61 (Schmidt-Zachmann *et al.*, 1987; Verheijen *et al.*, 1989; Zirwes *et al.*, 1997b; 2000). However, although all these nucleolar proteins appear to be associated with the meta- and anaphase chromosomes, NO66 immunostaining accentuates the chromosomal surfaces only during anaphase (our unpublished results). At present, the mechanisms and functions of these chromatin associations are elusive.

Recently, a series of possible links of proteins functioning in the nucleolus and certain other chromosomal regions has emerged. The two DNA helicases of the RecQ family, BLM and WRN, are involved in recombination and deficiencies in these proteins cause Bloom's and Werner's syndrome, respectively, each characterized by genomic instability and predisposition to cancer (reviewed by van Brabant *et al.*, 2000; Nakayama, 2002). BLM transitorily colocalizes with replication protein A (RPA) as well as PML bodies and at times enters the nucleolus (Gray *et al.*, 1998; Sanz *et al.*, 2000; Yankiwski *et al.*, 2000). WRN also shows in many cell types a marked accumulation in nucleoli (Marciniak *et al.*, 1998; von Kobbe and Bohr, 2002) but in addition may be enriched in replication foci (Baynton *et al.*, 2003; Rodriguez-Lopez *et al.*, 2003). Their biological functions remain elusive, but a growing body of evidence points to a function in the resolution of stalled replication forks, notably in repeated sequence elements such as telomeres and rDNA. Moreover, one of the major nucleolar proteins, nucleolin, has recently been shown to form a complex with RPA, pointing to another connection between ribosome formation and DNA metabolism (Daniely and Borowiec, 2000).

Finally, the mammalian protein pescadillo and its yeast homolog Yph1p/Nop7p are mainly located in nucleoli where they seem to be involved in the assembly and export of the large ribosomal subunit (Adams *et al.*, 2002; Oeffinger *et al.*, 2002). Immunoprecipitation experiments, however, have revealed that Yph1p is a component of different complexes, which on the one hand contain 60S ribosomal proteins and a number of nonribosomal nucleolar proteins, but on the other hand also components of the origin recognition complex (ORC), the mini-chromosome maintenance (MCM) proteins and other cell cycle-regulatory proteins. Therefore, it has been proposed that Yph1p may serve a critical role in coordinating the processes of ribosome biosynthesis and DNA replication with cell proliferation (Du and Stillman, 2002). So far our coimmunoprecipitation experiments performed with mAb No-5-1-1 directed against protein NO66 using cellular extracts of *Xenopus* XLKE-A6 cells did not permit the detection of proteins involved in the replication

process such as PCNA, XOrc1, XOrc2, XCdc6, XMcm3, and XMcm7 in the isolated immunocomplexes (our unpublished results). However, a yeast two-hybrid screen using NO66 as a bait, resulted in the identification of the putative human homologue of the yeast protein Nsa1p (Nop7-associated protein 1) recently described by Harnpicharnchai *et al.* (2001). This result confirms the functional role of protein NO66 in ribosome biogenesis, in particular in the synthesis of the large ribosomal subunit, and might also underscore its relationship to replication-related events (Supplemental Figures, Figure 4s, and our unpublished results).

The novel nucleolar protein NO66 presented here is obviously a further member of the category of "dual location proteins," which can occur in the nucleolus as well as in distinct nucleoplasmic entities. Whether, however, they serve truly different or related functions in the two locations and whether protein NO66 might link ribosome biogenesis to the replication of distinct chromatin regions requires further investigation.

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REFERENCES

Adams, C.C., Jakovljevic, J., Roman, J., Harnipicharnchai, P., and Woolford, J.L., Jr. (2002). Saccharomyces cerevisiae nucleolar protein Nop7p is necessary for biogenesis of 60S ribosomal subunits. RNA *8,* 150–165.

Andersen, J.S., Lyon, C.E., Fox, A.H., Leung, A.K., Lam, Y.W., Stehen, H., Mann, M., and Lamond, A.I. (2002). Directed proteomic analysis of the human nucleolus. Curr. Biol. *12,* 1–11.

Ayoub, N., Noma, K., Isaac, S., Kahan, T., Grewal, S.I., and Cohen, A. (2003). A novel jmjC domain protein modulates heterochromatization in fission yeast. Mol. Cell. Biol. *23,* 4356–4370.

Azum-Gélade, M.C., Noaillac-Depeyre, J., Caizergues-Ferrer, M., and Gas, N. (1994). Cell cycle redistribution of U3 snRNA and fibrillarin. Presence in the cytoplasmic nucleolus remnant and in the prenucleolar bodies at telophase. J. Cell Sci. *107,* 463–475.

Baynton, K., Otterlei, M., Bjoras, M., von Kobbe, C., Bohr, V.A., and Seeberg, E. (2003). WRN interacts physically and functionally with the recombination mediator protein RAD52. J. Biol. Chem. *278,* 36476–36486.

Benavente, R., Krohne, G., and Franke, W.W. (1985). Cell type-specific expression of nuclear lamina proteins during development of *Xenopus laevis.* Cell *41,* 177–190.

Bohmann, K., Ferreira, J.A., and Lamond, A.I. (1995). Mutational analysis of p80 Coilin indicates a functional interaction between coiled bodies and the nucleolus. J. Cell Biol. *131,* 817–831.

Brandner, J.M., Reidenbach, S., Kuhn, C., and Franke, W.W. (1998). Identification and characterization of a novel kind of nuclear protein occurring free in the nucleoplasm and in ribonucleoprotein structures of the "speckle" type. Eur. J. Cell Biol. *75,* 295–308.

Bridger, J.M., Kill, I.R., and Lichter, P. (1998). Association of Ki-67 with satellite DNA of the human genome in early G1 cells. Chromosome Res. *6,* 13–24.

Brown, D.C., and Gatter, K.C. (2002). Ki67 protein: the immaculate deception? Histopathology *40,* 2–11.

Carmo-Fonseca, M. (2002). The contribution of nuclear compartmentalization to gene regulation. Cell *108,* 513–521.

Carmo-Fonseca, M., Mendes-Soares, L., and Campos, I. (2000). To be or not to be in the nucleolus. Nat. Cell Biol. *2,* E107–E112.

Charroux, B., Pellizzoni, L., Perkinson, R.A., Young, J., Shevchenko, A., Mann, M., and Dreyfuss, G. (2000). Gemin4. A novel component of the SMN complex that is found in both gems and nucleoli. J. Cell Biol. *148,* 1177–1186.

Chen, D., and Huang, S. (2001). Nucleolar components involved in ribosome biogenesis cycle between the nucleolus and nucleoplasm in interphase cells. J. Cell Biol. *153,* 169–176.

Chirgwin, J.M., Przybyla, A.E., MacDonald, R.J., and Rutter, W.J. (1979). Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry *18,* 5294–5299.

Clissold, P.M., and Ponting, C.P. (2001). JmjC: cupin metalloenzyme-like domains in jumonji, hairless and phospholipase $A_2\beta$. Trends Biochem. Sci. *26,* 7–9.

Cooke, C.A., Bernat, R.L., and Earnshaw, W.C. (1990). CENP-B: a major human centromere protein located beneath the kinetochore. J. Cell Biol. *110,* 1475–1488.

Cordes, V.C., Reidenbach, S., Köhler, A., Stuurman, N., van Driel. R., and Franke W.W. (1993). Intranuclear filaments containing a nuclear pore complex protein. J. Cell Biol. *123,* 1333–1344.

Cordes, V.C., Reidenbach, S., and Franke, W.W. (1996). Cytoplasmic annulate lamellae in cultured cells: composition, distribution, and mitotic behavior. Cell Tissue Res. *284,* 177–191.

Daniely, Y., and Borowiec, J.A. (2000). Formation of a complex between nucleolin and replication protein A after cell stress prevents initiation of DNA replication. J. Cell Biol. *149,* 799–810.

Dimitrova, D.S., and Berezney, R. (2002). The spatio-temporal organization of DNA replication sites is identical in primary, immortalized and transformed mammalian cells. J. Cell Sci. *115,* 4037–4051.

Dingwall, C., and Laskey, R.A. (1991). Nuclear targeting sequences-a consensus?. Trends Biochem. Sci. *16,* 478–481.

Du, Y.C., and Stillman, B. (2002). Yph1p, an ORC-interacting protein: potential links between cell proliferation control, DNA replication, and ribosome biogenesis. Cell *109,* 835–848.

Dumont, J.N. (1972). Oogenesis in Xenopus laevis. J. Morphol. *136,* 153–179.

Eissenberg, J.C., and Elgin, S.C.R. (2000). The HP1 protein family: getting a grip on chromatin. Curr. Opin. Genet. Dev. *10,* 204–210.

Endl, E., and Gerdes, J. (2000). The Ki-67 protein: fascinating forms and unknown function. Exp. Cell Res. *257,* 231–237.

Evan, G.I., Lewis, G.K., Ramsay, G., and Bishop, J.M. (1985). Isolation of monoclonal antibodies specific for human c-myc proto-oncogene product. Mol. Cell. Biol. *5,* 3610–3616.

Evans, J.P., and Kay, B.K. (1991). Biochemical fractionation of oocytes. Methods Cell Biol. *36,* 133–148.

Fox, A.H., Lam, Y.W., Leung, A.K., Lyon, C.E., Andersen, J., Mann, M., and Lamond, A.I. (2002). Paraspeckles: a novel nuclear domain. Curr. Biol. *12,* 13–25.

Franke, W.W., Schmid, E., Winter, S., Osborn, M., and Weber, K. (1979). Widespread occurrence of intermediate-sized filaments of the vimentin-type in cultured cells from diverse vertebrates. Exp. Cell Res. *123,* 25–46.

Franke, W.W., Kleinschmidt, J.A., Spring, H., Krohne, G., Grund, C., Trendelenburg, M.F., Stoehr, M., and Scheer, U. (1981). A nucleolar skeleton of protein filaments demonstrated in amplified nucleoli of *Xenopus laevis.* J. Cell Biol. *90,* 289–299.

Fujita, M., Ishimi, Y., Nakamura, H., Kiyono, T., and Tsurumi, T. (2002). Nuclear organization of DNA replication initiation proteins in mammalian cells. J. Biol. Chem. 2 *77,* 10354–10361.

Gerbi, S.A., Borovjagin, A.V., and Lange, T.S. (2003). The nucleolus: a site of ribonucleoprotein maturation. Curr. Opin. Cell Biol. *15,* 318–325.

Görisch, S.M., Richter, K., Scheuermann, M.O., Herrmann, H., and Lichter, P. (2003). Diffusion-limited compartmentalization of mammalian cell nuclei assessed by microinjected macromolecules. Exp. Cell Res. *289,* 282–294.

Goldberg, I.G., Sawhney, H., Pluta, A.F., Warburton, P.E., and Earnshaw, W.C. (1996). Surprising deficiency of CENP-B binding sites in African green monkey α-satellite DNA: implications for CENP-B function at centromeres. Mol. Cell. Biol. *16,* 5156–5168.

Gray, M.D., Wang, L., Youssoufian, H., Martin, G.M., and Oshima, J. (1998). Werner helicase is localized to transcriptionally active nucleoli of cycling cells. Exp. Cell Res. *242,* 487–494.

Grummt, I. (2003). Life on a planet of its own: regulation of RNA polymerase I transcription in the nucleolus. Genes Dev. *17,* 1691–1702.

Hadjiolov, A.A. (1985). Cell Biology Monographs, Vol. 12. The Nucleolus and Ribosome Biogenesis. Vienna: Springer-Verlag.

Harlow, E., and Lane, D. (1998). Using Antibodies: A Laboratory Manual. Cold Spring Harbor, NY: Cold Spring Harbor Press, 63–99.

Harnpicharnchai, P. *et al.* (2001). Composition and functional characterization of yeast 66S ribosome assembly intermediates. Mol. Cell *8,* 505–515.

Hingorani, M.M., and O'Donnell, M. (2000). Sliding clamps: a (tail)ored fit. Curr. Biol. *10,* R25–R29.

Huang, S. (2002). Building an efficient factory: where is pre-rRNA synthesized in the nucleolus?. J. Cell Biol. *157,* 739–741.

Huang, S., Deerinck, T.J., Ellisman, M.H., and Spector, D.L. (1997). The dynamic organization of the perinucleolar compartment in the cell nucleus. J. Cell Biol. *137,* 965–974.

Hügle, B., Scheer, U., and Franke, W.W. (1985). Ribocharin: a nuclear Mr 40,000 protein specific to precursor particles of the large ribosomal subunit. Cell *41,* 615–627.

Kleinschmidt, J.A., and Franke, W.W. (1982). Soluble acidic complexes containing histones H3 and H4 in nuclei of *Xenopus laevis* oocytes. Cell *29,* 799–809.

Kneissel, S., Franke, W.W., Gall, J.G., Heid, H., Reidenbach, S., Schnölzer, M., Spring, H., Zentgraf, H., and Schmidt-Zachmann, M.S. (2001). A novel karyoskeletal protein: characterization of protein NO145, the major component of nucleolar cortical skeleton in *Xenopus* oocytes. Mol. Biol. Cell *12,* 3904–3918.

Köhler, G., and Milstein, C. (1975). Continuous cultures of fused cells secreting antibody of predefined specificity. Nature *256,* 495–497.

Krohne, G., Debus, E., Osborn, M., Weber, K., and Franke, W.W. (1984). A monoclonal antibody against nuclear lamin proteins reveals cell type-specificity in *Xenopus laevis.* Exp. Cell Res. *150,* 47–59.

Kuhn, J., Götting, C., Schnölzer, M., Kempf, T., Brinkmann, T., and Kleesiek, K. (2001). First isolation of human UDP-D-xylose: proteoglycan core protein β-D-xylosyltransferase secreted from cultured JAR choriocarcinoma cells. J. Biol. Chem. *276,* 4940–4947.

Lamond, A.I., and Earnshaw, W.C. (1998). Structure and function in the nucleus. Science *280,* 547–553.

Lennon, G., Auffray, C., Polymeropoulos, M., and Soares, M.B. (1996). The IMAGE Consortium: an integrated molecular analysis of genomes and their expression. Genomics *33,* 151–152.

Leonhardt, H., Rahn, H.-P., Weinzierl, P., Sporbert, A., Cremer, T., Zink, D., and Cardoso, M.C. (2000). Dynamics of DNA replication factories in living cells. J. Cell Biol. *149,* 271–279.

Leung, A.K., and Lamond, A.I. (2003). The dynamics of the nucleolus. Crit. Rev. Eukaryot. Gene Expr. *13,* 39–54.

Lin, D.Y., and Shih, H.M. (2002). Essential role of the 58-kDa microspherule protein in the modulation of Daxx-dependent transcriptional repression as revealed by nucleolar sequestration. J. Biol. Chem. *277,* 25446–25456.

Lohr, F., Wenz, F., Haas, S., and Flentje, M. (1995). Comparison of proliferating cell nuclear antigen (PCNA) staining and BrdUrd-labelling index under different proliferative conditions in vitro by flow cytometry. Cell Prolif. *28,* 93–104.

Madine, M.A., Swietlik, M., Pelizon, C., Romanowski, P., Mills, A.D., and Laskey, R.A. (2000). The roles of the MCM, ORC, and Cdc6 proteins in determining the replication competence of chromatin in quiescent cells. J. Struct. Biol. *129,* 198–210.

Mais, C., and Scheer, U. (2001). Molecular architecture of the amplified nucleoli of *Xenopus* oocytes. J. Cell Sci. *114,* 709–718.

Marciniak, R.A., Lombard, D.B., Johnson, F.B., and Guarente, L. (1998). Nucleolar localization of the Werner syndrome protein in human cells. Proc. Natl. Acad. Sci. USA *95,* 6887–6892.

Meier, U.T., and Blobel, G. (1990). A nuclear localization signal binding protein in the nucleolus. J. Cell Biol. *111,* 2235–2245.

Meier, U.T., and Blobel, G. (1994). NAP57, a mammalian nucleolar protein with a putative homolog in yeast and bacteria. J. Cell Biol. *127,* 1505–1514.

Mertens, C., Kuhn, C., and Franke, W.W. (1996). Plakophilins 2a and 2b: constitutive proteins of dual location in the karyoplasm and the desmosomal plaque. J. Cell Biol. *135,* 1009–1025.

Meßmer, B., and Dreyer, C. (1993). Requirements for nuclear translocation and nucleolar accumulation of nucleolin in *Xenopus laevis.* Eur. J. Cell Biol. *61,* 369–382.

Misteli, T. (2000). Cell biology of transcription and pre-mRNA splicing: nuclear architecture meets nuclear function. J. Cell Sci. *113,* 1841–1849.

Nakayama, H. (2002). RecQ family helicases: roles as tumor suppressor proteins. Oncogene *21,* 9008–9021.

Oeffinger, M., Leung, A.K., Lamond, A.I., and Tollervey, D. (2002). Yeast pescadillo is required for multiple activities during 60S ribosomal subunit synthesis. RNA *8,* 626–636.

Ochs, R.L., Lischwe, M.A., Spohn, W.H., and Busch, H. (1985). Fibrillarin: a new protein of the nucleolus identified by autoimmune sera. Biol. Cell *54,* 123–134.

Olson, M.O., Hingorani, K., and Szebeni, A. (2002). Conventional and nonconventional roles of the nucleolus. Int. Rev. Cytol. *219,* 199–266.

Pederson, T. (1998). The plurifunctional nucleolus. Nucleic Acids Res. *26,* 3871–3876.

Pederson, T. (2000). Half a century of "the nuclear matrix." Mol. Biol. Cell *11,* 799–805.

Peitsch, W.K., Grund, C., Kuhn, C., Schnölzer, M., Spring, H., Schmelz, M., and Franke, W.W. (1999). Drebrin is a widespread actin-associating protein enriched at junctional plaques, defining a specific microfilament anchorage system in polar epithelial cells. Eur. J. Cell Biol. *78,* 767–778.

Raska, I., Andrade, L.E.C., Ochs, R.L., Chan, E.K.L., Chang, C.M., Roos, G., and Tan, E.M. (1991). Immunological and ultrastructural studies of the nuclear coiled body with autoimmune antibodies. Exp. Cell Res. *195,* 27–37.

Rodriguez-Lopez, A.M., Jackson, D.A., Nehlin, J.O., Iborra, F., Warren, A.V., and Cox, L.S. (2003). Characterization of the interaction between WRN, the helicase/exonuclease defective in progeroid Werner's syndrome, and an essential replication factor, PCNA. Mech. Ageing Dev. *124,* 167–174.

Rose, O., Grund, C., Reinhardt, S., Starzinski-Powitz, A., and Franke, W.W. (1995). Contactus adherens, a special type of plaque-bearing adhering junction containing M-cadherin, in the granule cell layer of the cerebellar glomerulus. Proc. Natl. Acad. Sci. USA *92,* 6022–6026.

Sanz, M.M., Proytcheva, M., Ellis, N.A., Holloman, W.K., and German, J. (2000). BLM, the Bloom's syndrome protein, varies during the cell cycle in its amount, distribution, and co-localization with other nuclear proteins. Cytogenet. Cell Genet. *91,* 217–223.

Sasaki, K., Kurose, A., Ishida, Y., and Matsuta, M. (1994). Estimation of S-phase fraction in tumor tissue sections by immunohistochemical staining of PCNA. J. Histochem. Cytochem. *42,* 957–960.

Scalenghe, F., Buscaglia, M., Steinheil, C., and Crippa, M. (1978). Large scale isolation of nuclei and nucleoli from vitellogenic oocytes of *Xenopus laevis.* Chromosoma *66,* 299–308.

Scheer, U., and Hock, R. (1999). Structure and function of the nucleolus. Curr. Opin. Cell Biol. *11,* 385–390.

Scherl, A., Coute, Y., Deon, C., Calle, A., Kindbeiter, K., Sachez, J.C., Greco, A., Hochstrasser, D., and Diaz, J.J. (2002). Functional proteomic analysis of human nucleolus. Mol. Cell. Biol. *13,* 4100–4109.

Schmid, E., Franke, W.W., Grund, C., Schiller, D.L., Kolb, H., and Paweletz, N. (1983). An epithelial cell line with elongated myoid morphology derived from bovine mammary gland. Expression of cytokeratins and desmosomal plaque proteins in unusual arrays. Exp. Cell Res. *146,* 309–328.

Schmidt-Zachmann, M.S., and Nigg, E.A. (1993). Protein localization to the nucleolus: a search for targeting domains in nucleolin. J. Cell Sci. *105,* 799–806.

Schmidt-Zachmann, M.S., Hügle, B., Scheer, U., and Franke, W.W. (1984). Identification and localization of a novel nucleolar protein of high molecular weight by a monoclonal antibody. Exp. Cell Res. *153,* 327–246.

Schmidt-Zachmann, M.S., Hügle-Dörr, B., and Franke, W.W. (1987). A constitutive nucleolar protein identified as a member of the nucleoplasmin family. EMBO J. *6,* 1881–1890.

Schmidt-Zachmann, M.S., Knecht, S., and Krämer, A. (1998). Molecular characterization of a novel, widespread nuclear protein that colocalizes with spliceosome components. Mol. Biol. Cell *9,* 143–160.

Siegel, L.M., and Monty, K.J. (1966). Determination of molecular weights and frictional ratios of proteins in impure systems by use of gel filtration and density gradient centrifugation. Application to crude preparations of sulfite and hydroxylamine reductases. Biochim. Biophys. Acta *112,* 346–3662.

Smalheiser, N.R. (1996). Proteins in unexpected locations. Mol. Biol. Cell *7,* 1003–1014.

Spector, D.L., Fu. X. D., and Maniatis, T. (1991). Associations between distinct pre-mRNA splicing components and the cell nucleus. EMBO J. *10,* 3467–3481.

Starborg, M., Gell, K., Brundell, E., and Höög, C. (1996). The murine Ki-67 cell proliferation antigen accumulates in the nucleolar and heterochromatic regions of interphase cells and at the periphery of the mitotic chromosomes in a process essential for cell cycle progression. J. Cell Sci. *109,* 143–153.

Sullivan, G.J., Bridger, J.M., Cuthbert, A.P., Newbold, R.F., Bickmore, W.A., and McStay, B. (2001). Human acrocentric chromosomes with transcription-

ally silent nucleolar organizer regions associate with nucleoli. EMBO J. *20,* 2867–2877.

Thiry, M., Cheutin, T., O'Donohue, M.F., Kaplan, H., and Ploton, D. (2000). Dynamics and three-dimensional localization of ribosomal RNA within the nucleolus. RNA *6,* 1750–1761.

Thomas, J.O., and Kornberg, R.D. (1975). An octamer of histones in chromatin and free in solution. Proc. Natl. Acad. Sci. USA *72,* 2626–2630.

Tschochner, H., and Hurt, E. (2003). Pre-ribosomes on the road from the nucleolus to the cytoplasm. Trends Cell Biol. *13,* 255–263.

Tsuneoka, M., Koda, Y., Soejima, M., Teye, K., and Kimura, H. (2002). A novel myc target gene, mina53, that is involved in cell proliferation. J. Biol. Chem. *277,* 35450–35459.

van Brabant, A.J., Stan, R., and Ellis, N.A. (2000). DNA helicases, genomic instability, and human genetic disease. Annu. Rev. Genomics Hum. Genet. *1,* 409–459.

Verheijen, R., Kuijpers, H.J., van Driel, R., Beck, J.L., van Dierendonck, J.H., Brakenhoff, G.J., and Ramaekers, F.C. (1989). Ki-67 detects a nuclear matrix-associated proliferation-related antigen. II. Localization in mitotic cells and association with chromosomes. J. Cell Sci. *92,* 531–540.

von Kobbe, C., and Bohr, V.A. (2002). A nucleolar targeting sequence in the Werner syndrome protein resides within residues 949–1092. J. Cell Sci. *115,* 3901–3907.

Wachtler, F., Hopman, A.H.N., Wiegant, J., and Schwarzacher, H.G. (1986). On the position of nucleolus organizer regions (NORs) in interphase nuclei. Exp. Cell Res. *167,* 227–240.

Weipoltshammer, K., Schofer, C., Almeder, M., Sylvester, J., and Wachtler, F. (1996). Spatial distribution of sex chromosomes and ribosomal genes: a study on human lymphocytes and testicular cells. Cytogenet. Cell Genet. *73,* 108–113.

Wehner, K.A., Ayala, L., Kim, Y., Young, P.J., Hosler, B.A., Lorson, C.L., Baserga, S.J., and Francis, J.W. (2002). Survival motor neuron protein in the nucleolus of mammalian neurons. Brain Res. *945,* 160–173.

Wyman, C., and Botchan, M. (1995). A familiar ring to DNA polymerase processivity. Curr. Biol. *5,* 334–337.

Yankiwski, V., Marciniak, R.A., Guarente, L., and Neff, N.F. (2000). Nuclear structure in normal and Bloom syndrome cells. Proc. Natl. Acad. Sci. USA *97,* 5214–5219.

Zirwes, R.F., Kouzmenko, A.P., Peters, J.-M., Franke, W.W., and Schmidt-Zachmann, M.S. (1997a). Topogenesis of a nucleolar protein: determination of molecular segments directing nucleolar association. Mol. Biol. Cell *8,* 231–248.

Zirwes, R.F., Schmidt-Zachmann, M.S., and Franke, W.W. (1997b). Identification of a small, very acidic constitutive nucleolar protein (NO29) as a member of the nucleoplasmin family. Proc. Natl. Acad. Sci. USA *94,* 11387–11392.

Zirwes, R.F., Eilbracht, J., Kneissel, S., and Schmidt-Zachmann, M.S. (2000). A novel helicase-type protein in the nucleolus: protein NOH61. Mol. Biol. Cell *11,* 1153–1167.