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Topogenesis of a Nucleolar Protein: Determination of Molecular Segments Directing Nucleolar Association

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To identify the element(s) in nucleolar proteins which determine nucleolus-specific topogenesis, we have used different kinds of cDNA constructs encoding various chimeric combinations of mutants of the constitutive nucleolar protein NO38 (B23): 1) with an amino terminally placed short "myc tag"; 2) with two different carboxyl terminally attached large α-helical coiled coil structures, the lamin A rod domain or the rod domain of vimentin; 3) with the sequence-related nucleoplasmic histone-binding protein nucleoplasmin; and 4) with the soluble cytoplasmic protein pyruvate kinase. To avoid the problem of formation of complexes with endogenous wild-type (wt) molecules and "piggyback" localization, special care was taken to secure that the mutants and chimeras used did not oligomerize as is typical of protein NO38 (B23). Using microinjection and transfection of cultured cells, we found that the segment comprising the amino-terminal 123 amino acids (aa) alone was sufficient to effect nucleolar accumulation of the construct molecules, including the chimeras with the entire rod domains of lamin A and vimentin. However, when the amino-terminal 109 aa were deleted, the molecules still associated with the nucleolus. The results of further deletion experiments and of domain swaps with nucleoplasmin all point to the topogenic importance of two independent molecular regions located at both the amino- and carboxyl-terminal end. Our definition of dominant elements determining the nucleolar localization of protein NO38 (B23) as well as of diverse nonnucleolar proteins will help to identify its local binding partner(s) and functions, the construction of probes examining other proteins or sequence elements within the nucleolar microenvironment, and the generation of cells with an altered nuclear architecture.

INTRODUCTION

Protein import into the cell nucleus occurs through the pore complexes of the nuclear envelope (NPC[1]) which act as a molecular sieve, allowing a relatively unrestricted diffusion of molecules up to 40–60 kDa and effecting the specific nuclear accumulation of karyophilic proteins of various sizes containing a nuclear location signal (NLS; for recent reviews, see Fabre and Hurt, 1994; Gerace, 1994; Panté and Aebi, 1996). NLSs have been identified and characterized in a number of proteins of diverse species and in general appear as oligopeptide segments usually but not always rich in basic amino acids (aa) which either occur singly or, as in the nuclear protein studied best in this respect, the histone-binding protein nucleoplasmin, as two interrupted basic domains forming a more extended bipartite NLS (reviewed in Dingwall and Laskey, 1991; Robbins *et al.*, 1991; Makkerh *et al.*, 1996). A prototype NLS consensus sequence, however, has not emerged. In addition, soluble factors have been identified and

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[1] Abbreviations used: NLS, nuclear location signal; NPC, nuclear pore complex; wt, wild type.

characterized in molecular terms, which can bind NLSs and NPCs and are essentially involved in the nuclear accumulation process (for recent reviews, see Moore and Blobel, 1994; Görlich and Mattaj, 1996; Hurt, 1996).

After nuclear uptake, the different nuclear proteins distribute differently. Some appear to be dispersed throughout the diffusion-accessible space of the nucleoplasm. Others accumulate in and at specific intranuclear structures such as the nuclear lamina, the NPC, certain chromatin regions, interchromatinous nuclear bodies, and nucleoli. In most cases, the principles and mechanisms of subnuclear topogenesis are not clear. Only the binding of some proteins to nucleic acids, be it sequence-specific or broadly selective, has been studied to some extent (Bandziulis *et al.*, 1989; Van Holde, 1989; Churchill and Travers, 1991; Li and Bingham, 1991; Tafuri and Wolffe, 1993). Moreover, sequence requirements for the assembly of karyoskeletal proteins of the lamin group into a lamina associated with the inner nuclear membrane have been determined (Loewinger and McKeon, 1988; Holtz *et al.*, 1989; Krohne *et al.*, 1989; Kitten and Nigg, 1991; Smith and Blobel, 1993; Soullam and Worman, 1993; Meier and Georgatos, 1994).

Otherwise, little is known about the topogenic principles governing the association of proteins with distinct nuclear structures. Some sequence requirements for an accumulation at the nucleoli, the main site of ribosome biosynthesis (for reviews, see Hadjiolov, 1985; Scheer and Benavente, 1990; Scheer and Weisenberger, 1994), have been reported for certain viral proteins such as proteins Tat and Rev of human immunodeficiency virus (Dang and Lee, 1989; Cochrane *et al.*, 1990; Hatanaka, 1991), protein Rex of human T-cell leukemia virus (Siomi *et al.*, 1988), the ICP27 regulatory protein of herpes simplex virus (Mears *et al.*, 1995) and for the stress protein hsp70 under heat shock condition (Munro and Pelham, 1984; Dang and Lee, 1989; Milarski and Morimoto, 1989). None of these proteins, however, is a regular nucleolar constituent and therefore the biological significance and function of such nucleolar associations remains obscure.

For studying the molecular requirements for nucleolar association of a typical nucleolar protein in a systematic way, we have chosen the major nonribosomal protein NO38, a constitutive molecule of the granular component (GC) of the nucleolus (Schmidt-Zachmann *et al.*, 1987). The aa sequence of *Xenopus laevis* protein NO38 contains two large acidic domains rich in aspartic and glutamic acid residues, one of them flanked by two putative karyophilic signals, and shows a high sequence homology, particularly in the amino-terminal region, to nucleoplasmin. Like nucleoplasmin, protein NO38 tends to form oligomeric complexes, in vitro and in vivo. Analysis of the murine counterpart of protein NO38 (Schmidt-Zachmann and Franke, 1988) has revealed that it is highly homologous to the mammalian nucleolar protein B23 (Busch, 1984; Chan *et al.*, 1986a,b, 1990), also named numatrin (Feuerstein *et al.*, 1988) or nucleophosmin (Chan *et al.*, 1989).

In the present study, we report on principles of subnuclear localization recognized with deletion mutants and point mutations of protein NO38 (B23) in chimeric constructs with different kinds of well-defined protein structures as reporters. We show that two independent molecular domains located at both ends of the molecule are involved in the nucleolar targeting process.

MATERIALS AND METHODS

Antibodies

Production and characterization of monoclonal antibodies (mAbs) No-185 and No-63, both specific for *Xenopus* protein NO38 but not reactive with the mammalian counterpart, have been reported (Schmidt-Zachmann *et al.*, 1987). The mAb 9E10 (ATCC CRL 1729) specifically recognizes an epitope in the decapeptide EQKLISEEDL of the human c-myc protein (Evan *et al.*, 1985), and mAb L3-4B4 (kindly provided by Dr. R. Stick, Max Planck Institute for Biophysical Chemistry, Göttingen, Germany) reacts with chicken and mammalian lamin A, but not with *Xenopus* lamins (Lehner *et al.*, 1987; Stick *et al.*, 1988). In one set of experiments, mAb b7-1A9-M9 directed against the histone-binding protein nucleoplasmin from *Xenopus* was used (kindly provided by Dr. C. Dreyer, Max Planck Institute for Developmental Biology, Tübingen, Germany). Moreover, we applied mAbs against the human nucleolar protein B23 (kindly provided by Dr. P.-K. Chan, Baylor College for Medicine, Houston, TX), mAb 14.13.8 directed against *Xenopus* vimentin (supplied by Dr. H. Herrmann, this institute) and, for comparison, antibodies recognizing mammalian vimentin (Progen Biotechnics, Heidelberg, Germany). For immunodetection experiments, either supernatants of hybridoma cultures (undiluted) or ascites fluid (1:1000) were used. The nucleolus-specific human autoimmune serum SclC (Reimer *et al.*, 1987) was used in a 1:100 dilution, whereas the guinea pig antiserum ($L_{A386}I$) against *Xenopus* lamin A (kindly provided by Dr. G. Krohne, University of Würzburg, Würzburg, Germany) was diluted 1:400 before use. It should be noted that antiserum $L_{A386}I$ specifically reacts with *Xenopus* lamin A but does not recognize the corresponding protein in human cells. Since $L_{A386}I$ shows no background staining on cultured human cells transfected with lamin- or non-lamin-containing constructs, it was suitable for the detection of chimeric proteins fused to the *Xenopus* lamin A rod upon expression in PLC cells.

Construction of NO38 Mutants

All mutated clones (Figure 1) were derived from the wild-type (wt) NO38 cDNA clone λ NO38-185 (Schmidt-Zachmann *et al.*, 1987). Only a selection of representative examples of the most often used and crucial constructs is described here in some detail.

Amino-terminal deletions (e.g., N1, Δ4aa; N2, Δ31aa) were introduced by *Bam*HI linearization of the phagescript expression vector (Stratagene, Heidelberg, Germany) containing the NO38 cDNA. Subsequently, the cDNA was shortened by partial digestion with the exonuclease *Bal*31 (Maniatis *et al.*, 1982), and the termini were repaired with T4 DNA polymerase to generate blunt ends. These DNA molecules were ligated with the reannealed product of two complementary oligonucleotides containing a *Bam*HI restriction site and translation start codons (ATG) in all three reading frames. The

shortened cDNA fragments attached to the linker were released from the remaining vector sequences by digestion with *Bam*HI and *Eco*RI and recloned into the Bluescript expression vector (Stratagene).

Carboxyl-terminal deletion mutants were generated using appropriate restriction sites. For the construction of mutant N3 (Δ54aa), the cDNA coding for wt protein NO38 was digested with *Dde*I. After filling in with nucleotides to create blunt ends, the cDNA fragment was isolated by digestion with *Eco*RI and finally subcloned into the Bluescript vector previously cut with *Eco*RI and *Sma*I. In mutant N4 (Δ179aa), the codon for aa residue 121 was changed to a stop codon by oligonucleotide-directed mutagenesis, using the Amersham System (Amersham Buchler, Braunschweig, Germany) based on the method of Taylor *et al.* (1985).

Internal deletion mutants (N5, Δaa5-14; N6, Δaa24-30; N7, Δaa56-59; N9, Δaa153-159 and Δaa189-195) and point mutations (N8) were also generated by mutagenesis reactions. In mutant N8 the codon for Cys^{22} was altered into a serine codon. All mutants were identified by nucleotide sequencing (Sanger *et al.*, 1977), and mutated cDNA inserts were subcloned into the Bluescript vector.

Generation of Epitope-tagged Protein NO38 Constructs

The sequence containing the epitope of mAb KT3 against SV40 T-antigen (MacArthur and Walter, 1984) was introduced into the NO38 cDNA by directed mutagenesis using an oligonucleotide coding for the aa sequence KPPTPPPEPET. The insertion was performed at the carboxyl-terminal end of wt protein NO38, just in front of the internal stop codon.

In addition, protein NO38 was fused in-frame to the carboxyl-terminal part of synaptophysin containing the epitope of the mAb SY-38 (Wiedenmann and Franke, 1985). For this, the wt NO38 cDNA clone was cut with *Dde*I, filled in, redigested with *Eco*RI, and ligated blunt end to a fragment of synaptophysin cDNA clone pSR5 (Leube *et al.*, 1987, 1989) obtained by digestion with *Nar*I, followed by creation of blunt ends and final digestion with *Xho*I. The resulting cDNA construct was subcloned into the *Eco*RI and *Xho*I sites of Bluescript. The polypeptide encoded lacks the last 53 aa residues of NO38 which are replaced by the carboxyl-terminal 74 residues of synaptophysin.

Some constructs (MN1-MN7) were tagged with an epitope of the human c-myc protein using the BT-myc-vector (Schmidt-Zachmann and Nigg, 1993) which contains several restriction sites downstream of the myc-tag, and cDNA fragments isolated by using appropriate restriction enzymes were inserted in-frame.

Generation of Chimeric Proteins

Combinations of NO38 Protein with the Lamin Rod Domain. cDNA fragments coding for different parts of protein NO38 were combined with the rod domain of lamin A derived from a cDNA coding for a "tailless" mutant of the *Xenopus* protein ($L_{A1\text{-}386}BT$) comprising aa 1–386 due to a stop codon introduced directly after the rod domain (Gieffers and Krohne, 1991; see Figure 4).

Construct L, coding for the rod domain alone, was obtained from plasmid $L_{A1\text{-}386}BT$ by cutting off the head-encoding segment with *Eco*RI and *Bgl*II. The remaining plasmid DNA was religated using as a linker annealed oligonucleotides with *Eco*RI- and *Bgl*II-compatible ends, a translation start codon and a *Mlu*I site.

For construction of NL0, we introduced a *Bam*HI restriction site at the end of the coding sequence of mutant N8 via polymerase chain reaction-mediated in vitro mutagenesis, thereby destroying the internal stop codon. The resulting cDNA was isolated by *Hin*dIII and *Bam*HI digestion. A second cDNA fragment coding for the lamin A rod was obtained by digestion of $L_{A1\text{-}386}BT$ with *Cla*I, end filling, and recutting with *Bgl*II. Both inserts were ligated together in the eukaryotic expression vector pRcCMV previously linearized with *Hin*dIII and *Not*I blunt.

The NO38 cDNA fragment used to construct NL1 was obtained by digestion of the N8-containing Bluescript plasmid with *Dde*I and *Eco*RI. The resulting fragment was cloned into plasmid $L_{A1\text{-}386}BT$ opened with *Eco*RI and *Bgl*II using a linker fragment containing *Dde*I- and *Bgl*II-compatible ends and an internal *Hin*dIII site. For construction of NL2, plasmid $L_{A1\text{-}386}BT$ was digested with *Cla*I and *Bgl*II and the purified 2-kb insert was ligated to plasmid N8 previously cut by *Cla*I and *Bgl*II. NL3 was produced by digestion of NO38-mutant N8 with *Bfa*I, filling in, and redigestion with *Eco*RI. The resulting fragment was ligated to plasmid $L_{A1\text{-}386}BT$, previously cut with *Bgl*II, end filled, and restricted with *Eco*RI. For construction of NL4, the NO38-encoding part was obtained by *Dde*I digestion of a NO38 mutant lacking aa 165–186 (ΔA2), followed by creation of blunt ends and digestion with *Spe*I. The resulting fragment was cloned into plasmid L previously cut with *Mlu*I, end filled, and recut by *Spe*I.

Construction of NP L was performed by *Ear*I digestion of plasmid npl-BT containing the cDNA of *Xenopus* nucleoplasmin (kindly provided by Dr. J. Kleinschmidt, this center), end filling, and *Eco*RI redigestion of an isolated 0.58-kb fragment. The resulting 0.39-kb fragment coding for the first 127 aa of nucleoplasmin was subcloned into plasmid L previously digested with *Mlu*I, followed by end filling and redigestion with *Eco*RI.

Combinations of NO38 Protein with Nucleoplasmin. For the generation of fusion proteins containing either the amino-terminal half of nucleoplasmin fused to the carboxyl-terminal half of protein NO38 (NP/NO) or vice versa (NO/NP), the corresponding cDNA fragments were combined by using appropriate restriction sites within the wt cDNA clones (*Hin*cII in nucleoplasmin and *Ava*II in NO38). The corresponding constructs encoding the first 115 aa of nucleoplasmin, followed by aa 110–299 of NO38 (NP/NO), or the first 109 aa of protein NO38, followed by aa 116–200 of nucleoplasmin (NO/NP) were subcloned into the BT-myc-vector, resulting in the final constructs M-NP/NO and M-NO/NP (our unpublished results).

Construction of MN6 was achieved through *Dde*I digestion of npl-BT, end filling, and redigestion with *Eco*RI. The resulting 625-bp fragment was ligated together with a second 307-bp insert, derived from wt NO38 by digestion of NO38-BT with *Dde*I, end filling, and redigestion with *Hin*dIII, into *Eco*RI- and *Hin*dIII-opened Bluescript vector.

To obtain MN8, plasmid npl-BT was digested with *Eco*RV and *Pst*I. The isolated insert was treated with T4 polymerase and ligated into plasmid NO38-BT that had been linearized with *Bgl*II, end filled, and recleaved with *Eco*RV.

Combination of NO38 Protein with Pyruvate Kinase. Construction of MN7 was performed by digestion of NO38-BT with *Dde*I, end filling, and ligation of the resulting fragment into *Bst*XI-digested and T4 DNA-polymerase-treated plasmid pGEM2-myc-NLS-PK (Schmidt-Zachmann and Nigg, 1993).

Combination of NO38 Protein with the *Xenopus* Vimentin Rod. MN9 was constructed by digestion of plasmid $Vim_{\Delta T}$ NLS (*Sac*II/*Eco*47III)-pDS5 (kindly provided by Dr H. Herrmann, this center) with *Sac*II and *Sal*I. The recovered insert was subcloned into plasmid NO38-BT previously digested with *Bgl*II and *Sal*I using a linker fragment containing *Bgl*II- and *Sac*II-compatible ends.

Expression of Cloned cDNAs In Vitro

In vitro transcription and translation reactions of wt protein NO38, mutated forms of protein NO38, and fusion proteins were performed according to the manufacturer's (Promega via Serva, Heidelberg, Germany) protocol (cf. Kleinschmidt *et al.*, 1986). Cotranslation experiments were performed in the same way, except that two different in vitro-transcribed RNAs were added to the translation reaction. In some experiments the coupled transcription/translation system (T_NT-system) of Promega was used. Translation products were analyzed with SDS-PAGE and autoradiography. In vitro translation assays to be used for microinjection into *Xenopus* oocytes

(see below) were dialyzed against injection buffer (10 mM PIPES, pH 7.4, 40 mM KCl, 10 mM NaCl) and stored at −20°C until use.

Gel Electrophoresis and Immunoprecipitation of In Vitro Translation Products

SDS-PAGE was done according to Thomas and Kornberg (1975). For detection of oligomeric forms of in vitro-translated proteins, aliquots of translation mixtures were added to sample buffer containing only 0.1% SDS and were separated by electrophoresis without previous boiling.

For immunoprecipitations, translation mixtures were incubated for 1 h at 4°C either with the NO38-specific mAb No-63 or with the lamin antibodies of serum $L_{A386}I$ in immunoprecipitation buffer (0.1% SDS, 0.5% DOC, 1% NP-40, 20 mM methionine, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA). The immune complexes were then bound to protein A-Sepharose (Pharmacia, Freiburg, Germany) for 1 h at 4°C. After centrifugation (15 s, 10,000 × *g*) the resulting supernatant was precipitated with six volumes of acetone and prepared for SDS-PAGE. The Sepharose beads with bound immune complexes were washed several times with immunoprecipitation buffer, then once each with phosphate-buffered saline (PBS) containing 0.1% Triton X-100 and with PBS, boiled in SDS-sample buffer, and solubilized proteins were analyzed with SDS-PAGE and autoradiography.

Immunoprecipitation from Cellular Extracts of Human PLC Cells

Confluently grown PLC cells that were either untransfected or stably transfected with the chimeric cDNA MN9 were washed three times with PBS and then lysed directly onto the culture dishes with 1 ml lysis buffer (0.5% Triton X-100, 0.02% NaN_3, 100 mM Na-MES, pH 6.5, 250 mM NaCl, 0.5 mM $MgCl_2$, 1 mM dithiothreitol, and for inhibition of proteases 100 mM Pefablock, 20 mM pepstatin, and 20 mM leupeptin) per 10-cm dish for 2 min at room temperature. The lysates were transferred to a 1.5-ml reaction tube and incubated for 15 min on ice. After centrifugation of the insoluble cell debris (15 min, 14,000 × *g*, 4°C), the supernatant was precleared with protein G-Sepharose (1 h, 4°C) and subsequently reacted with α-NO38 mAb NO-185 (1 h, 4°C) that had been covalently coupled to protein G-Sepharose as described (Gersten and Marchalonis, 1978). Bound immunocomplexes were sedimented (30 s, 10,000 × *g*), washed three times briefly with lysis buffer and once with PBS, and solubilized by boiling in SDS-sample buffer. Proteins that remained in the supernatant fractions were precipitated and solubilized by boiling in SDS-sample buffer. The resulting protein fractions were separated by SDS-PAGE and blotted onto nitrocellulose filters. The distribution of protein MN9 and wt protein NO38 (B23) was assayed by immunoblotting using species-specific mAbs directed against *Xenopus* vimentin or human B23 (Figure 9). Bound first antibodies were fluorographically visualized using peroxidase-coupled secondary antibodies and the ECL detection system (Amersham-Buchler).

Microinjection and Subfractionation of Oocytes

Microinjection of in vitro translation products and the manual isolation of nuclei and cytoplasms from *X. laevis* oocytes were performed as described (Krohne *et al.*, 1989).

For sedimentation analyses of in vitro translation products after microinjection into the cytoplasm of oocytes, 60–70 nuclei were isolated manually 18 h after injection, collected in 5:1 isolation-medium (83 mM KCl, 17 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, containing additional 100 mM KCl) and homogenized. The homogenates were incubated for 15 min on ice and cleared by centrifugation (10 min, 10,000 × *g*). The supernatant fractions containing the salt-extracted nuclear proteins were layered on top of 5–30% (w/v) sucrose gradients (in 5:1 buffer). Upon centrifugation (cf. Hügle *et al.*, 1985) fractions of 0.4 ml were collected, proteins were precipitated with 20% trichloroacetic acid, and separated by SDS-PAGE. Bovine serum albumin (BSA), catalase, and thyroglobulin (Pharmacia) were used as S-value reference proteins in parallel gradients.

Cell Culture and Microinjection of RNA

The human hepatoma-derived PLC cells (ATCC CRL 8024) used for microinjection and transfection experiments were grown in DMEM (Life Technologies, Inc., Karlsruhe, Germany) supplemented with 10% fetal calf serum. Microinjection into cultured cells was performed as described (Ansorge, 1982; Kreis and Birchmeier, 1982; Leube *et al.*, 1989). Briefly, RNA was obtained by in vitro transcription of 5 μg linearized DNA template in the presence of 5 mM GpppG and 0.5 mM GTP to obtain capped mRNAs. The extracted and precipitated RNA was dissolved in PBS and centrifuged for 10 min (10,000 × *g*) before use. The final RNA concentration was about 1 μg/μl. After injection, cells were grown for 16 to 18 h and examined by immunofluorescence microscopy.

Transfection Experiments

For transfection experiments, all constructs were subcloned into the eukaryotic expression vector pRcCMV (Invitrogen via ITC Biotechnology, Heidelberg, Germany).

Transfections, usually with 7.5 μg of DNA per 3.5-cm cell culture dish, were carried out essentially as described (Krek and Nigg, 1991) using the method of Chen and Okayama (1987). Transfected cells were analyzed 24 h after removal of the DNA-Ca^{2+} precipitate by immunofluorescence microscopy.

Stably transfected cells were obtained by continuous culture of the transfected cells after removal of the DNA-Ca^{2+} precipitate in DMEM (Life Technologies, Inc.) supplemented with 10% fetal calf serum and geniticin (G418; Life Technologies, Inc.; 0.7 mg/ml final concentration). G418-resistant clones were isolated, analyzed for the maintained expression of the transfected constructs by immunofluorescence microscopy, and further cultured in the same medium for at least 10 passages.

Immunofluorescence Microscopy

Cells grown on coverslips were washed three times with PBS and then fixed for 10 min in methanol and for 30 s in acetone, both at −20°C. The cells were briefly air dried and rehydrated in PBS prior to immunocytochemistry (Hügle *et al.*, 1983; Leube *et al.*, 1989; Troyanovsky *et al.*, 1993) using secondary antibodies conjugated either with FITC or with Texas Red (Dianova, Hamburg, Germany).

Immunoelectron Microscopy

Human PLC cells grown on coverslips were briefly washed with PBS and then fixed for 5 min in −20°C methanol, followed by three brief dippings in −20°C acetone for 2 s each and transferred to PBS. Incubation of the first antibody (vimentin-specific mAb 14.13.8, undiluted hybridoma supernatant) was in a humid chamber for 2 h at room temperature. After three washes with PBS for 5 min each, bound antibodies were reacted with 5 nm in diameter gold particles coated with anti-mouse IgG (Biotrend, Aurion Gold Reagents, Cologne, Germany) overnight. Silver enhancement was carried out as described (Blessing *et al.*, 1993). Dehydration and embedding were according to Franke *et al.* (1978).

RESULTS

Mutational Analysis of NO38

To define the sequence requirements of NO38 responsible for specific nucleolar accumulation of this protein, we constructed a series of mutants of the *X. laevis*

cDNA (Schmidt-Zachmann *et al.*, 1987) suitable for microinjection and transfection experiments. Using restriction enzymes, exonuclease digestions, and directed in vitro mutagenesis, we constructed series of amino-terminal, carboxyl-terminal, and internal deletions as well as point mutations and various double mutants containing two internal deletions (Figure 1). In pilot transfection experiments we had encountered two major kinds of problems that could give rise to misinterpretations of results obtained with such transfected cells.

The first problem became obvious from our observation that several constructs containing a small tag epitope, notably in the carboxyl-terminal part, gave false-negative results, probably due to antibody inaccessibility resulting from an altered conformation. This has been overcome by examining the same tag in various positions and also by the use of extended tags in the form of chimeric proteins with a stable and defined conformation such as the lamin A rod domain (Figure 4) and nucleoplasmin.

Even more problematic was the oligomerization of protein NO38 which could have led to the formation of hybrid oligomers, including endogenous wt NO38, that consequently could give false-positive results. Therefore, we have performed a series of special controls for these artifacts and have studied the requirements for oligomer formation.

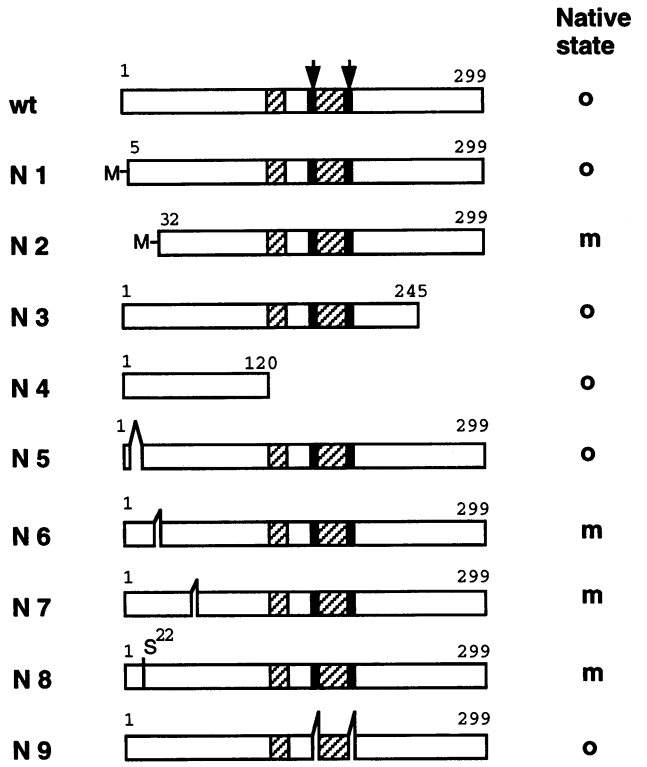


Figure 1. Mutational analysis of nucleolar protein NO38. Schematic drawing of the domains of wt protein NO38 from *X. laevis* based on the amino acid sequence previously published (Schmidt-Zachmann *et al.*, 1987) and various mutated forms (N1-9). The first and the last aa residues are indicated by numbers. Hatched areas represent acidic domains and the black bars (denoted by arrow) represent the two putative NLS sequences (Schmidt-Zachmann *et al.*, 1987). Internal deletions in mutants N5-N7 and N9 are shown as gaps. Ser^{22} in mutant N8 indicates the substitution of Cys^{22} in wt NO38 by Ser. The native forms of association of the proteins as determined by various methods (see text and Figure 2) are given in the column on the right. m, monomeric state; o, oligomeric state.

Oligomerization Properties of NO38 Mutants

Protein NO38 forms, in vivo and in vitro, homo-oligomeric complexes containing five or six molecules (Schmidt-Zachmann *et al.*, 1987; Yung and Chan, 1987). Therefore, we first characterized the oligomerization properties of the various mutants produced because of the possibility that specific mutants form heterotypic oligomer complexes with endogenous protein NO38 present in the microinjected or transfected host cells, thus allowing piggyback transport of the mutated protein.

First, the in vitro translation products of the individual mutants were analyzed by electrophoresis at low SDS concentrations known to preserve the oligomeric state of nucleoplasmin and protein NO38 (Figure 2A; cf. Dingwall *et al.*, 1987; Schmidt-Zachmann *et al.*, 1987). Under the same conditions, mutants missing 20 or more aa residues at the amino-terminus (e.g., N2) or containing short internal deletions in the amino-terminal region (e.g., N6, Δ24-30; N7, Δ56-59) did not form oligomers. On the other hand, the short mutant N4 containing only the amino-terminal part of protein NO38 formed "superstable" oligomers, as compared with the wt protein, which even upon heating in the presence of 3% SDS were not completely dissociated (Figure 2A). However, deletion of aa 5–14 (N5) or carboxyl-terminal truncations down to the remaining length of only 120 aa residues (N4) did not prevent oligomerization. These results suggest that the segment crucial for oligomerization of protein NO38 is located between aa 15 and 120, i.e., in the amino-terminal region (for partially compatible results with mammalian protein B23, see Liu and Chan, 1991).

Even the exchange of a single aa residue within this domain could inhibit oligomerization: When Cys^{22}, an aa residue present in this position in protein NO38 from different species as well as in nucleoplasmin (Dingwall *et al.*, 1987; Schmidt-Zachmann *et al.*, 1987; Schmidt-Zachmann and Franke, 1988; Chang and Olson, 1989), was replaced by serine, no oligomeric forms were detected (N8; Figure 2A). Oligomerization of mutant N8 was also not observed in vivo. When we microinjected radiolabeled protein N8 into the cytoplasm of *Xenopus* oocytes and analyzed this protein from manually isolated oocyte nuclei by sucrose gradient centrifugation, we found the mutant protein to

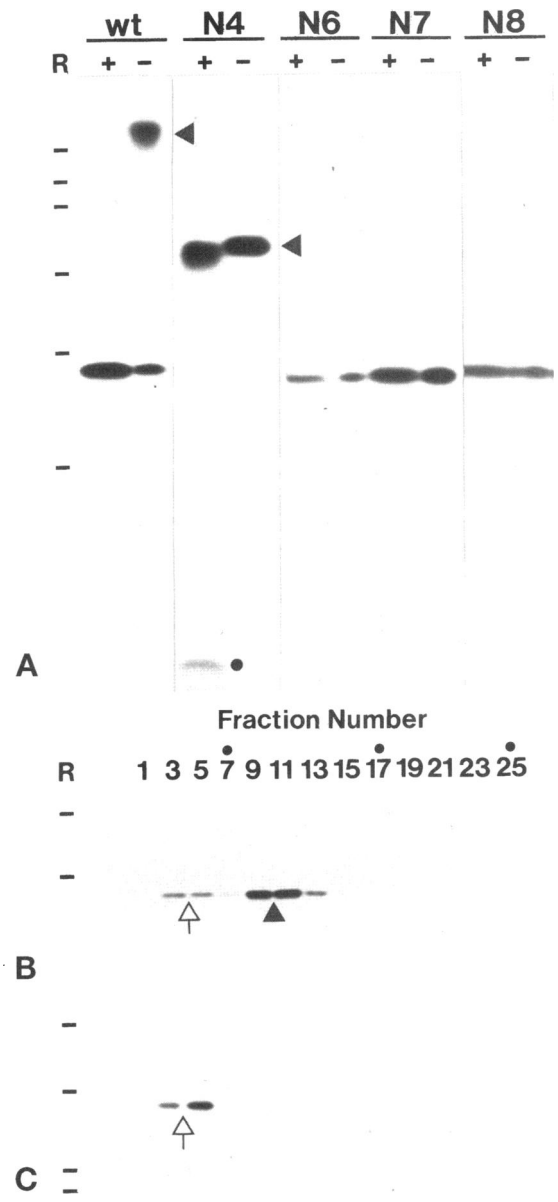


Figure 2. Biochemical analyses of oligomerization properties of wt and mutated NO38 proteins. (A) Autoradiogram of in vitro translated [^{35}S]methionine-labeled wt and mutated forms of NO38 after separation by SDS-PAGE. Prior to electrophoresis, samples of the translation mixtures were either heated for 5 min to 95°C in sample buffer with 3% SDS (lanes denoted +) or were mixed with sample buffer containing 0.1% SDS at room temperature (lanes denoted −). The oligomeric forms of wt NO38 and of mutant N4 are indicated by arrowheads. Note that the oligomeric form of N4 is even stable after being heated in the presence of 3% SDS, so that only a small amount of monomeric N4 can be detected (marked by a point). For mutant N8, a longer exposure autoradiogram is shown. Horizontal bars in lane R denote the positions of the myosin heavy chain (223 kDa), β-galactosidase (116 kDa), phosphorylase b (97 kDa), BSA (66 kDa), ovalbumin (45 kDa), and carbonic anhydrase (29 kDa) used as reference proteins. (B and C) Sucrose gradient centrifugation analysis of [^{35}S]methionine-labeled proteins wt NO38 (B) and mutant N8 in which Cys^{22} is substituted by Ser (C), as obtained after cDNA transcription and translation in vitro. Translation mixtures were microinjected into the cytoplasm of *X. laevis* oocytes and nuclei were isolated manually after 18 h. Salt-extracted nuclear proteins were analyzed by sucrose gradient centrifugation, followed by SDS-PAGE and autoradiography. In B, the oligomeric state of wt protein NO38 is denoted by a black arrowhead and monomeric forms of wt NO38 and mutant N8 are marked by arrows in B and C, respectively. No oligomeric state of N8 can be detected (C). Dots above the fraction numbers in B indicate the peak position of BSA (4.5S), catalase (11.3S), and thyroglobulin (16.5S) separated on a parallel gradient as S-value marker proteins. Horizontal bars in lanes R denote the positions of BSA, ovalbumin, and carbonic anhydrase.

sediment with ~3S, i.e., in a position expected for a monomer (Figure 2C), compared with a value of ~7S for the wt protein oligomer (Figure 2B). Moreover, these results show that no hetero-oligomers are formed between mutant N8 and endogenous wt protein under our in vivo conditions.

It should be emphasized, however, that oligomerization of protein NO38 apparently does not involve formation of a disulfide bond between Cys^{22} residues of adjacent polypeptide chains, as expected from the strong reducing conditions prevailing in the nucleocytoplasmic space (e.g., Hwang *et al.*, 1992 and refs. cited therein), and in agreement with our observation that protein NO38 obtained by translation in vitro could not be dissociated into subunits by reducing agents such as 20 mM dithiothreitol.

Microinjection of mRNAs into Human Cultured Cells

In vitro transcribed and GpppG-capped mRNAs encoding *Xenopus* wt protein NO38 or its mutated forms (cf. Figure 1) were injected into the cytoplasm of human hepatoma cells of line PLC, and the intracellular locations of their translation products were determined 18 h later by immunofluorescence microscopy using *Xenopus*-specific NO38-antibodies (Figure 3). All resulting proteins were found, exclusively or predominantly, in the nucleolus, which probably reflects the formation of heterospecific *Xenopus* NO38 complexes with the corresponding endogenous human protein.

In such experiments, significant non-nucleolar staining could only be seen in cells injected with mRNA-encoding mutants in which both putative NLS sequences had been deleted (e.g., N9; cf. Figure 1). These cells showed, in addition to nucleolar staining, a strong cytoplasmic fluorescence (Figure 3B, inset). Since in *Xenopus* oocytes both NLS sequences are required for efficient nuclear accumulation (our unpublished results), the nuclear uptake of at least a part of the double NLS deletion mutant N9 might have been due to the formation of heterotypic complexes with endogenous host cell wt protein NO38 (B23). To test this possibility, wt protein NO38 tagged with the SV40

T-antigen epitope of mAb KT3 was cotranslated in vitro along with the carboxyl-terminal deletion mutant N4 or with the NLS double-deletion mutant N9. Immunoprecipitation with mAb KT3 resulted in the coprecipitation of mutants N4 and N9, along with the wt protein, indicating that hetero-oligomer complexes had formed in the translation mixture (our unpublished results).

When mRNAs encoding the nonoligomerizing NO38 mutants N2, N6, N7, and N8 (Figure 1) were injected into cultured cells and analyzed by immunofluorescence microscopy, no significant fluorescence could be seen in different series of experiments. And when these mutants were translated in vitro, no product could be immunoprecipitated with two different NO38 antibodies. Instead, the proteins remained in the supernatant fraction. This suggested that the specific epitopes were either lost due to the mutation or masked due to changes of conformation and binding of other proteins.

Epitope Tags at the Carboxyl Terminus

Since monomeric forms of NO38 mutants could not be analyzed by microinjection experiments using NO38 antibodies, two different sequences bearing epitopes for other antibodies, i.e., mAb KT3 directed against the SV40 T antigen (MacArthur and Walther, 1984) and mAb Sy-38 directed against the vesicle membrane protein synaptophysin (Leube *et al.*, 1987, 1989), were introduced as tags near the carboxyl terminus of protein NO38.

When the various tagged forms of wt protein NO38 were analyzed by transcription in vitro, microinjection of mRNA into cultured cells and subsequent immunofluorescence microscopy, the resulting translation products could be detected in nucleoli with both types of antibodies. By contrast, no fluorescence could be detected with mAbs KT3 or Sy-38, when monomeric tagged mutants of NO38 (see above) were analyzed in the same way. These results may be explained by an unusual conformation of the monomeric NO38 mutants, resulting in an inaccessibility of the tags to the specific antibodies.

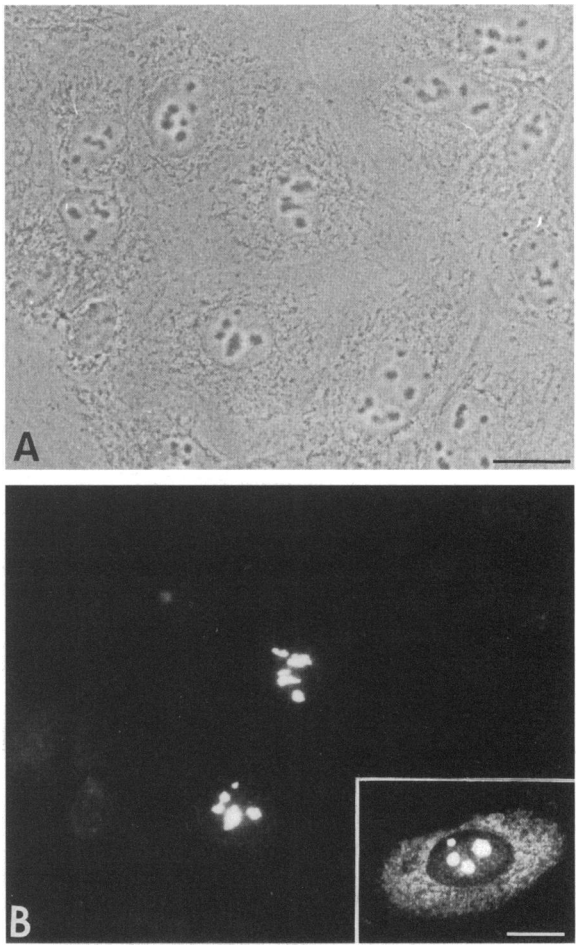


Figure 3. Localization of *Xenopus* wt protein NO38 and of the NLS-lacking double mutant N9 in cultured human PLC cells microinjected with the specific mRNAs. Phase-contrast (A) and immunofluorescence (B) micrographs of the same PLC cells injected with wt NO38 mRNA transcribed in vitro and stained with mAb No-63 specific for the *Xenopus* protein. The nucleoli of injected cells are brightly stained, whereas noninjected cells are completely negative. The inset in the lower right in B shows an immunofluorescence micrograph of a PLC cell injected with mRNA encoding the NLS-lacking double mutant N9. Here, in addition to nucleolar immunostaining, a strong cytoplasmic fluorescence is seen. Bars, 20 μm.

Constructs Encoding Chimeras of Protein NO38 and a Lamin Rod

Because of the masking problems described and the general uncertainty with negative results obtained with mutants containing small tags, we decided to use alternatively as reporter structure a large protein domain with a well-defined conformation known to be stable in the nucleus. For this, we chose the rod domain of *Xenopus* lamin A, to which specific antibodies were available that did not cross-react with mammalian lamins. This α-helical domain is known to dimerize and form rod-shaped coiled coil structures of ~50 nm in length, to which antibodies should have access even when fused to unusually folded proteins. We therefore constructed a series of plasmids coding for chimeric proteins containing various parts of protein NO38 fused to the lamin A rod (Figure 4).

Besides their specific detectability and stability, the large NO38-lamin rod chimeras also had the advantage that they no longer formed protein NO38 oligomers. When the corresponding DNA constructs were transcribed and translated in vitro and the products analyzed by electrophoresis at low SDS concentrations without boiling, only products of the size expected for the monomeric protein were found.

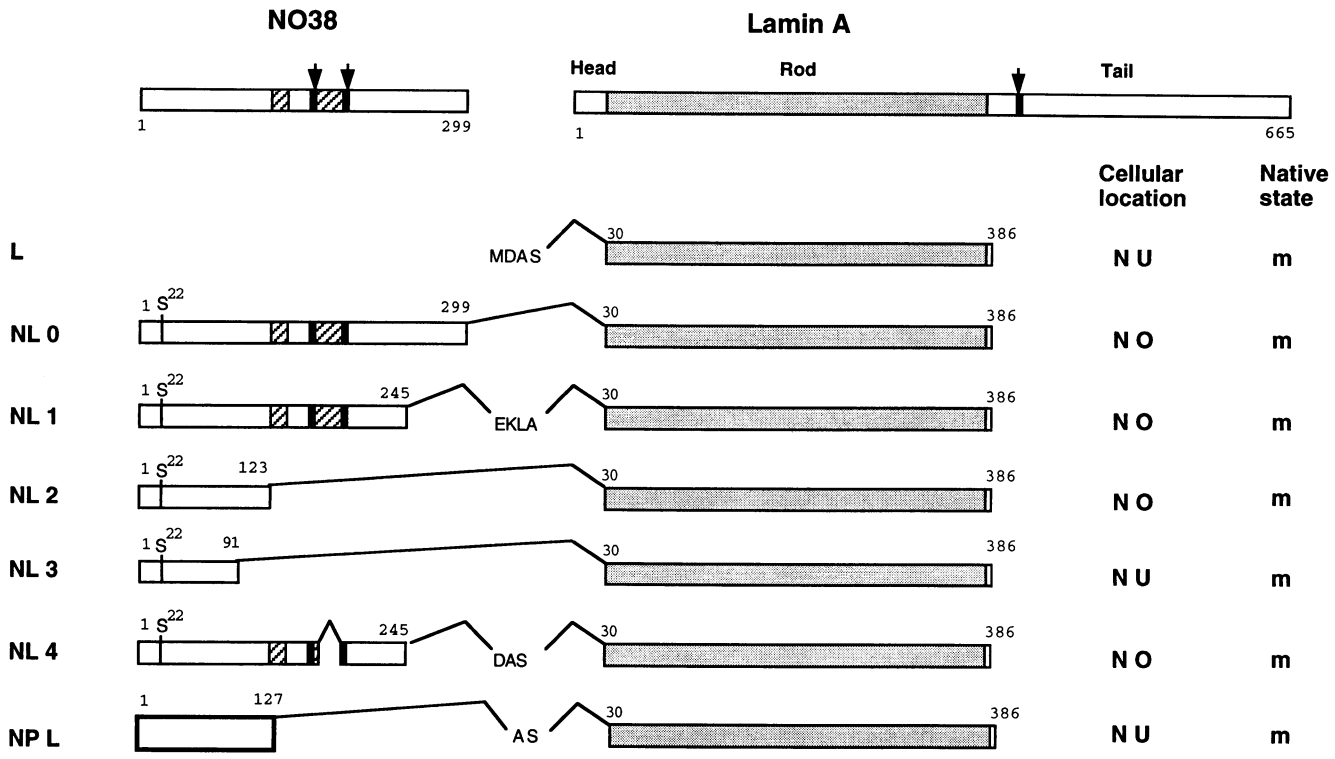


Figure 4. Constructs encoding chimeric proteins for analyses of intracellular distribution in cell transfections. Schematic drawing of chimeric proteins containing domains of nucleolar protein NO38 (as in Figure 1) fused to the rod (L) domain of *Xenopus* lamin A (NL0-4) whose major domains are denoted Head, Rod, and Tail. The first and last aa residues of the specific constituent polypeptides are given by numbers, internal deletions and substitutions of Cys^{22} in wt protein NO38 by Ser are indicated. The gray region in lamin A indicates the α-helical rod domain, and the NLS sequence identified in the tail region (cf. Loewinger and McKeon, 1988) is shown as a black bar (arrow). Linker sequences are given in one-letter codes. Construct NP-L consists of the amino-terminal half of *Xenopus* nucleoplasmin, which shows high sequence homology to protein NO38, fused to the lamin A rod domain.

Protein NL1 and wt NO38 were cotranslated in vitro and NL1 was immunoprecipitated from the translation mixture with lamin antibodies $L_{A386}I$. No wt protein NO38 was coprecipitated, indicating that no hetero-oligomeric complexes had been formed. Moreover, when the chimeric proteins translated in vitro were analyzed by sucrose gradient centrifugation, they sedimented with 3–4S, again indicative of a monomeric state (our unpublished results).

Localization Analyses Using Protein NO38-Lamin Chimeras

To determine the regions in protein NO38 required for nucleolar accumulation, we transiently transfected human PLC cells with DNA encoding the various chimeric proteins and examined their intracellular distribution with lamin antibodies $L_{A386}I$, which do not cross-react with human lamins. Some of our results obtained by immunofluorescence microscopy are shown in Figure 5. First, we analyzed the intracellular distribution of full-length NO38 fused to the lamin A rod. The chimeric protein was found almost exclusively in nucleoli of transfected cells (Figure 5, A and A′). Chimera NL1, which consists of NO38 aa 1–245 fused to the lamin A rod, resulted in a typical nucleolar immunostaining (Figure 5B′), as demonstrated by colabeling with nucleolus-specific human antibodies (Figure 5B).

This result clearly indicates that the far carboxyl terminus of protein NO38 is not required for nucleolar accumulation, at variance with the report of Peculis and Gall (1992). Practically identical results were obtained with chimera NL2 containing only the amino-terminal 123 aa of protein NO38. Obviously, this chimeric protein, which lacks all of the protein NO38 characteristics such as the two large acidic clusters and the NLS (Schmidt-Zachmann *et al.*, 1987), still contains a sufficient nucleolus-addressing element (Figure 5, C and C′). The efficiency of the nucleolar uptake might be somewhat reduced since here in some experiments a weak nucleoplasmic staining was also visible. By contrast, no nucleolar location was observed when the amino-terminal domain was further shortened on its carboxyl-terminal side. For example, chimera NL3, in

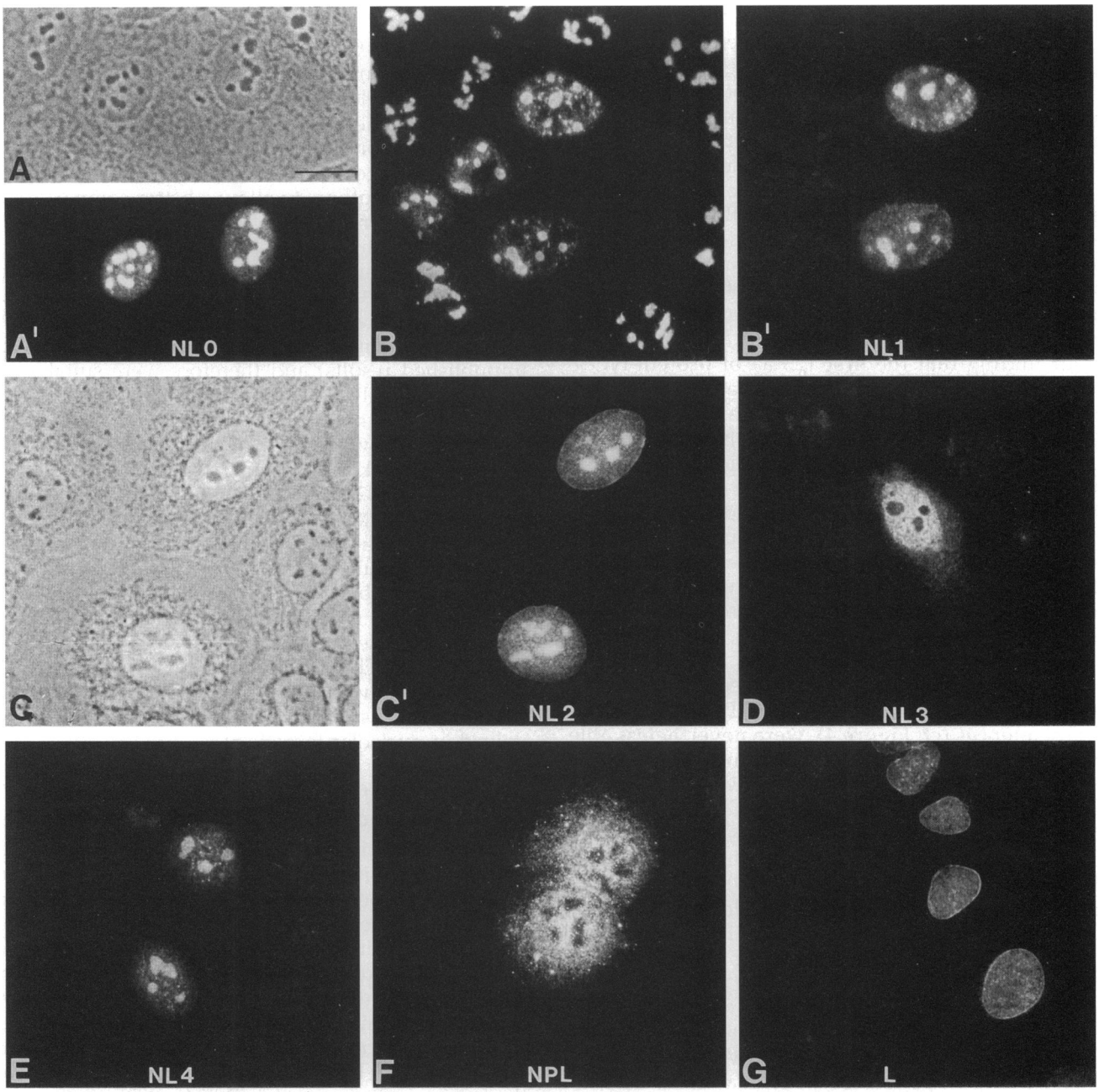


Figure 5. Micrographs presenting results of analyses showing topogenic differences between different NO38-lamin rod chimeric proteins upon transfection of PLC cells. Phase-contrast (A) and corresponding immunofluorescence (A′) microscopy of cultured human PLC cells transiently transfected with cDNA encoding chimera NL0 consisting of full-length NO38 (aa 1–299) fused to the lamin rod (cf. Figure 4). The chimeric protein is strongly enriched in nucleoli. Also, chimera NL1 lacking 54 aa from the carboxyl terminus of NO38 accumulates in nucleoli, showing that the monomeric form of protein NO38 lacking its carboxyl terminus directs the large lamin rod to the nucleolus. Here, for immunodetection nucleolus-specific human autoantibodies (SclC in B) and *Xenopus* lamin-specific guinea pig antibodies (L_{A386}I in B′) were used. A similar localization was observed for a chimera containing only the first 123 aa of protein NO38 (NL2; C, phase contrast; C′, same field in epifluorescence optics), indicating that the amino-terminal domain of NO38 is sufficient for nucleolar accumulation. In contrast, immunofluorescence microscopy of cells transfected with NL3-encoding DNA (protein NO38 aa 1–91 plus lamin rod) revealed that the protein no longer accumulated at the nucleolus but diffusely distributed throughout the nucleoplasm (D). Deletion of the large acidic domain of NO38 (NL4 in E) cannot prevent the corresponding chimeric protein from entering the nucleolus. In addition, however, small punctate nucleoplasmic aggregates are visible. When the lamin rod was fused to the amino-terminal domain of nucleoplasmin (NP-L), the resulting chimera accumulated in nuclei but was practically excluded from nucleoli (F). The lamin rod alone (mutation designated L in Figure 4) showed a diffuse nucleoplasmic staining in addition to a typical lamina staining but negative nucleoli (G). Bar, 15 μm (A, identical magnification in all photographs).

which aa 1–91 of protein NO38 were fused to the lamin A rod, showed a diffuse nucleoplasmic distribution without nucleolar binding (Figure 5D).

That the large acidic domain of protein NO38 is not necessary for nucleolar accumulation was also confirmed by the nucleolar immunostaining of chimera NL4 in which this region had been deleted (Figure 5E). Here, however, in addition to a strong nucleolar staining, distinct punctate nucleoplasmic fluorescence was also seen in many, but not all transfected cells. The formation of such small nucleoplasmic aggregates, which was occasionally also observed with other chimeras, may be due to overexpression of the specific proteins.

Our finding that the first 123 aa of protein NO38 were sufficient to direct the large lamin rod to the nucleolus was then controlled by transfections with lamin rod chimeras in which the NO38 part was replaced by the homologous portion (127 aa) of nucleoplasmin (Schmidt-Zachmann *et al.*, 1987). As shown in Figure 5F, cells transfected with this construct (NP-L) showed lamin immunofluorescence throughout the nucleoplasm, with negative nucleoli. This indicates that the different subnuclear distribution of these two closely related proteins is determined by aa sequence differences in the amino-terminal domain.

It was also quite surprising to see that chimeric proteins such as NL2, NL3, and NP-L, all without NLS sequences (cf. Figure 4), were nevertheless able to enter the nucleus. Moreover, transfections introducing the lamin rod alone (L) revealed that this domain was also able to reach the nucleus in the absence of the lamin NLS which is located in the carboxyl-terminal tail domain (Loewinger and McKeon, 1988; Krohne *et al.*, 1989). Interestingly, however, the protein showed, in addition to an enrichment at the lamina (i.e., rim-like staining), a significant uniform immunostaining throughout the entire nucleoplasm (Figure 5G).

To examine whether the nuclear uptake of such NLS-lacking proteins was due to transport as hybrid complexes of the chimeric protein with endogenous intact lamins, we performed double-label immunofluorescence microscopy using the *Xenopus*-specific antibodies $L_{A380}I$ and mAb L3-4B4 specific for human lamin A protein. In both cases, a bright nucleolar fluorescence was observed in cells expressing these chimeric proteins (our unpublished results), suggesting that this staining was due to hybrid complexes formed between the *Xenopus* lamin rod chimera and the complete endogenous human lamin (for discussion, see Holtz *et al.*, 1989; Loewinger and McKeon, 1989). This result also demonstrates the dominance of the nucleolus-addressing element in protein NO38 over the lamina assembly forces of intact lamin molecules.

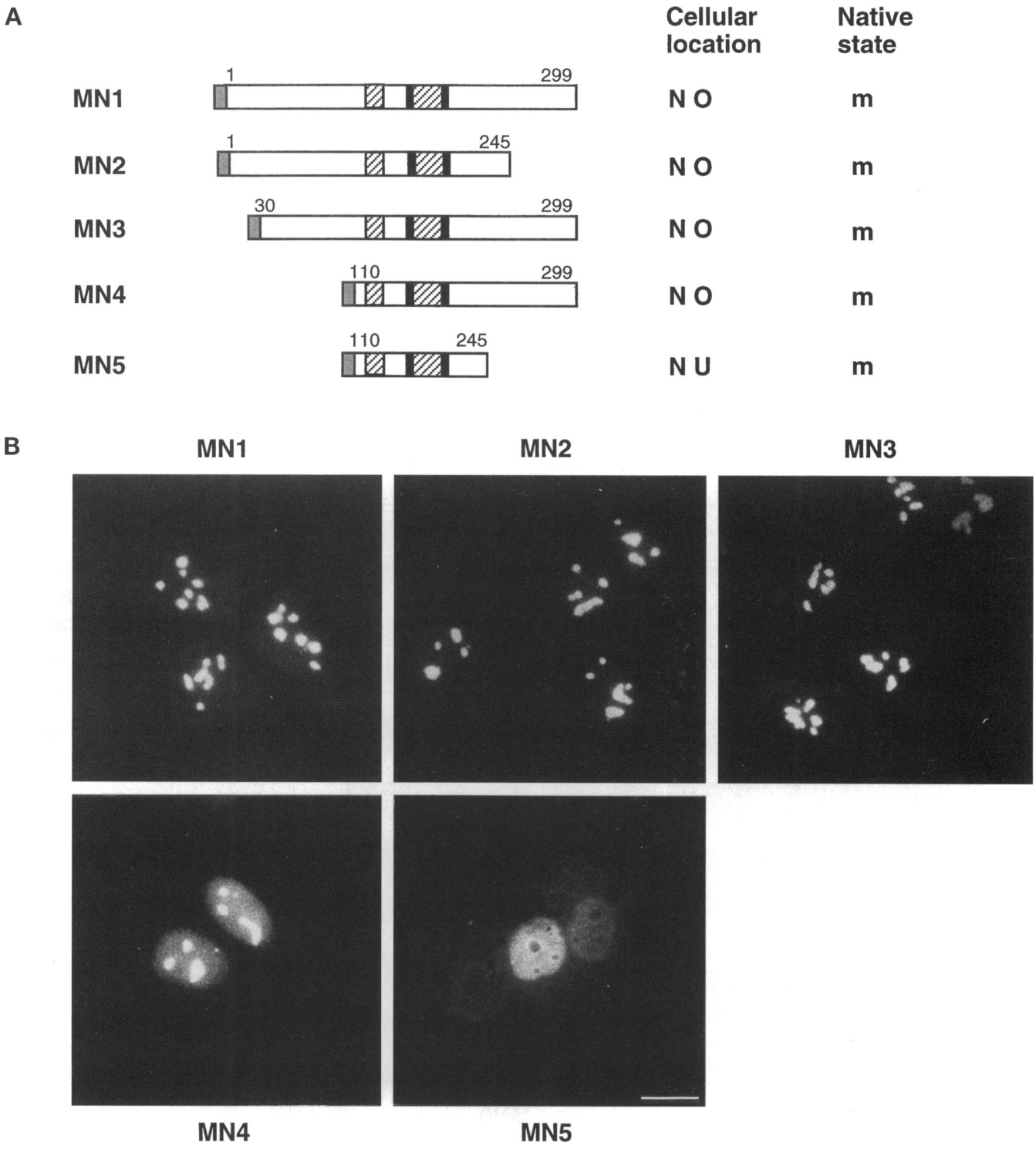
Localization Analyses of Protein NO38 Mutants with Myc Tags at the Amino-Terminus

Because in a study by Peculis and Gall (1992) a carboxyl-terminal domain of ~24 aa had been reported to direct the nucleolar localization of protein NO38, seemingly in contradiction to our results (see above), we decided to generate the same type of constructs as used by these authors to allow a better comparison of the results. Therefore, we fused the same myc tag to the amino-terminus of various NO38 mutants (e.g., MN1-MN5; Figure 6). In vitro translation and immunoprecipitation experiments showed, to our surprise, that these myc-tagged proteins were no longer able to form the oligomeric structures described above (our unpublished results) and therefore were suitable for transfection analysis. The results presented in Figure 6 clearly demonstrate that the myc tag did not alter the distribution of wt protein NO38 (MN1) and of mutated forms lacking different aa numbers at the carboxyl- (e.g., MN2, Δ54 aa) terminus or the amino- (e.g., MN3, Δ29 aa; MN4, Δ109 aa) terminus. The corresponding proteins still localized efficiently to nucleoli. Interestingly, further truncation of mutant MN4 by deleting the carboxyl-terminal 54 aa residues resulted in protein MN5 which was no longer able to accumulate in the nucleolus, but remains exclusively in the nucleoplasm. The intracellular distribution of the MN5 protein suggested to us that probably two different distinct structural domains of NO38 are involved in nucleolar targeting.

Identification of Two Structural Elements Required for Nucleolar Accumulation

Since the definition of a "signal localization sequence" requires its positive identification by a chimeric construct which consists of the minimal signal sequence and of a reporter protein, we analyzed the nucleolar targeting capacities of the two molecular domains of protein NO38 under question by generating fusion proteins containing either the amino- or the carboxyl-terminal domain of protein NO38. To assess whether the carboxyl-terminal region of NO38 does influence the intracellular localization of non-nucleolar proteins, the corresponding sequence (aa 246–299) was fused to nucleoplasmin and the cytoplasmic protein pyruvate kinase, modified to contain the SV40-NLS (MN6 and MN7; Figure 7A). Remarkably, PLC cells expressing these chimeric proteins showed a strong nucleolar fluorescence when probed with the myc antibody,

Figure 6 (on facing page). Construction and subcellular localization studies of myc-tagged deletion mutants of the nucleolar protein NO38. (A) Schematic drawing of myc-tagged mutants of protein NO38 (first and last aa of NO38 are given in numbers). The myc tag fused in-frame to the amino-terminus of NO38 is indicated as a gray box. The cellular location and the native state of the individual



(Figure 6 cont). polypeptides are listed on the right (NO, nucleolus; NU, nucleoplasmic, m, monomeric). (B) Subcellular localization of different mutated forms of protein NO38 synthesized in PLC cells transfected with the specific construct cDNA and detected after 24 h with mAb 9E10 against the myc tag. Fusion of the myc tag to the wt protein NO38 did not alter its intracellular distribution because it was still found exclusively in the nucleolus (MN1). Deletions at both ends of protein NO38 (MN2; MN3) also did not prevent the resulting truncated proteins from entering the nucleolus. Even after removal of the amino-terminal half, including almost the entire oligomerization domain, the residual protein still localized to the nucleolus (MN4). Here, a weak but significant nucleoplasmic staining is also visible. In contrast, a protein lacking considerable portions of both terminal domains (MN5) accumulated in the nucleoplasm but not in the nucleolus. Bar, 20 μm.

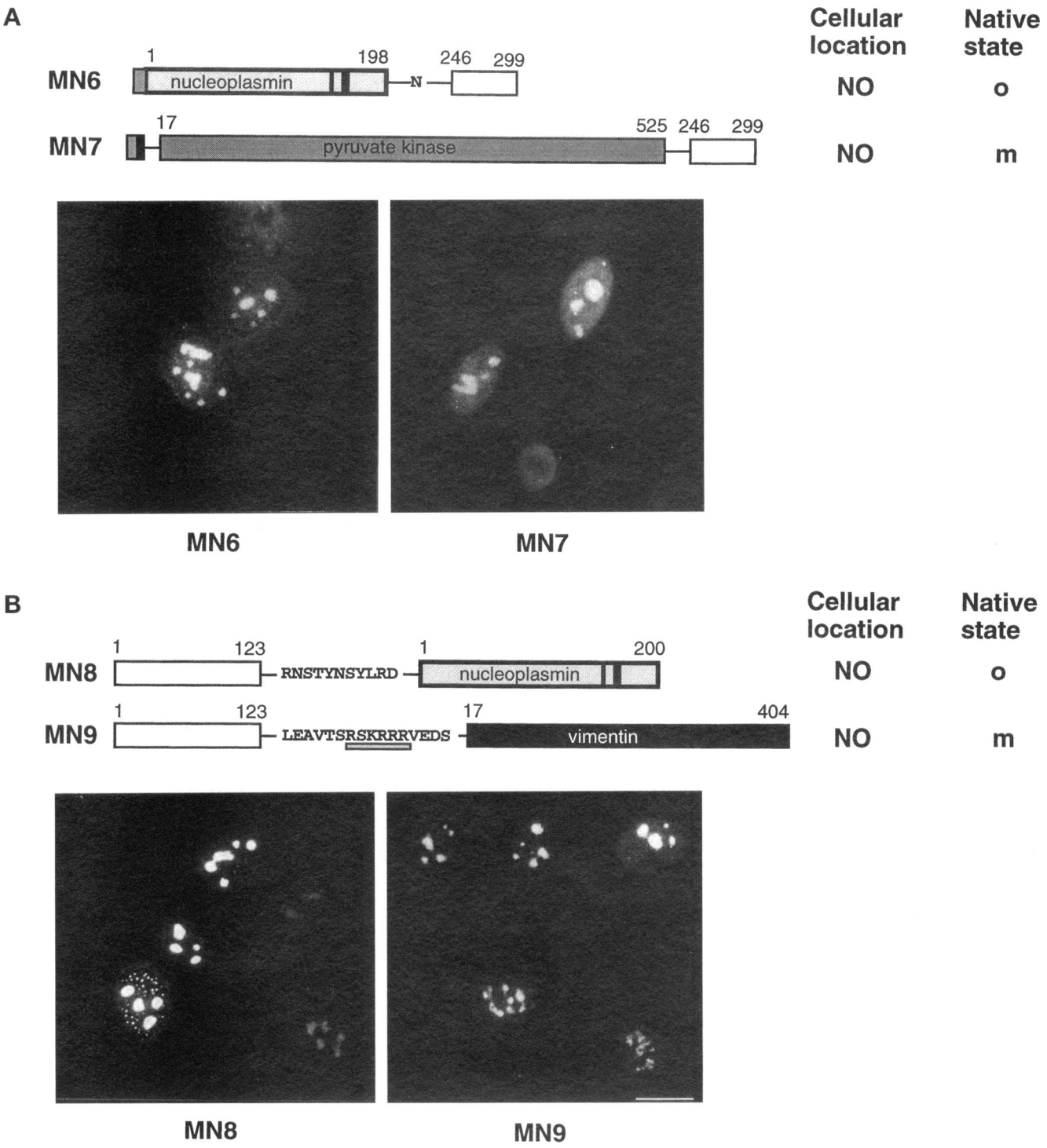


Figure 7. Identification of two distinct molecular segments of protein NO38 involved in nucleolar accumulation. (A) Schematic presentation of fusion protein constructs containing the carboxyl terminal element of NO38 (aa 246–299) fused either to the histone-binding protein, nucleoplasmin (MN6), or to the cytoplasmic protein pyruvate kinase (MN7) which also contains the SV40-NLS (black box) to guarantee nuclear uptake of the fusion protein. Both constructs carried the myc tag (gray box). Amino acid positions at termini and junctions are indicated by numbers. Cellular localization and native forms are summarized on the right (NO, nucleolar; o, oligomeric; m, monomeric). Immunofluorescence micrographs of PLC cells transfected with these cDNAs using mAB 9E10 revealed that both fusion proteins were directed to the nucleolus, also confirming the effect of the carboxyl terminus of protein NO38 for nucleolar localization (cf. Peculis and Gall, 1992). (B) Schematic drawing of fusion protein constructs containing the amino-terminal region of protein NO38 (aa 1–123) fused to

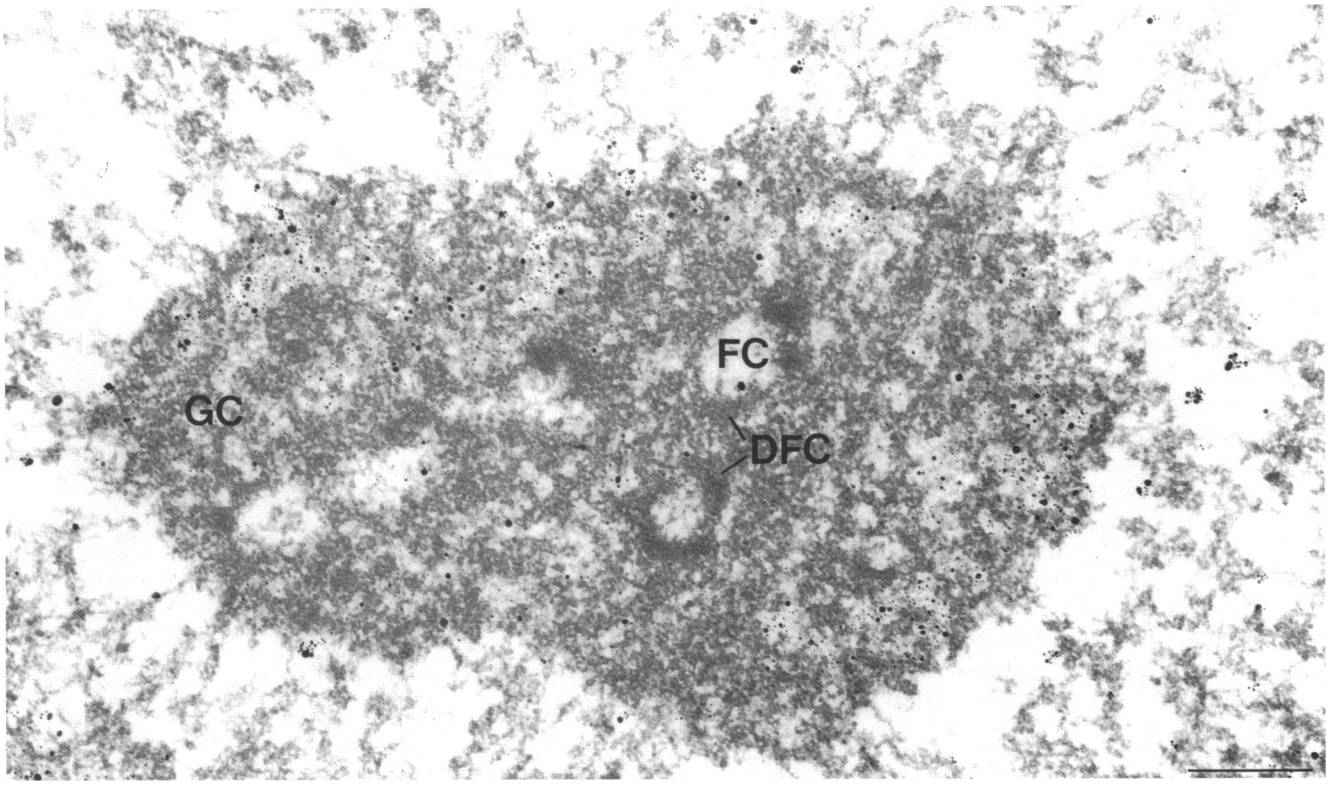


Figure 8. Immunoelectron microscopy of mutant MN9 in the nucleolus of human PLC cells stably transfected to synthesize the chimeric protein containing the *Xenopus* vimentin rod. The mAb 14.13.8 directed against *Xenopus* vimentin was detected by secondary antibodies coupled to 5 nm gold particles. The GC is specifically labeled, whereas fibrillar centers (FC) and dense fibrillar components (DFC) are practically free of gold particles. Bar, 0.5 μm.

indicating that this molecular region of NO38 does play an important role for the nucleolar accumulation of the protein.

Because we knew that the amino-terminus of protein NO38 is able to redirect a lamin rod to the nucleolus (cf. NL2, Figure 5, C and C'), we constructed further chimeric proteins consisting of aa 1–123 of protein NO38 fused to nucleoplasmin (MN8) or to the rod domain of the cytoskeletal filament protein vimentin (MN9). Again, the two reporter proteins were redirected to the nucleolus (Figure 7B).

Subnucleolar Localization of the Chimeric Protein MN9

To investigate in greater detail the intranucleolar distribution of chimeric proteins containing one of the nucleolar-addressing elements identified in protein NO38, immunoelectron microscopy was performed on PLC-cells stably transfected with the construct MN9 combining the amino-terminal region of NO38 with the rod domain of vimentin (cf. Figure 7B). The chimeric protein, detected by a mAb against *Xenopus* vimentin and 5 nm immunogold particles, was mostly concentrated in the GC of the nucleolus, whereas the fibrillar centers and the dense fibrillar component were practically devoid of gold particles (Figure 8). This result clearly indicates that the amino-terminal part of NO38 is not only able to direct a cytoskeletal protein to the nucleolus but also determines its specific intranucleolar distribution, indistinguishable from the intranucleolar localization of native protein NO38 (cf. Schmidt-Zachmann *et al.*, 1987).

Chimeric Protein MN9 Does Not Interact with Endogenous wt-NO38 In Vivo

Because the region of protein NO38 (B23) essential for its oligomerization is located near the amino-terminus (aa 15–123; see above), one might argue that the appearance of the chimeric protein MN9, consisting of the rod domain of vimentin fused to the amino-termi-

(Figure 7 cont). nucleoplasmin (MN8) and the vimentin rod domain (MN9). Construct MN9 also contains a lamin-type NLS denoted in the linker region. Immunofluorescence microscopy of PLC cells transiently producing the resulting chimeric proteins was carried out using either mAb b7-1A9-M9 against nucleoplasmin (MN8) or mAb 14.13.8 against *Xenopus* vimentin (MN9). Notice that both reporter proteins were specifically and efficiently accumulated in the nucleolus. Bar, 20 μm.

nal part (aa 1–123) of NO38, could be simply explained by the formation of heterotypic complexes between protein MN9 and the endogenous wt protein NO38 present in the transfected cells. Although none of our in vitro assays (gel electrophoresis, coimmunoprecipitations) supported this possibility, we nevertheless examined the oligomerization properties of mutant MN9 in vivo. The fusion protein from PLC cells stably transfected with construct MN9 was immunoprecipitated using mAb No-185 directed against *Xenopus* NO38. On analysis of the resulting fractions by immunoblotting using mAB 14.13.8 against *Xenopus* vimentin (Figure 9A), the MN9 protein was exclusively detected in the immunoprecipitate obtained from transfected cells (Figure 9A, lane 5). When the same nitrocellulose filter was reprobed with the mAb against human NO38 (B23), the endogenous wt protein was found in the supernatant fractions of untransfected as well as transfected cells (Figure 9B, lanes 3 and 6). We conclude that the chimeric protein MN9 and wt NO38 (B23) do not form hybrid complexes in vivo and therefore the appearance of the vimentin rod in the nucleolus is correlated to the presence of the amino-terminal nucleolar localization element of protein NO38.

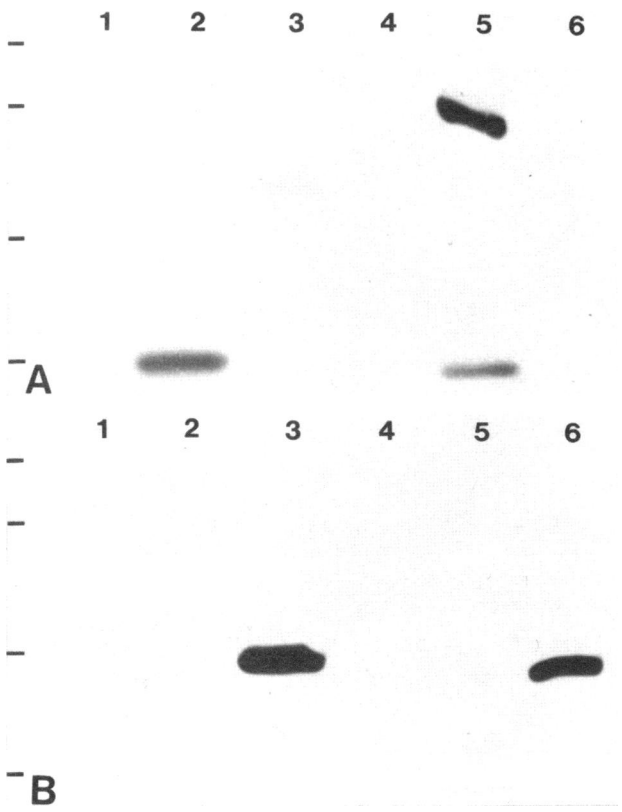


Figure 9. Analysis of the oligomerization properties of fusion protein MN9 in vivo. Cellular extracts from untransfected human PLC cells (lanes 1–3) and cells stably transfected with DNA coding for the chimeric protein MN9 (lanes 4–6) were subjected to immunoprecipitation experiments using mAb No-185 against *Xenopus* NO38. The resulting fractions (10% of the preclearing fraction, lanes 1 and 4; 10% of the pellet fraction, lanes 2 and 5; 4% of the supernatant fraction, lanes 3 and 6) were separated by SDS-PAGE, transferred to nitrocellulose, and probed with mAbs 14.13.8 (A) and subsequently with α-human B23 (B). The fusion protein MN9 (55 kDa) is detectable in the pellet fraction obtained from transfected cells (A, lane 5), whereas endogenous wt protein NO38 (B23) is exclusively present in the supernatant fractions (B, lanes 3 and 6); i.e., it does not coprecipitate with the fusion protein. The faint bands in A represent low amounts of the IgG light chain of the antibody used for immunoprecipitation. Bars indicate the positions of reference proteins from top to bottom: phosphorylase b (97.4 kDa), BSA (66 kDa), ovalbumin (45 kDa), and carbonic anhydrase (29 kDa).

DISCUSSION

Although the chemical prerequisites and the structural components responsible for the specific nuclear uptake of a given protein are relatively well understood (for recent reviews, see INTRODUCTION), the molecular and structural principles governing the intranuclear topogenesis of a specific nuclear protein are still unclear. In particular, it is not known whether the distribution of a protein within the nucleus, notably its association with a specific structure, is determined by a distinct feature of its primary amino acid sequence in the sense of a topogenic signal sequence. To address this question for a ubiquitous major nucleolar hallmark protein, we have constructed a series of mutants and chimeras of the nucleolar protein NO38 (B23) and analyzed them with respect to their subcellular localization. Using deletion mutants and different reporter protein strategies including chimeras with large stable structures such as the ~50-nm long α-helical coiled coil rod domains of the nuclear lamina protein, lamin A, and the cytoplasmic filament protein, vimentin, or with short labels such as the myc tag, we have determined that two independent domains of protein NO38, located at the amino- and the carboxyl-terminus, are each necessary and sufficient for the accumulation in the nucleolus. The significance of the existence of two independent nucleolar address sequence regions in the same protein, NO38 (B23), is especially clear from our chimeras with the closely related "sister" protein, nucleoplasmin, which despite the overall sequence homology is notoriously nucleoplasmic, excluding the nucleolus. It becomes obvious from our results and the studies of others that nucleolar localization is at least a two-step process: NLS-mediated nuclear translocation and subsequent nucleolar accumulation requiring protein domains promoting binding to nucleolar structures. Moreover, our study has also shown that after nuclear uptake of a given protein the principles responsible for nucleolar accumulation of eu- and ectopic proteins are not necessarily dependent on the presence of an established NLS sequence (cf. con-

structs NL2, NL3), indicative of a dominance of these nucleolar addresses.

In the course of our studies, we found it critically important to consider that the topogenic information in nucleolar protein NO38 (B23), as in any other nuclear protein, is not dependent on the oligomerization of the specific protein construct or on any other form of complex with endogenous NO38 (B23) protein or other nuclear proteins. Although our results exclude a piggyback nuclear uptake and nucleolar accumulation of stable complexes, homotypic or heterotypic, we cannot exclude an involvement of short-lived transient carrier complexes (for NLS-binding proteins, see Adam and Gerace, 1991; Moore and Blobel, 1992; Görlich *et al.*, 1994, 1995).

Obviously, our results are partly confirmatory to, and partly somewhat at variance with, those reported by Peculis and Gall (1992) who concluded from injections of myc-tagged constructs of wt and deleted protein NO38 into newt oocytes that the nucleolar association was dependent on a 24-aa long region preceding the 11-aa carboxyl-terminal piece. We have no compelling explanation for this difference but cannot exclude that they may be due to the different cell systems used. We emphasize, however, that we have positively shown, in diverse deletions and protein combinations, the nucleolar accumulation potential of both structural domains identified in protein NO38, the amino- and the carboxyl-terminal one, whereas the assumed nucleolar-addressing element proposed by Peculis and Gall (1992) was reportedly not sufficient to effect the nucleolar accumulation of non-nucleolar reporter proteins.

Detailed analysis of our deletion experiments has revealed that the first 123 aa of protein NO38 (B23) are sufficient to guarantee nucleolar accumulation, whereas upon truncation of further 32 aa the remaining piece of 91 aa could no longer effect nucleolar enrichment of the reporter protein. On the other hand, the amino-terminal 29 aa are not needed for nucleolar topogenesis and even the deletion of as much as the amino-terminal 109 aa did not interfere with the appearance of the residual protein in the nucleolus. This indicates that aa residues 110–123 are essential for this topogenic function, but we have also noticed that this short element alone is not sufficient to secure nucleolar accumulation. In contrast, the last 54 aa of protein NO38 (B23) are able to direct the cytoplasmic protein pyruvate kinase as well as nucleoplasmin into the nucleolus.

What is clear from a comparison of the aa sequences of the two nucleolar address-contributing regions of protein NO38 (B23) is that the sequences under question do not comprise any possible NLS-like element, as reported for the putative nucleolar-addressing sequences in viral proteins of HIV (Rev and Tat) and human T-cell leukemia virus (Rex; for references, see INTRODUCTION), the human nucleolar protein p120 (Valdez *et al.*, 1994), and the ribosomal proteins L31 (Quaye *et al.*, 1996) and S6 (Schmidt *et al.*, 1995). Moreover, the regions containing the nucleolar address segment of protein NO38 do not reveal any clustering of basic aa or any known nucleic acid-binding motif as reported for other nucleolar proteins such as transcription factor mUBF (Maeda *et al.*, 1992), nucleolin (Créancier *et al.*, 1993; Meßmer and Dreyer, 1993; Schmidt-Zachman and Nigg, 1993) and yeast nucleolar proteins NSR1 (Yan and Mélèse, 1993) and GAR1 (Girard *et al.*, 1994).

From these comparisons, we conclude that there is no "consensus sequence element" responsible for the specific nucleolar enrichment of these different proteins, nor is there any indication of a common mechanistic principle. Although some of these proteins may bind directly to rDNA or its transcription products, others may be enriched and retained in the nucleolus through protein–protein interactions. The nature of the molecules that keep protein NO38 (B23) in the nucleolus and the factors regulating its dissociation, e.g., during mitosis (Schmidt-Zachmann *et al.*, 1987) and upon drug-induced inhibition of rDNA transcription (Chan, 1992) as well as in the process of nucleocytoplasmic shuttling (Borer *et al.*, 1989), remain to be identified. Studies to identify possible binding partners of protein NO38 (B23) are currently performed.

It is remarkable that the topogenic nucleolar accumulation power located in protein NO38 (B23) is so strong that it even overrides other topogenic principles such as, in lamin rod chimeras, the tendency of sizable α-helical coiled coils to assemble in the nuclear lamina or the assembly drive of vimentin rods to associate with vimentin filaments (for nucleoplasmic accumulation of typical intermediate filament structures, see Bader *et al.*, 1991; Eckelt *et al.*, 1992; Rogers *et al.*, 1995), the cytoplasmic retention of pyruvate kinase, and the specific nucleoplasmic accumulation of nucleoplasmin. This opens the possibility to probe by constructing specific chimeras with protein NO38 local reactions in the nucleolar microenvironment and to introduce changes of nuclear and nucleolar architecture by transfection with engineered DNA molecules.

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