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A constitutive nucleolar protein identified as a member of the nucleoplasmin family

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Using monoclonal antibodies we have localized a polypeptide, appearing on gel electrophoresis with a M_r of ~38 000 and a pI of ~5.6, to the granular component of the nucleoli of *Xenopus laevis* oocytes and a broad range of cells from various species. The protein (NO38) also occurs in certain distinct nucleoplasmic particles but is not detected in ribosomes and other cytoplasmic components. During mitosis NO38-containing material dissociates from the nucleolar organizer region and distributes over the chromosomal surfaces and the perichromosomal cytoplasm; in telophase it re-populates the forming nucleoli. With these antibodies we have isolated from a *X. laevis* ovary λgt11 expression library a cDNA clone encoding a polypeptide which, on one- and two-dimensional gel electrophoresis, co-migrates with authentic NO38. The amino acid sequence deduced from this clone defines a polypeptide of 299 amino acids of mol. wt 33 531 which is characterized by the presence of two domains exceptionally rich in aspartic and glutamic acid, one of them flanked by two putative karyophilic signal heptapeptides. Comparison with other protein sequences shows that NO38 is closely related to the histone-binding, karyophilic protein nucleoplasmin: the first 124 amino acids have 58 amino acid positions in common. Protein NO38 also shows striking homologies to the phosphopeptide region of rat nucleolar protein B23 and the carboxy-terminal region of human B23. We propose that protein NO38, which forms distinct homo-oligomers of ~7S and M_r of ~230 000, is a member of a family of karyophilic proteins, the 'nucleoplasmin family'. It is characterized by its specific association with the nucleolus and might be involved in nuclear accumulation, nucleolar storage and pre-rRNA assembly of ribosomal proteins in a manner similar to that discussed for the role of nucleoplasmin in histone storage and chromatin assembly.

Key words: nucleolus/karyophilic proteins/nucleoplasmin/ribosomal proteins/ribosome assembly

Introduction

The proteins of the nucleocytoplasmic phase can be divided into three major groups according to their distribution (Gurdon, 1970; Bonner, 1975,1978; DeRobertis *et al.*, 1978; DeRobertis, 1983): (i) those remaining in the cytoplasm, be it due to assembly into large structures, to associations with other molecules or to 'karyophobic exclusion'; (ii) those equilibrating between both the cytoplasm and the nucleus: and (iii) those accumulating in the nucleus, be it by association with other nucleus-specific structural components or by 'karyophilic principles' (e.g. Dabauvalle and Franke, 1982,1986; Dingwall *et al.*, 1982; Feldherr *et al.*, 1984; Paine, 1984; Dingwall and Laskey, 1986). Of special importance in our understanding of the latter category have been the findings of relatively short amino acid sequences in a number of karyophilic proteins ('karyophilic signals'; Hall *et al.*, 1984; Kalderon *et al.*, 1984; Lanford and Butel, 1984; Krippl *et al.*, 1985; Lanford *et al.*, 1986; Richardson *et al.*, 1986).

Enrichment of a given protein in the nucleus, however, does not explain the intranuclear topological specificities of many proteins. Certain nuclear proteins are known to be located, predominantly or exclusively, in the chromosomes or special chromosome portions (e.g. centromeres, heterochromatin, specific transcriptional loops, scaffold structures; for review see Gasser and Laemmli, 1987), in the nuclear lamina (reviewed by Krohne and Benavente, 1986) and the pore complexes (e.g. Davis and Blobel, 1986). A nuclear structure of special complexity for which more detailed information of protein topology is available is the nucleolus. Recent immunolocalization studies have permitted assignment of certain proteins to the various nucleolar subcompartments as originally defined by electron microscopy, *in situ* hybridization of rDNA and autoradiographic studies of nucleotide incorporation into rRNA precursors (for reviews see Busch and Smetana, 1970; Goessens, 1984; Hadjiolov, 1985; Fakan and Hernandez-Verdun, 1986).

The 'fibrillar centers' (FC) are the interphase equivalent structures to the nucleolar organizer region (NOR) of metaphase chromosomes in that they contain rDNA as well as RNA polymerase I and certain argyrophilic proteins (Arroua *et al.*, 1982; Williams *et al.*, 1982; Derenzini *et al.*, 1983; Scheer and Rose, 1984; Spector *et al.*, 1984; Moreno *et al.*, 1985). The 'dense fibrillar component' (DFC), which is widely believed to contain ribonucleoprotein during pre-rRNA processing, is characterized by the presence of specific proteins such as a M_r 110 000 polypeptide, i.e. 'nucleolin' (also referred to as 'protein C23'; Prestayko *et al.*, 1974; Bugler *et al.*, 1982; Spector *et al.*, 1984; Escande *et al.*, 1985; Lapeyre *et al.*, 1986), an M_r 180 000 polypeptide (Schmidt-Zachmann *et al.*, 1984), 'fibrillarin' of M_r 34 000 (Lischwe *et al.*, 1985; Ochs *et al.*, 1985b) and several other proteins (e.g. Kistler *et al.*, 1984). The 'granular component' (GC) of the nucleolus has long been assumed to contain pre-ribosomal precursor particles in different stages of maturation (Fakan and Bernhard, 1971). This view has more recently been supported by *in situ* localizations of ribosomal proteins (Hügle, 1985; Hügle *et al.*, 1985a) and of ribocharin, an acidic protein of M_r 40 000 associated with the precursor of the large ribosomal subunit (Hügle *et al.*, 1985b; compare also Kistler *et al.*, 1984). In addition, an abundant phosphoprotein of M_r 37 000, termed B23 (Prestayko *et al.*, 1974; Busch, 1984; Spector *et al.*, 1984; Morris *et al.*, 1985; Fields *et al.*, 1986), has been localized to this region. The nucleolar periphery also contains a special kind of 8–12 nm 'cortical fibrils' (Moreno *et al.*, 1982) and is characterized by a major polypeptide of M_r ~145 000 (Franke *et al.*, 1981; Krohne *et al.*, 1982; Benavente *et al.*, 1984).

To gain more insight into the principles of the nucleolar topo-

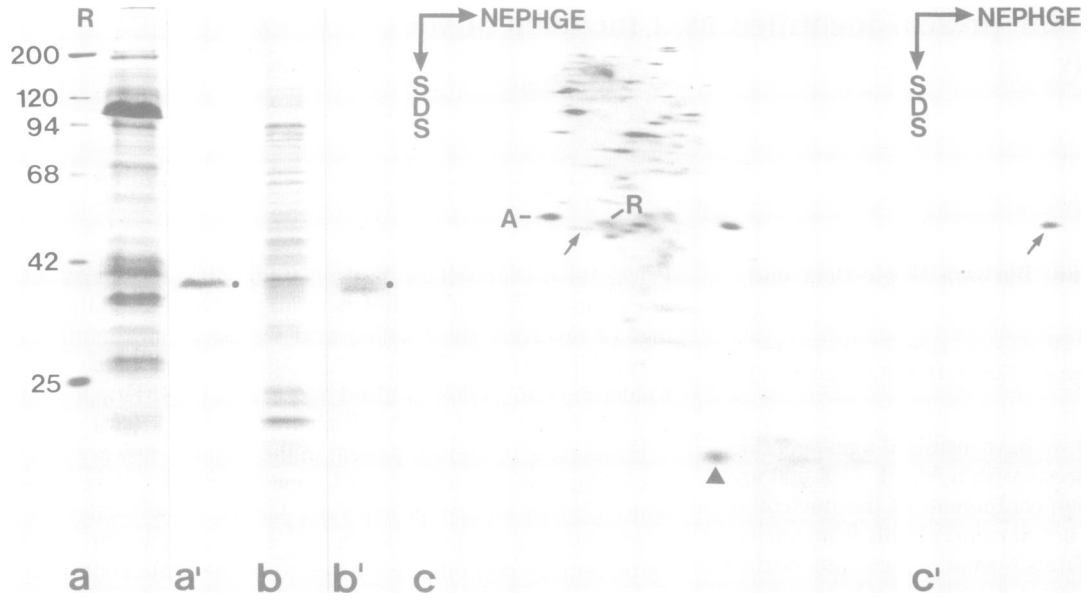

Fig. 1. Identification of the polypeptide recognized by No-185 in different nuclear fractions from *X. laevis* oocytes and in cultured cells by SDS−PAGE and immunoblotting. **(a)** Coomassie blue staining of proteins extracted from low speed pellet (LSP) with PBS containing 0.1 M KCl. Reference proteins (R) are, from top to bottom ($M_r \times 10^{-3}$): myosin heavy chain, β-galactosidase, phosphorylase a, bovine serum albumin (BSA), α-actin and chymotrypsinogen. **(a')** Corresponding autoradiograph obtained after immunoblotting with culture supernatant from hybridoma clone No-185. Note that antibody No-185 reacts selectively with a polypeptide of M_r 38 000. **(b)** Coomassie blue staining of proteins extracted from the LSP of cultured kidney epithelial cells (line A6) of *X. laevis* with PBS containing 0.1 M KCl. **(b')** Corrsponding autoradiograph after immunoblotting with No-185 antibody showing the same antigen which here appears as a doublet (M_r 38 000 and M_r 40 000). The second band may represent a different degree of phosphorylation. **(c)** Polypeptides of the high speed pellet (1 h; 100 000 *g*) from nuclear homogenates of *X. laevis* oocytes were separated by two-dimensional gel electrophoresis (first dimension, non-equilibrium pH gradient, NEPHGE; second dimension, electrophoresis in 18% polyacrylamide gels in the presence of SDS) and stained with Coomassie Blue. A, α-actin; R, ribocharin; the arrowhead denotes added pancreatic RNase. **(c')** Corresponding immunoblot to the gel shown in **(c)**. The position of the antigenic polypeptide of M_r 38 000 with an apparent pI of 5.6 is indicated by arrows in **(c)** and **(c')**.

genesis and the functional organization within the nucleolus, we have produced a panel of monoclonal antibodies against nucleolar proteins from oocytes of *Xenopus laevis*, the classic object of nucleolar research because of the high number and activity of amplified extrachromosomal nucleoli. In the present study we introduce two monoclonal antibodies which specifically react with a nucleolar non-ribosomal protein of M_r ~38 000 located in the granular component of the interphase nucleolus and in precursor particles of both the small and large ribosomal subunits. Analysis of the amino acid sequence deduced from a cDNA clone encoding this protein has revealed an unexpected homology to nucleoplasmin, an abundant histone-binding karyophilic protein. This is the first non-ribosomal protein of the nucleolus whose complete amino acid sequence has been determined.

Results

Monoclonal antibodies reacting with protein NO38

Two monoclonal antibodies (No-63, IgG2a; No-185, IgG1) which reacted with the same nucleolar protein were used. In immunoblotting experiments of SDS−PAGE-separated proteins, both antibodies reacted with a polypeptide of M_r ~38 000 present in oocytes as well as in various somatic cells of *X. laevis* (Figures 1a,a',b, b'). In cultured epithelial cells (line A6) of *X. laevis* an additional minor polypeptide of M_r ~40 000 was reactive (Figure 1b,b'). Immunoblot analysis of nuclear proteins separated by two-dimensional gel electrophoresis showed that the polypeptide (NO38) was acidic (Figure 1c,c') with an isoelectric pH (in 9.5 M urea) of ~5.6, as determined by isoelectric focusing (data not shown).

When different nuclear fractions from *X. laevis* oocytes were analysed by enzyme-linked immunosorbant assay (ELISA) and immunoblotting for the presence of the antigen, ~52% of protein NO38 was recovered in the nucleoli-enriched low speed pellet (LSP), ~33% in the subsequent high speed pellet (HSP) that also contained the nucleoplasmic pre-ribosomal particles and ~15% in the high speed supernatant (HSS) containing the soluble nuclear proteins. The protein could be extracted from pellets at elevated ionic strength (0.2−0.3 M alkali salt).

The specificities of the antibodies were also determined by immunoprecipitation of HSP proteins solubilized in buffers of elevated ionic strength. While No-185 precipitated only protein NO38, immunoprecipitates obtained with No-63 (data not shown) contained, to our surprise, NO38 and an additional polypeptide of M_r ~30 000 which we identified as nucleoplasmin, the major nucleoplasmic protein of *X. laevis* oocytes (Laskey *et al.*, 1978; Earnshaw *et al.*, 1980; Krohne and Franke, 1980a,b; Mills *et al.*,1980). These results also suggest that No-63 and No-185 recognize two different epitopes in NO38.

Immunolocalization

Immunofluorescence microscopy using antibodies No-63 or No-185 on cultured epithelial cells of *X. laevis* (Figure 2a,a'), chicken fibroblasts (Figure 2b,b') and marsupial PtK_2 cells (data not shown) as well as on frozen sections of tissues of various species (Figure 3a−d) revealed the nucleolar localization of protein NO38. No reaction was seen in late spermatids and spermatozoa, in agreement with the absence of nucleoli in these cells (Figure 3b,b'). Amphibian erythrocytes (Figure 3c,c') which contain only 'residual nucleolar structures' and are believed to be transcriptionally inactive, revealed one or two small immunoreactive dots that seemed to correspond to the 'residual nucleo-

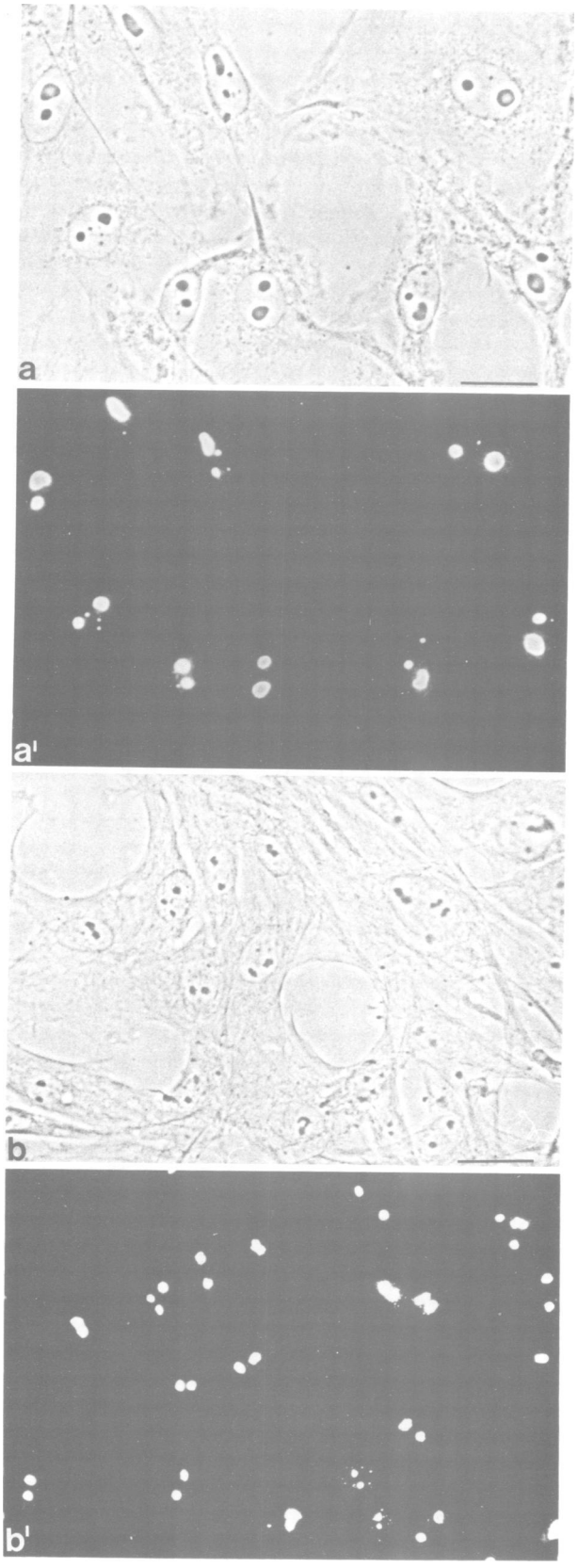

Fig. 2. Immunofluorescence microscopy using a monoclonal antibody (No-63) to protein NO38 on cultured kidney epithelial cells of line A6 of X. *laevis* **(a,a')** and chicken fibroblasts **(b,b')**. Corresponding phase contrast **(a,b)** and epifluorescence **(a',b')** pictures are shown. Bright nucleolar fluorescence is seen which often appears to be more concentrated in the nucleolar periphery. In addition, small fluorescent dot-like nucleoplasmic structures are sometimes seen. Bars, 20 μm.

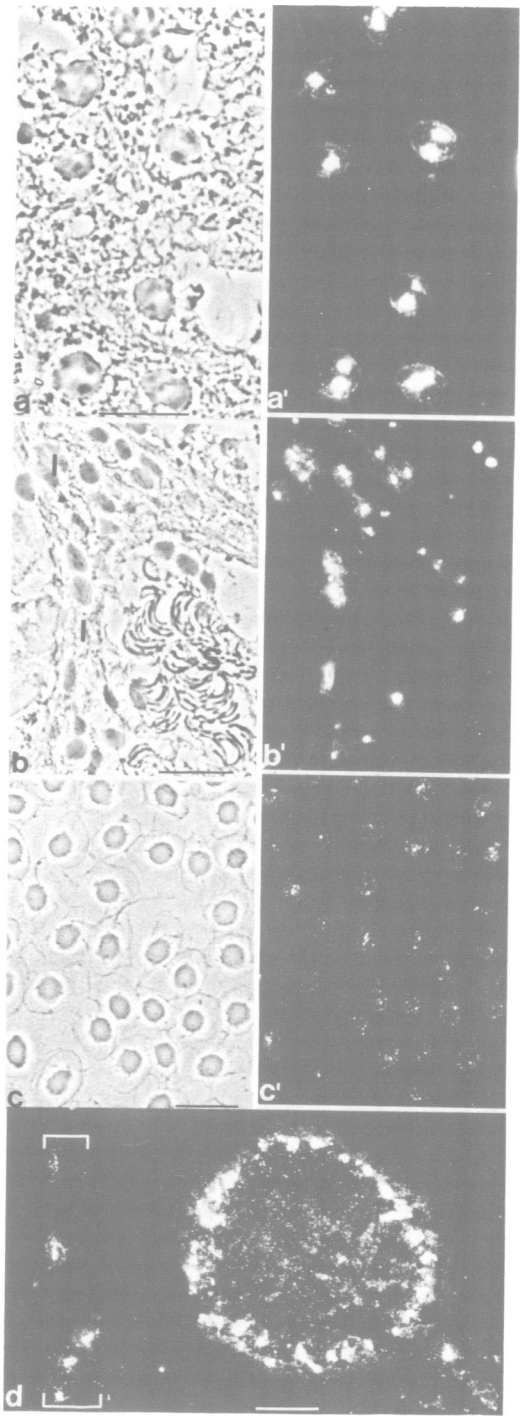

Fig. 3. Immunofluorescence microscopy showing the localization of protein NO38 in frozen sections of amphibian tissues and in blood smears of *X. laevis*. **(a,a')** Frozen section of liver tissue of *Pleurodeles waltlii* in phase-contrast **(a)** an epifluorescence **(a')** optics, showing bright fluorescence of nucleoli and a few small particles in the nucleoplasm. **(b,b')** In frozen sections of testicular tissue of *X. laevis* (**b**, phase contrast; **b'**, epifluorescence) positively stained nucleoli and small nucleoplasmic granules are seen in interstitial (I, interstitial space) and Sertoli cells, as well as spermatogonia and spermatocytes, whereas spermatids and spermatozoa (S) are negative. **(c,c')** Nuclei of *X. laevis* erythrocytes (**c**, phase-contrast) contain one or two brightly fluorescent small dots (**c'**, epifluorescence optics) corresponding to the 'residual nucleolar structures' (antibody No-185). **(d)** Fluorescence micrograph of a frozen section through ovary tissue of *Triturus cristatus*, showing the strongly stained amplified nucleoli, most of them located at the periphery of the oocyte, and some fluorescent smaller nucleoplasmic entities. In addition, the nucleoli of the surrounding epithelial cell layer (demarcated by brackets) are also stained. Bars, 20 μm.

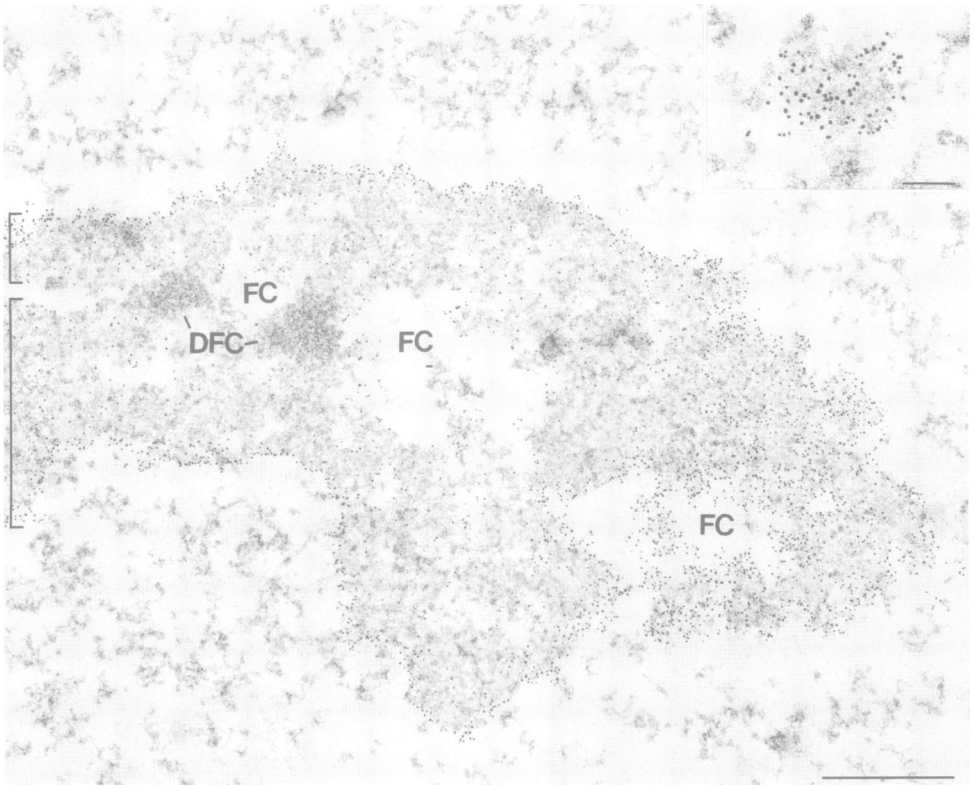

Fig. 4. Immunoelectron microscopy of protein NO38 in the nucleolus of a hepatocyte in a frozen section through *T. cristatus* liver tissue reacted with monoclonal antibody No-185 (pre-embedding, 5 nm gold particles). The granular component (indicated by brackets) is specifically labelled, whereas fibrillar centres (FC) and dense fibrillar components (DFC) are free of gold particles. In addition, some distinct granular aggregates in the nucleoplasm are also decorated by the gold particles (insert in the upper right). Bars denote 0.4 μm and 80 nm (insert).

lar structures' described in erythrocytes of amphibia and birds (Laval *et al.*, 1981; Schmidt-Zachmann *et al.*, 1984). The latter have been reported to be inactive in nucleolar transcription and lack RNA polymerase I (e.g. MacLean *et al.*, 1973; Zentgraf *et al.*, 1975; Krüger and Seifart, 1977; Hentschel and Tata, 1978; MacLean and Gregory, 1981).

To determine the localization of protein NO38 with greater precision, immunoelectron microscopy was performed by using secondary antibodies coupled with colloidal gold particles. The antibody label was exclusively associated with the GC, often showing an enrichment at the nucleolar surface (Figure 4 presents, for example, the nucleolus of a hepatocyte of *Triturus cristatus*). Outside of the nucleolus, certain distinct small aggregates found to be decorated by the gold particles (insert in Figure 4) are probably identical to similar structures described as containing ribosomal proteins and ribocharin (Hügle *et al.*, 1985a,b).

The specific GC localization of NO38 was confirmed by immunolocalization in segregated nucleoli of *X. laevis* A6 cells treated with actinomycin D (data not shown; cf. Schmidt-Zachmann *et al.*, 1984; Hügle *et al.*, 1985a).

Biochemical characterization of protein NO38 in soluble form and in pre-ribosomal particles

When protein NO38 solubilized from LSP or HSP fractions at elevated ionic strength was analysed by sucrose gradient centrifugation, it appeared with a sedimentation coefficient of ~7S, indicative of an oligomeric state (data not shown). Gel filtration revealed a M_{app} of ~550 000 and, using the equation of Monty and Siegel (1966), a mol. wt of 200 000 for the solubilized protein was calculated from these values. Protein NO38 was also found in pre-ribosomal particles fractionated by sucrose gradient centrifugation. NO38 was present in both 40S and 65S particles, i.e. the precursors for the small and large ribosomal subunits, respectively (Figure 5; Hügle *et al.*, 1985b).

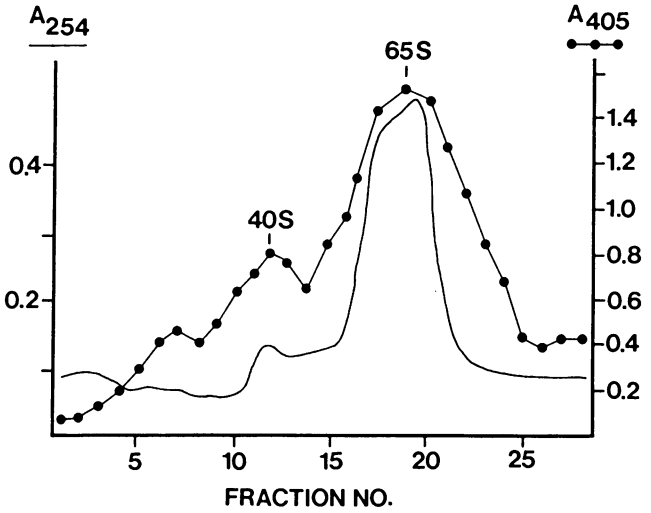

Fig. 5. Immunological identification of the nucleolar protein NO38 in free nucleoplasmic 40S and 65S pre-ribosomal particles by sucrose gradient (10–40%) centrifugation using monoclonal antibody No-63. Material of high speed pellets (HSP) from oocyte nuclear homogenates was re-dispersed in buffer and fractionated by sucrose gradient centrifugation. Pre-ribosomal particles appear with 40S and 65S (A_{254}, absorbancy at 254 nm; cf. Hügle *et al.*, 1985b). The antigen recognized by antibody No-63 is recovered in both the 40S and 65S particles (A_{405}, absorbancy at 405 nm, for determination of ELISA activity).

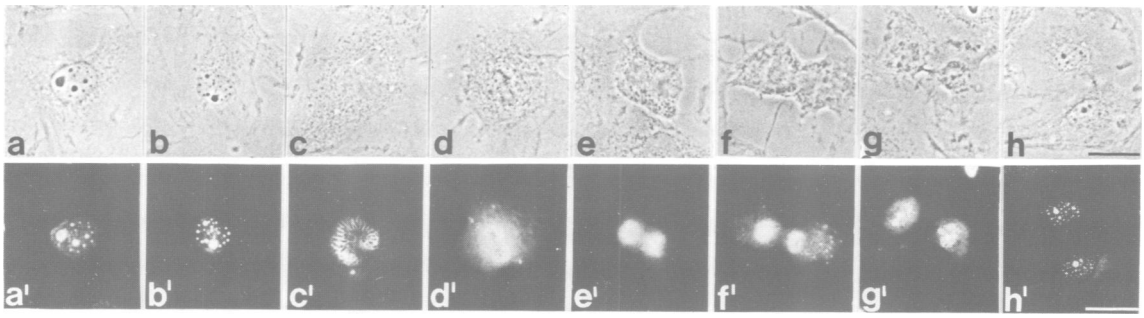

Fig. 6. Distribution of nucleolar protein NO38 in kidney epithelial cells (line A6) of *X. laevis* during mitosis as visualized by immunofluorescence microscopy using antibody No-63. Phase-contrast images **(a–h)** and corresponding immunofluorescence micrographs **(a'–h')** are shown. In interphase **(a,a')**, brightly stained nucleoli and small nucleoplasmic dots can be observed. In early prophase **(b,b')** the antibody stains the still compact nucleolus and an increased number of small nucleoplasmic structures, probably resulting from the disintegration of the nucleolus. In early metaphase **(c,c')**, mid-metaphase **(d,d')** and various anaphase stages **(e,e'** and **f,f')**, most of the antigen is recognized at the surfaces of the chromosomes. Additionally, weak fluorescence in the perichromosomal cytoplasm can be seen. In early telophase **(g,g')**, several 'pre-nucleolar bodies' are brightly stained, whereas in late telophase **(h,h')** the protein is again recovered in the reformed nucleoli and in small nucleoplasmic structures of the daughter cells. Bars, 20 μm.

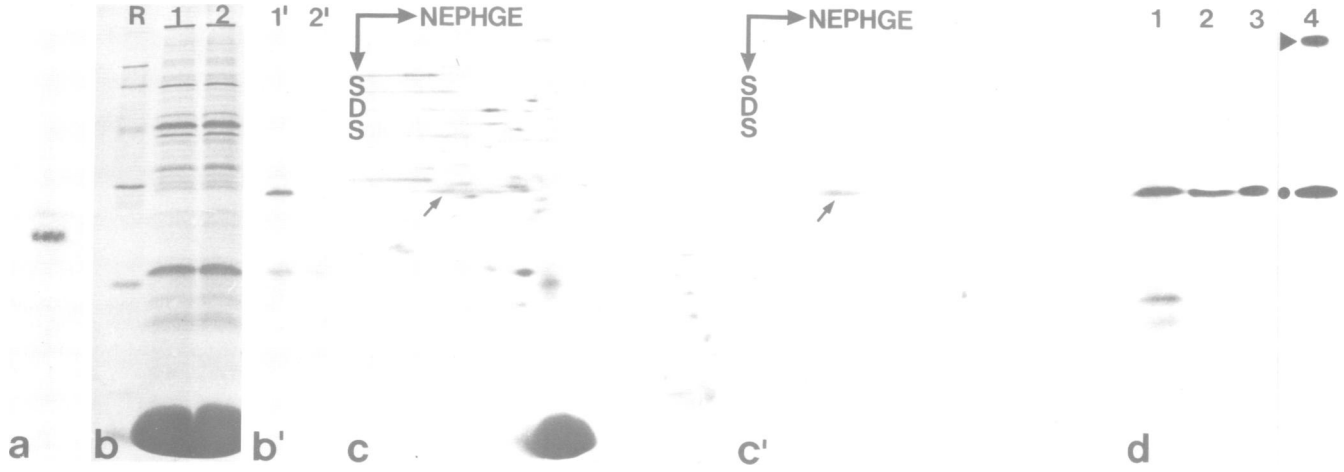

Fig. 7. Molecular characterization and identification of a cDNA clone coding for the nucleolar protein NO38. **(a)** Autoradiograph showing Northern blot hybridization with clone λNO38-185 on poly(A)$^+$ RNA from *X. laevis* ovaries. Note the reaction with an ~1.25-kb RNA (the significance of a weakly cross-hybridizing band at ~1.5 kb is not clear). **(b)** Coomassie blue staining of SDS–PAGE-separated proteins of rabbit reticulocyte lysate after *in vitro* translation using mRNA selected by hybridization with poly(A)$^+$ RNA from *X. laevis* ovaries (**lane 1; lane 2**, control without mRNA added). Reference proteins (lane R) are as in Figure 1a but without myosin heavy chain. **(b')** Fluorograph of the same gel showing [^{35}S]methionine-labelled translation product from the hybrid-selected mRNA **(lane 1')** which has the same mobility as protein NO38. **(c)** Coomassie blue-stained gel after two-dimensional gel electrophoresis (conditions and symbols as in Figure 1c), showing the separation of *X. laevis* oocyte nuclear proteins (HSP fraction) in co-electrophoresis with the [^{35}S]methionine-labelled translation products. **(c')** Corresponding fluorograph, showing that the polypeptide encoded by the hybrid-selected mRNA co-migrates with the native protein (for comparison see Figure 1c,c'). **(d)** Fluorograph presenting the comparison of the [^{35}S]methionine-labelled *in vitro* translation products of mRNA obtained from hybrid-selected poly(A)$^+$ RNA of *X. laevis* ovaries **(lane 1)** and of mRNA transcribed from the cDNA clone λNO38-185 in 'bluescribe' vector **(lanes 2–4)**. Note identical SDS–PAGE mobility of both translation products (**lanes 2** and **3** show different loadings). When the translation product is analysed in the presence of 0.1% SDS without boiling, an oligomer (arrowhead) of M_r ~230 000 is recognized in addition to the NO38 monomer (indicated by dot), indicating that this polypeptide has a tendency to form a distinct oligomers.

Distribution of protein NO38 during mitosis

During mitosis the location of protein NO38 changes drastically (Figure 6a–h'). In prophase, the major nucleolar units were still positive but an increasing number of small NO38-positive nucleolar units appeared, illustrating the fragmentation of the nucleolus. In metaphase and anaphase, NO38 immunofluorescence was noted on the surfaces of the chromosomes, in addition to a diffuse staining of the perichromosomal cytoplasm (Figure 6c–f'). In telophase, the antigen appeared in the 'pre-nucleolar bodies' and in the reforming nucleoli of the daughter cells (Figure 6g–h'). This pattern of distribution during mitosis is similar to those described for mammalian nucleolar protein B23 (Ochs *et al.*, 1983) and ribocharin (Hügle *et al.*, 1985b) and also resembles the distribution of some ribosomal proteins, part of which are enriched on chromosomal surfaces and re-populate the newly forming nucleoli, besides ribosome-bound material that remains in the cytoplasm (Hügle *et al.*, 1985a).

Isolation of cDNA clone encoding protein NO38

A λgt11 expression library of *X. laevis* ovary mRNAs was screened with both antibodies No-63 and No-185. Thirteen positive clones were picked, plaque-purified and phage DNA was isolated. The majority of phages reacting with both antibodies contained cDNA inserts of ~1.1 kb, as judged from their electrophoretic mobilities; one of these clones (λNO38-185) was used for further analyses.

Northern blot hybridization on poly(A)$^+$ RNA from *X. laevis* ovaries showed an intense band in a position corresponding to ~1.25 kb (Figure 7a). *In vitro* translation of cDNA hybrid-selected mRNAs from *X. laevis* ovary poly(A)$^+$ RNA yielded a single polypeptide that co-migrated, on one- and two-

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   1                                                                      GTATTTGGTTTGTGGAAAAA

  21 ATGGAAGATTCAATGGACATGGATAACATCGCTCCACTCCGCCCACAGAATTTCCTCTTTGGATGCGAGCTTAAAGCAGACAAAAAAGAA
   1  M  E  D  S  M  D  M  D  N  I  A  P  L  R  P  Q  N  F  L  F  G  C  E  L  K  A  D  K  K  E

 111 TATTCATTTAAAGTCGAAGATGATGAAAATGAGCATCAGTTGTCCTTGAGAACAGTTAGTTTGGGTGCTTCTGCTAAAGATGAACTTCAT
  31  Y  S  F  K  V  E  D  D  E  N  E  H  Q  L  S  L  R  T  V  S  L  G  A  S  A  K  D  E  L  H

 201 GTTGTTGAGGCTGAAGGAATAAATTATGAGGGTAAAACCATTAAAATTGCCCTTGCCTCACTGAAGCCGTCTGTGCAACCAACGGTATCT
  61  V  V  E  A  E  G  I  N  Y  E  G  K  T  I  K  I  A  L  A  S  L  K  P  S  V  Q  P  T  V  S

 291 CTAGGAGGGTTTGAGATAACACCACCAGTAATTTTGCGGTTGAAATCTGGTTCAGGACCAGTATATGTCAGTGGGCAACATCTTGTGGCT
  91  L  G  G  F  E  I  T  P  P  V  I  L  R  L  K  S  G  S  G  P  V  Y  V  S  G  Q  H  L  V  A

 381 TTAGAAGATCTTGAATCCTCTGATGATGAAGATGAGGAACATGAACCCAGTCCAAAAAATGCAAAAAGGATAGCACCTGATTCAGCATCA
 121  L [E  D  L  E  S  S  D  D  E  D  E  E] H  E  P  S  P  K  N  A  K  R  I  A  P  D  S  A  S

 471 AAAGTTCCTCGGAAAAAAACACGTCTAGAAGAAGAGGAAGAAGATTCAGACGAGGATGATGATGATGACGAGGATGATGATGATGAAGAT
 151  K  V  P  R  K  K  T  R  L [E  E  E  E  E  D  S  D  E  D  D  D  D  D  E  D  D  D  D  E  D

 561 GATGATGAAGAGGAGGAAGAAACACCTGTTAAAAAGACTGACTCTACAAAATCGAAGGCAGCTCAAAAGTTGAATCACAATGGGAAGGCT
 181  D  D  E  E  E  E  E] T  P  V  K  K  T  D  S  T  K  S  K  A  A  Q  K  L  N  H  N  G  K  A

 651 TCTGCATTGTCCACAACACAAAAGACTCCAAAAACTCCAGAGCAGAAAGGAAAACAAGATACAAAGCCTCAAACACCAAAGACACCAAAA
 211  S  A  L  S  T  T  Q  K  T  P  K  T  P  E  Q  K  G  K  Q  D  T  K  P  Q  T  P  K  T  P  K

 741 ACTCCTTTGTCTTCTGAGGAAATAAAAGCAAAGATGCAAACTTATTTAGAAAAGGGTAATGTTCTTCCTAAAGTGGAAGTAAAGTTTGCA
 241  T  P  L  S  S  E  E  I  K  A  K  M  Q  T  Y  L  E  K  G  N  V  L  P  K  V  E  V  K  F  A

 831 AACTATGTGAAGAATTGCTTCAGGACTGAAAATCAGAAGGTAATTGAAGATCTTTGGAAATGGAGACAATCCCTGAAAGATGGAAAATAA
 271  N  Y  V  K  N  C  F  R  T  E  N  Q  K  V  I  E  D  L  W  K  W  R  Q  S  L  K  D  G  K  *

 921 ACCTGCCTCCTGTTTAAATGTTAATTTGTTTTTTTTTTTTAAATGTTTATCTTGGGCCAAGTGTTTTGAAATGTAAGACTAAACTGCATG

1011 CTTTATGAAAATAAAATTTGTTCTTACATAAAAAAAAAA
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Fig. 8. Nucleotide sequence of cDNA clone λNO38-185 and the amino acid sequence deduced therefrom (one-letter code). The AUG in position 21 has been selected as the start of translation because it is preceded by an adenosine in position −3 (cf. Kozak, 1986). The asterisk denotes the termination codon; the 3′-untranslated region includes a canonical polyadenylation signal (double underlined sequence) 14 nucleotides in front of the poly(A) tail. The two domains rich in Glu and Asp are denoted by boxes. Two potential nuclear accumulation signals homologous to the SV40 T antigen signal are denoted by dotted lines. Four complete repeats of a Lys-Thr-Pro tripeptide are underlined by brackets.

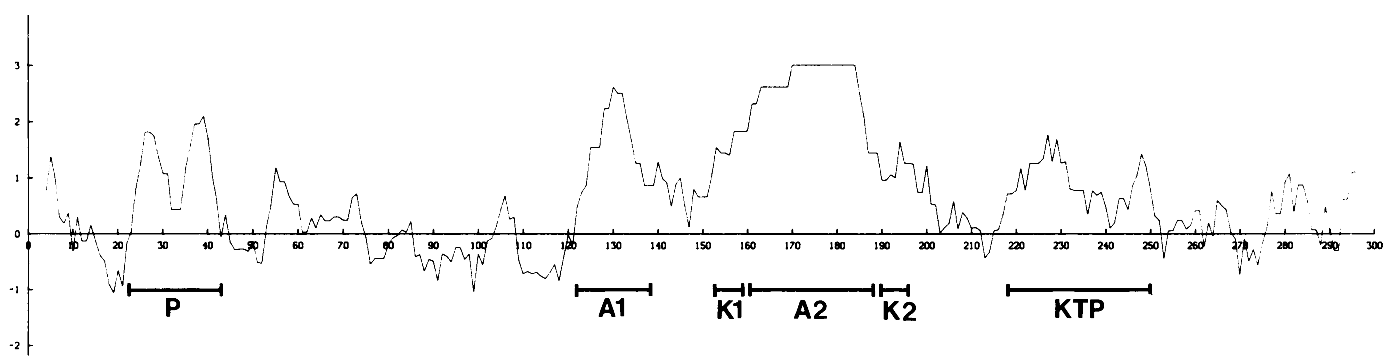

Fig. 9. Hydrophilicity plot of the protein NO38 amino acid sequence according to Hopp and Woods (1981). Several major hydrophilic domains are obvious (bars). The proximal hydrophilic domain (P) is localized at positions 23−42, the first acidic domain (A1) at positions 122−137 and the second larger acid domain (A2) at positions 160−187. A2 is flanked by two heptapetides resembling putative 'karyophilic signals' (K1 and K2). The fourth hydrophilic domain (KTP) is characterized by four complete repeats, and one incomplete, of the tripeptide Lys-Thr-Pro.

dimensional gel electrophoresis, with the authentic nucleolar protein NO38 (Figure 7b−c′). When this cDNA clone was transcribed by T7 polymerase in 'bluescribe' expression vector, and the resulting RNA examined by translation *in vitro* using the reticulocyte lysate system, a translation product of the same size was obtained (Figure 7d, lanes 1−3). Interestingly, this *in vitro* polypeptide synthesized from the cDNA clone formed spontaneously distinct oligomeric assemblies as demonstrated by SDS−PAGE of non-denatured protein (Figure 7d, lane 4).

Nucleotide sequence and amino acid sequence deduced therefrom

Figure 8 presents the nucleotide sequence of λNO38-185 and the amino acid sequence deduced therefrom. The clone contains 20 untranslated nucleotides at the 5′ end and 129 non-coding nucleotides at the 3′ end, with a poly(A)$^+$ addition signal 14 nucleotides in front of the poly(A) tail, of which only a short segment (10 nucleotides) was included. Comparison with the mRNA size estimated from Northern blots (see above) indicated that the clone does not represent the complete mRNA but lacks a total of ~200 nucleotides distributed over both the poly(A) tail and the 5′ end.

We assume that translation starts at the first methionine codon because this is the only AUG that meets the −3 = A requirement of Kozak (1986). The resulting polypeptide contains 299 amino acid residues including the initial methionine, correspon-

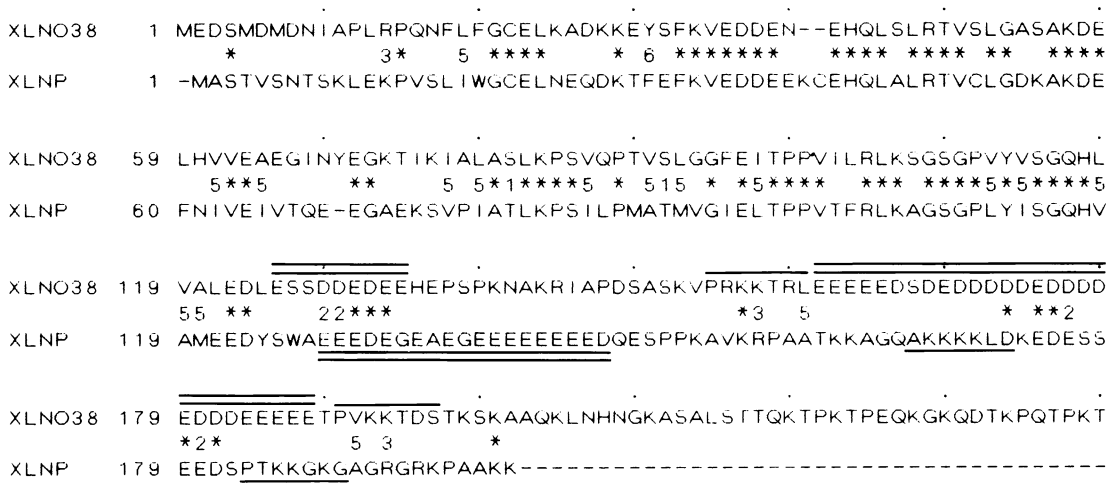

Fig. 10. Comparison of the relevant portion of the amino acid sequence of the nucleolar protein NO38 (XLNO38) with that of nucleoplasmin from the same species (XLNP; taken from Dingwall *et al.*, 1987). The N-terminal part of protein NO38, i.e. the region preceding the first acid domain, shows high sequence homology to nucleoplasmin. Common amino acid residues are denoted by asterisks, whereas conservative exchanges are designated by numbers (1, hydroxyl; 2, acidic; 3, basic; 5, aliphatic). The acidic domains are demarcated by double lines, the putative karyophilic signals by single lines.

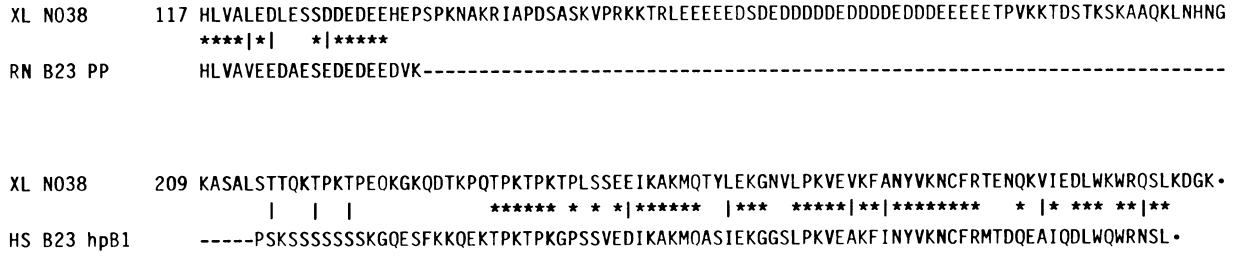

Fig. 11. Comparisons of the relevant parts of the amino acid sequence of protein NO38 of *X. laevis* (XLNO38; the first amino acid position in each comparison is given on the left margin) with partial sequences of rat and human nucleolar protein B23. The upper part shows that *X. laevis* protein NO38 has 11 amino acids in common (denoted by asterisks) with the sequence of a phosphopeptide fragment of nucleolar protein B23 (RNB23PP) from rat hepatoma ceils (cf. Chan *et al.*, 1986a). The lower part shows that the carboxy-terminal 86 amino acids positions of protein NO38 have 42 amino acids in common with a sequence reported for the carboxy-terminal fragment of human B23 (HSP23hpB1; cf. Chan *et al.*, 1986b). Note that the homology is concentrated in the last 65 residues, whereas the preceding segment does not reveal any homology. Conservative exchanges are denoted by vertical bars.

ding to a total mol. wt of 33 531. As this methionine is probably lost in the mature polypeptide, the final polypeptide might be of mol. wt 33 400, i.e. smaller than the M_r value of ~38 000 estimated from SDS−PAGE. This deviation is probably due to an anomalously low mobility on SDS−PAGE, as expected from the presence of extended clusters of negatively charged amino acids (cf. Kaufmann *et al.*, 1984; Kleinschmidt *et al.*, 1986).

The most conspicuous feature of the amino acid sequence of nucleolar protein NO38 is the presence of two negatively charged clusters of glutamic and aspartic acid residues located at positions 122−133 and 160−187 (Figure 8), the latter presenting as many as 27 Glu/Asp out of 28 residues. As shown in Figure 9 these two acidic domains are also the most hydrophilic ones in this protein. Other remarkable features of protein NO38 are four complete (and one incomplete) repeats of the tripeptide Lys-Thr-Pro in the hydrophilic domain between positions 218 and 247 (Figures 8 and 9) and two possible 'karyophilic signals' similar to those identified in several nuclear proteins (Hall *et al.*, 1984; Kalderon *et al.*, 1984; Goldfarb *et al.*, 1986; Kleinschmidt *et al.*, 1986; Lanford *et al.*, 1986; Richardson *et al.*, 1986; Dingwall *et al.*, 1987). Both these positively charged heptapeptides (PRKKTRL and PVKKTDS) are adjacent to the large negatively charged domain (A2 of Figure 9) and display significant homology to the 'nuclear location signal' of the SV40 large T antigen (Kalderon *et al.*, 1984).

Comparison of the amino acid sequence of protein NO38 with those of other proteins disclosed two important homologies (Figure 10). The N-terminal part of NO38, i.e. the 124 amino acids preceding the first negatively charged domain (A1), has 58 amino acid positions (and an additional 18 conservative amino acid exchanges) in common with the recently published sequence of nucleoplasmin of the same species (cf. Dingwall *et al.*, 1987), resulting in a total of 61% homology of this intercept. Further down the protein, no significant sequence homology between the two proteins was detected (Figure 10). Moreover, like nucleolar protein NO38, nucleoplasmin and another karyophilic, histone-binding protein of *X. laevis* (protein N1/N2) also contain extended Glu/Asp-rich domains, one in nucleoplasmin (Figure 10; cf. Dingwall *et al.*, 1987) and two in protein N1/N2 (Kleinschmidt *et al.*, 1986).

Moreover, as shown in Figure 11, positions 117−136 of protein NO38 have 11 amino acids in common with the partial sequence of a phosphopeptide fragment of the nucleolar protein B23 from rat hepatoma cells (Chan *et al.*, 1986a), and the carboxy-terminal 86 amino acid positions of NO38 have 42 amino acids in common with a sequence reported for the carboxy-terminal fragments of rat and human proteins B23 (Chan *et al.*, 1986b). Here the homology is concentrated in the last 65 residues (65%) whereas the preceding 21 positions do not reveal any homology.

Discussion

Protein NO38 is a non-ribosomal nucleolar protein located in structural complexes known as a locus of later stages of pre-rRNA processing and the assembly of pre-ribosomal particles, such as the nucleolar GC and nucleoplasmic precursor particles to both the small and the large ribosomal subunits. This pattern of occurrence is different from those reported for other nucleolar proteins localized to the FC and DFC (see Introduction) or those GC components that are mutually exclusive to the small or the large subunit precursor particles (cf. Hügle *et al.*, 1985a,b; Warner *et al.*, 1980). Protein NO38 may also occur in a 'soluble', i.e. non-ribonucleoprotein-associated form, as suggested by the recovery of 15% in the 100 000 *g* supernatant fraction of nucleoplasmic material, but the physical and biochemical properties of this soluble form are unknown.

Like several other nucleolar components, protein NO38 dissociates from the NOR during mitosis, and at least a considerable proportion of it distributes over the surfaces of all chromosomes, i.e. not only the NOR-containing ones (for other examples see Ochs *et al.*, 1983,1985a; Busch, 1984; Hügle *et al.*, 1985a,b). However, this mitotic distribution is by no means characteristic of all nucleolar proteins (Busch, 1984; Scheer and Rose, 1984; Schmidt-Zachmann *et al.*, 1984; Ochs *et al.*, 1985b; Freeman *et al.*, 1987), nor is it restricted to nucleolar proteins (for an example of non-nucleolar proteins see Chaly *et al.*, 1984). The mitotic distribution of protein NO38 is also remarkable as it indicates the existence of an as yet not understood mechanism that effectively re-accumulates these proteins, or the particles that contain them, first into the re-forming nucleus and then into the re-forming nucleoli.

The most important result of this study is perhaps the high sequence homology of a nucleolar protein, NO38, to nucleoplasmin, the abundant karyophilic protein that occurs in an apparently diffusible state throughout the nuclear sap (Krohne and Franke, 1980a,b; Mills *et al.*, 1980; Dingwall *et al.*, 1982; Dingwall and Laskey, 1986). Dingwall *et al.* (1987) have presented evidence that at least two nucleoplasmin species coexist in the same cell (cf. Krohne and Franke, 1980a,b). The addition of protein NO38 to these nucleoplasmins indicates that there is a family of nucleoplasmin-related proteins. Moreover, protein NO38 contains two highly negatively charged domains of almost uninterrupted stretches of aspartic and glutamic acid residues and therefore, like the karyophilic proteins nucleoplasmin and protein N1/N2 (Kleinschmidt *et al.*, 1986; Dingwall *et al.*, 1987), belongs to the group of proteins presenting extended Asp/Glu clusters. This group includes other nuclear proteins such as nucleolar protein, nucleolin (C23; Mamrack *et al.*, 1979; Busch, 1984), the chromatin components HMG1 and 2 (Walker, 1982), the adenovirus E1A gene product (for review see Moran and Mathews, 1987), *myc* oncogene products (Watt *et al.*, 1983; DePinho *et al.*, 1986; Stanton *et al.*, 1986) as well as diverse types of other proteins such as certain homeo-box proteins (Colberg-Poley *et al.*, 1985), prothymosin (Goodall *et al.*, 1986), neurofilament proteins (Geisler *et al.*, 1983; Lewis and Cowan, 1985), chromogranin, a major component of neuroendocrine vesicles (Benedum *et al.*, 1986; Iacangelo *et al.*, 1986), the precursor of amyloid protein of Alzheimer's disease (Kang *et al.*, 1987) and an acrosomal protein of sea urchin spermatozoa (Gao *et al.*, 1986).

From the finding that the karyophilic *Xenopus* proteins nucleoplasmin and N1/N2 effectively bind histones *in vivo* and *in vitro* and promote histone transfer to DNA, i.e. nucleosome assembly (Laskey *et al.*, 1978; Earnshaw *et al.*, 1980; Kleinschmidt and Franke, 1982; Kleinschmidt *et al.*, 1985; Cotten *et al.*, 1986) and that the Glu/Asp-rich domains are involved in histone-complex formation (Kleinschmidt *et al.*, 1986; Dingwall *et al.*, 1987) it has been proposed that these proteins function in the nuclear accumulation of histones and in chromatin assembly. In the same vein, we propose that protein NO38 serves similar but nucleolus-specific functions, be it the formation of special histone complexes, resulting in the local enrichment of certain histones specific for transcriptionally active nucleolar chromatin (e.g. Allis *et al.*, 1982), or the build-up of a perinucleolar pool of NO38 complexes with ribosomal proteins promoting the assembly of ribonucleoproteins on rRNA precursors. After all, most ribosomal proteins are positively charged and, thus, have the same problem as histones of existence in an environment of negatively charged molecules. If complexes of this kind exist in the nucleolus they would explain the association of protein NO38 with both GC structures and pre-ribosomal particles as well as the observations of Warner (1979) that the concentration of ribosomal proteins in the nucleus and nucleolus is, to some degree, independent from ongoing pre-rRNA synthesis, at least in HeLa cells (for *X. laevis* see, however, the data of Pierandrei-Amaldi *et al.*, 1985). Future studies will have to show whether ribosomal proteins exist in complexes with protein NO38, and experiments involving deletion and mutation of the acidic domains A1 and A2 are underway to clarify the specific role of these regions. Clearly, our extraction experiments show that protein NO38 is bound to nucleolar and free pre-ribosomal particles by relatively weak ionic forces.

Our finding of a high sequence homology between nucleolar protein NO38 and nucleoplasmin also provides an explanation of the reaction, in immunoprecipitation experiments, of one of our monoclonal antibodies (No-63) with both proteins, which indicates that the epitope recognized might be located in the first part of the molecule. The sequence relationship of these two proteins also explains the appearance of nucleolar immunoreactivity with antisera against nucleoplasmin, although most of the nucleoplasmin could be recovered in the soluble protein pool (cf. Krohne and Franke, 1980a). It will also be interesting to find out whether the observation of nucleoplasmin antibody reactivity on lampbrush chromosome loops, i.e. in association with ribonucleoproteins of nascent RNA polymerase II transcripts (Moreau *et al.*, 1986), represents the abundant 'general' nucleoplasmin or another member of the nucleoplasmin protein family that may be specifically involved in the assembly of products of polymerase II transcription.

Like nucleoplasmin and several other nuclear proteins, protein NO38 also contains oligopeptide sequences homologous to heptapeptides identified in certain viral proteins as 'nuclear localization signals'. Experiments to examine the involvement of the two putative karyophilic signals in the nuclear accumulation of protein NO38 are in progress, as are experiments to determine the regions of NO38 that are responsible for its nucleolar topogenesis. In connection with the question whether protein NO38 might also be involved, by forming complexes, in the effective nuclear transport and accumulation of newly synthesized ribosomal proteins (Warner, 1979), it will be important to find out whether the putative karyophilic signals that may exist in some ribosomal proteins (e.g. Moreland *et al.*, 1985), are independent or interact with other co-transported molecules.

The sequence of *Xenopus* protein NO38 also shows regions of homology with rat and human nucleolar protein B23 for which a similar SDS−PAGE mobility (M_r ~37 000) and charge (pI ~5.1 in 9.5 M urea) but a somewhat different intranucleolar

location (Daskal *et al.*, 1980; Spector *et al.*, 1984) have been reported and only partial sequence information is available (Chan *et al.*, 1986a,b). More sequence data on protein B23 would be needed to clarify whether this protein also contains one or several Asp/Glu-rich domains and the characteristic N-terminal portion common to nucleoplasmin and protein NO38 and, thus, might also be a member of the nucleoplasmin family.

Like nucleoplasmin (Earnshaw *et al.*, 1980; Dingwall *et al.*, 1982) and rat protein B23 (Chan *et al.*, 1986c), nucleolar protein NO38 also forms, *in vivo* and *in vitro*, distinct oligomeric complexes. In the case of NO38 the data presently available do not allow us to decide whether this form is a hexamer, as reported for protein B23, or a pentamer as proposed for nucleoplasmin. And like the histone-binding proteins nucleoplasmin and N1/N2 (Krohne and Franke, 1980a,b; Dabauvalle and Franke, 1982; Cotten, 1986), nucleolar protein NO38 can also be phosphorylated to a considerable degree (data not shown). Mammalian nucleolar protein B23 is also known to be highly phosphorylated (Mamrack *et al.*, 1977; Busch, 1984; Chan *et al.*, 1986a), and at least one sequence identified as a phosphorylation site in B23 is also contained in protein NO38 (Figure 11, upper sequence). Obviously, the state of phosphorylation of such proteins can influence the efficiency of binding to basic proteins, and Cotten *et al.* (1986) have shown that phosphorylated nucleoplasmin is more active in nucleosome assembly. Such observations suggest that the function of proteins of the nucleoplasmin family, including protein NO38, may be regulated by post-translational modification.

Materials and methods

Animals and cells

Clawed toads (*X. laevis*) were purchased from the South African Snake Farm (Fish Hoek, South Africa). Newts (*Triturus cristatus*) and salamanders (*Pleurodeles waltlii*) were reared in our laboratory. Cell cultures and procedures used for snap-freezing of tissue samples have been described (cf. Krohne and Franke, 1980b).

Monoclonal antibodies

Spleen cells from female BALB/c mice immunized with the pellet obtained after low speed centrifugation of a homogenate of mass-isolated *Xenopus* oocyte nuclei (for details see Schmidt-Zachmann *et al.*, 1984) were used for the preparation of hybridoma cells. Hybridoma cell lines producing antibodies No-63 and No-185 were selected (Schmidt-Zachmann *et al.*, 1984). Immunoglobulins were classified and produced from ascites fluid as described (Schmidt-Zachmann *et al.*, 1984) and purified by ammonium sulphate precipitation, followed by DE52 chromatography.

Cell fractionation and protein analyses

Large-scale isolation of nuclei from *X. laevis* oocytes and the fractionation of nuclear contents into a LSP, HSP and the final HSS have been described (Hügle *et al.*, 1985b). Homogenates of cultured cells were fractionated in the same way. In some experiments, proteins were extracted from the various pellets by gentle resuspension in phosphate-buffered saline (PBS) with additional 100 mM KCl for 20 min at 0–4°C, cleared by centrifugation under the same conditions and fractionated by sucrose gradient centrifugation as described (Schmidt-Zachmann *et al.*, 1984). HSP were resuspended in 0.5 ml TBS (100 mM NaCl, 1 mM $MgCl_2$, 1 mM β-mercaptoethanol, 10 mM Tris–HCl, pH 7.4) and fractionated in 10–40% sucrose gradients made up in TBS (Hügle *et al.*, 1985b).

Gradient fractions of 0.4 ml were collected and either used directly for ELISA, or proteins were precipitated in 15% trichloroacetic acid and analysed by SDS–PAGE (Hügle *et al.*, 1985b).

Gel filtration experiments on Sephacryl S-300 (Pharmacia, Uppsala, Sweden) columns (1.0 × 90 cm) were performed at 4°C with a flow-rate of 0.5 ml/min and a fraction volume of 2.8 ml in 83 mM KCl, 17 mM NaCl, 2 mM $MgCl_2$, 0.5 mM phenylmethyl sulfonylfluoride (PMSF), 2.5 mM dithiothreitol, 10 mM Tris–HCl (pH 7.2), using reference proteins in a parallel separation. Fractions were analysed by ELISA, SDS–PAGE and immunoblot. By combining data from gel filtration with those from sucrose gradient centrifugation, mol. wts of proteins were estimated according to Monty and Siegel (1968), using catalase as standard.

Gel electrophoresis and immunoblotting experiments

Conditions used for SDS–PAGE and two-dimensional gel electrophoresis as well as for immunoblotting were as described, using Tween 20 as blocking reagent (Schmidt-Zachmann *et al.*, 1984; Hügle *et al.*, 1985b).

Immunolocalization

Procedures used for immunofluorescence microscopy on frozen sections (~5 μm) as well as on cultured cells have been described (Krohne and Franke, 1980a,b; Schmidt-Zachmann *et al.*, 1984), using secondary antibodies coupled to fluorescein in combination with Texas Red- or rhodamine-coupled antibodies. The pre-embedding labelling technique of electron microscopic immunolocalization on cryosections used in this study was that described by Scheer and Rose (1984; cf. Hügle *et al.*, 1985a,b), using secondary antibodies coupled to 5 nm gold particles (from Janssen Life Sciences, Beerse, Belgium) and a Zeiss electron microscope.

Preparation and characterization of cDNA clones

The λgt11 expression library from RNA of *X. laevis* ovary and the screening procedures have been described (Kleinschmidt *et al.*, 1986). Both monoclonal antibodies (No-63; No-185) were used, and 13 positive clones were selected and plaque-purified. The clone selected (λNO38-185) was subcloned into M13 and sequenced by the dideoxy chain termination method (Sanger *et al.*, 1977) or into the *Eco*RI site of pUC8 (Vieira and Messing, 1982) and sequenced according to Maxam and Gilbert (1980). Standard computer programs were used for sequence comparisons and hydrophilicity analyses were done according to Hopp and Woods (1981).

Poly(A)$^+$-RNA from *X. laevis* ovaries was used for hybrid-selection and *in vitro* translation experiments and for RNA (Northern) blot analyses, using rRNAs from *Escherichia coli* and *X. laevis* electrophoresed in an adjacent lane for size comparisons (for references see Kleinschmidt *et al.*, 1986).

Expression of cloned cDNA in vitro

For expression *in vitro* the cDNA insert was subcloned into 'bluescribe' expression vector (Vector Cloning Systems, San Diego, CA), transcribed and the resulting RNA translated *in vitro* as described (Kleinschmidt *et al.*,1986).

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Recently the amino acid sequence of nucleolin (protein C23) of hamster (CHO) cells has been published (Lapeyre,B., Bourbon,H. and Amalric,F. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 1472–1476). This nucleolar, non-ribosomal protein contains three extended domains rich in glutamic and aspartic acid residues.