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Identification and cDNA cloning of a *Xenopus* nucleolar phosphoprotein, xNopp180, that is the homolog of the rat nucleolar protein Nopp140

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SUMMARY

The monoclonal antibody G1C7, recognises both *Xenopus* nucleolin and a protein of 180 kDa present in *Xenopus* oocyte nucleoli. This antibody was used to obtain a cDNA clone encoding the 180 kDa protein now called xNopp180 (*Xenopus* nucleolar phosphoprotein of 180 kDa). Analysis of the deduced amino acid sequence from this cDNA shows that xNopp180 is almost entirely composed of alternating acidic and basic domains. We show that xNopp180 is heavily phosphorylated and that it contains multiple consensus sites for phosphorylation by casein kinase II and cdc2 kinase. In addition we show that xNopp180 is the 180 kDa antigen recognised by the monoclonal antibody No-114, thus allowing reinterpretation of previous work with this antibody.

xNopp180 appears to be the *Xenopus* homolog of the rat nucleolar protein Nopp140. Nopp140 is a nuclear localisation signal binding protein that shuttles on curvilinear tracks between the nucleolus and the cytoplasm. Possible roles for xNopp180/Nopp140 in ribosome biogenesis are discussed.

Key words: *Xenopus*, nucleolar phosphoprotein, xNopp180

INTRODUCTION

Nucleoli are the sites of ribosome biogenesis. Each nucleolus contains a tandem array of ribosomal genes that are transcribed by RNA polymerase I to produce a precursor rRNA (pre rRNA), which is 45 S in mammals, 40 S in *Xenopus* and 35 S in yeast. Pre rRNA is then processed to give mature 18 S, 28 S and 5.8 S rRNAs, which are then packaged with ribosomal proteins to form preribosomal particles. The nucleolus can be divided into three morphologically distinct components and it is currently thought that each of these components correlates with a specific step in ribosome biogenesis (see Scheer and Benavente, 1990, for review). Despite their level of organisation nucleoli are dynamic structures. Nucleoli disappear at the onset of mitosis and re-form at the end of mitosis at specific chromosomal loci called nucleolar organizing regions (NORs) (Hadjiolov, 1985). NORs contain the tandemly repeated ribosomal genes. Prior to re-formation of nucleoli, so-called prenucleolar bodies are formed during telophase (Ochs et al., 1985). Similar prenucleolar bodies occur in early cleavage nuclei of *Xenopus* embryos prior to the onset of ribosomal gene transcripton (Hay and Gurdon, 1967).

A detailed understanding of ribosome biogenesis requires identification of the non-ribosomal nucleolar proteins that are required for transcription, processing and assembly of preribosomal particles. Two such proteins are nucleolin and Nopp140. Nucleolin is a nucleolar specific phosphoprotein that has been implicated in regulating RNA polymerase I transcription and binding to pre rRNA. Indeed, deletion of the yeast nucleolin-like gene *NSR 1* impairs 35 S pre-rRNA processing and ribosome biogenesis (Lee et al., 1992; Kondo and Inouye, 1992). Nucleolin is a highly modular protein, its amino-terminal third is composed of alternating acidic and basic domains, and the carboxy-terminal two thirds of the protein are composed of four RNA binding domains (reviewed by Dreyfuss et al., 1993) followed by a glycine-arginine rich (GAR) domain (Lapeyre et al., 1987; Lischwe et al., 1985). This structure is conserved among the vertebrates. Nucleolin is phosphorylated during interphase by casein kinase II (CKII) (Belenguer et al., 1989) and during mitosis by cdc2 kinase (Peter et al., 1990). Serine residues in acidic domains at the amino terminus are likely targets for CK II phosphorylation and TPXKK motifs in basic domains at the amino terminus are probable targets for cdc2 kinase phosphorylation. Phosphorylation of nucleolin by cdc2 kinase in mitosis suggests a role for nucleolin in nucleolar structure (Peter et al., 1990).

The rat nucleolar protein Nopp140 also contains multiple interspersed acidic and basic domains that contain potential CKII and cdc2 kinase phosphorylation sites, respectively (Meier and Blobel, 1992). Recent work on the human homolog of Nopp140, p130, has demonstrated that it can be phosphorylated by both of these kinases in vitro (Pai et al., 1995). Nopp140 is composed almost entirely of these alternating acidic and basic blocks of residues but unlike nucleolin it contains no recognisable RNA binding motifs.

Both nucleolin and Nopp140 have been localised to the dense fibrillar component of the nucleolus, both and have been

shown to bind to nuclear localisation signal peptides and both have been demonstrated to shuttle between the nucleolus and the cytoplasm (for review see Xue and Melese, 1994). Indeed, Nopp140 has been shown to move along curvilinear tracks between the nucleolus and the cytoplasm (Meier and Blobel, 1992).

A monoclonal antibody G1C7 (mAbG1C7) recognises an epitope in the amino-terminal 88 residues of *Xenopus* nucleolin that comprise one acidic and two basic domains (B. McStay, unpublished observation). Besides recognising *Xenopus* nucleolin, this monoclonal antibody also recognises a protein of 180 kDa apparent molecular mass. Here we describe the cloning of a full length cDNA encoding this 180 kDa protein, which we have named xNopp180 (*Xenopus* nucleolar phosphoprotein of 180 kDa). We show that this protein is the *Xenopus* homolog of Nopp140. We demonstrate that xNopp180 is a phosphoprotein and that it is nucleolar in location.

The monoclonal antibody No-114 (mAb No-114) recognises a *Xenopus* nucleolar protein of 180 kDa (Schmidt-Zachmann et al., 1984). This antibody has been used to study the distribution of the 180 kDa protein by immunoelectron microscopy and its distribution in the cell throughout the cell cycle (Schmidt-Zachmann et al., 1984). This 180 kDa protein has also been detected in prenucleolar bodies assembled in vitro with the use of *Xenopus* egg extract (Bell et al., 1992; Bauer et al., 1994). We demonstrate that xNopp180 is the antigen recognised by mAb No-114.

MATERIALS AND METHODS

Antibodies

The hybridoma G1C7 was cultured in RPMI with 10% foetal bovine serum. Cells were removed by centrifugation and the supernatant was used directly for western blots and immunolocalisation. The anti-*Xenopus* nucleolin antibody was raised by immunising rabbits with full length recombinant *Xenopus* nucleolin expressed in a baculovirus system (B. McStay, unpublished data). The monoclonal antibody No-114 in the form of ascites fluid was a gift from Dr Marion Schmidt-Zachmann.

Western and northern blots

SDS-polyacrylamide gels (Laemmli, 1970) were blotted onto nitrocellulose membranes. Following blocking in TBST (140 mM NaCl, 3 mM KCl, 25 mM Tris-HCl, pH 8.0, 0.05% Tween-20) with 10% nonfat dried milk and incubation with the primary antibody followed by secondary antibody coupled to horseradish peroxidase, blots were developed using ECL (Amersham).

Total RNA was prepared from stage 1 and 2 oocytes using Trizol (Gibco BRL). Northern blots were performed as described by Maniatis et al. (1982).

Cloning of x Nopp cDNA

A λ unizap cDNA (Stratagene) expression library constructed using stage 1 and 2 *Xenopus* oocyte mRNA (Cheng et al., 1993) was screened using mAbG1C7. Positive plaques were identified using secondary antibody coupled to horseradish peroxidase (Vectastain ABC, Vector Labs) and developed using 4-chloronaphthol. Inserts from positive plaques were rescued from the λ ZAP vector using Ex Assist helper phage (Stratagene). After preliminary DNA sequencing, one positive clone, P2.1, was chosen for further analysis. P2.1 contains an insert of 2.6 kb. The entire DNA sequence from both strands of this insert was determined by constructing a series of ordered deletions (Henikoff, 1984) and then sequencing by the dideoxy method using the enzyme sequenase (USB).

5′ RACE (rapid amplification of cDNA ends) was employed to obtain a full length cDNA clone (Clontech). A 25mer oligonucleotide corresponding to the sequence that was 300 nucleotides internal to the 5′ end of P2.1 insert was used as a primer in a reverse transcription reaction with total RNA prepared from stage 1 and 2 oocytes. Subsequent to this an anchor oligonucleotide was ligated to the 3′ end of the cDNA with T4 RNA ligase and amplified by PCR using a primer internal to that of primer used for reverse transcription and a primer that was complimentary to the ligated anchor. PCR reactions were performed using either Pfu or Taq polymerases. The resulting 1.1 kb products were subcloned as *Eco*RI to *Hin*dIII fragments into the vector pBluescript SK+ (Stratagene). Inserts from PCR reaction with each polymerase were sequenced on both strands and shown to be identical with each other and with the overlapping sequence in P2.1. The cloned 5′RACE product and P2.1 were fused at their common *Tth*III I site and cloned as an *Eco*RI to *Xho*I fragment into pBluescript SK+. The resulting plasmid pxNopp180 contains a 3.5 kb insert that encodes a full length cDNA.

In vitro translation

The translation initiation codon of the xNopp180 open reading frame present in the 3.5 kb cDNA was converted to an *Nco*I restriction site using oligonucleotide directed mutagenesis (Kunkel et al., 1987). The 5′ untranslated region was then removed as an *Eco*RI to *Nco*I restriction fragment and replaced with a 596 nucleotide *Eco*RI to *Nco*I restriction fragment from the vector pCITE 1 (Novagen) This fragment contains the internal ribosome entry site (IRES) from encephalomyocarditis virus (EMCV). The resulting plasmid is called pCITE xNopp.

pCITE xNopp was linearised with *Xho*I, transcribed in vitro with T3 RNA polymerase and translated in rabbit reticulocyte lysate (Promega and Novagen) as descibed by the manufacturer. In vitro translations were performed at 30°C for 2 hours unless otherwise stated. Translation reactions that contained [^{35}S]methionine were analysed by electrophoresis on SDS-polyacrylamide gels and then fixed in 40% methanol with 10% acetic acid, dried down and autoradiographed. Translation reactions without [^{35}S]methionine were electrophoresed on SDS-polyacrylamide gels and analysed by western blotting.

Localisation

The DNA sequence that encodes amino acid residues 877 and 878 of xNopp180 in the plasmid pCITE xNopp was converted to a *Bgl*II restriction site by site directed mutagenesis (Kunkel et al., 1987) using the oligonucleotide 5′GGTAAAAGGAAAAGATCTAG-TACAGGCAAT3′. This novel *Bgl*II restriction site was then used as the site of insertion of two copies of a DNA sequence that encodes the amino acid sequence MEQKLISEEDLN from the human c-myc, which is the epitope recognised by the monoclonal antibody 9E10 (Evan et al., 1985). The resulting plasmid pCITE xNoppmyc was linearised with *Xho*I and transcribed in vitro with phage T3 RNA polymerase. Transcripts were then injected into the cytoplasm of stage 5 and 6 *Xenopus laevis* oocytes (approx. 40 ng in 40 nl/oocyte). Injected oocytes were then incubated at 18°C for 40 hours. In order to determine nuclear localisation of epitope tagged xNopp180, oocyte nuclei were disected under paraffin oil (Pikaard et al., 1989) and probed by western blotting with the 9E10 monoclonal antibody. In order to determine the nucleolar localisation of epitope tagged xNopp180, oocyte nuclear contents were dispersed and centrifuged onto prepared microscope slides as previously described (Callan et al., 1987). Epitope tagged xNopp was detected on these spreads by indirect immunofluorescence with the 9E10 antibody (see figure legends for details).

Dephosphorylation

Xenopus oocyte nuclei were dissected out under paraffin oil, resus-

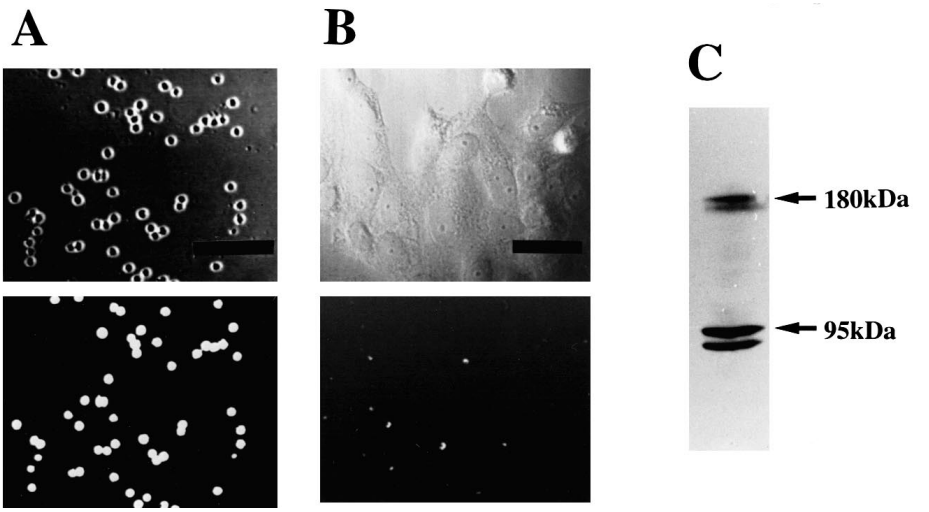

Fig. 1. Immunofluorescent staining and western blotting with mAbG1C7. (A) *Xenopus* oocyte nuclear contents were isolated and centrifuged onto microscope slides as described in Materials and Methods. Spreads were visualised by phase-contrast (top panel) and by indirect immunofluorescence staining with mAbG1C7 and TRITC labelled second antibody (bottom panel). Bar, 50 μm. (B) *Xenopus* cells (Xl K2) were grown on glass coverslips and after fixation were observed by phase-contrast microscopy or by indirect immunofluorescence staining with mAbG1C7 and TRITC labelled second antibody (bottom panel). Bar, 20 μM. (C) An extract from oocyte nuclei was electrophoresed on an SDS-8% polyacrylamide gel, western blotted and probed with the G1C7 antibody. The 95 kDa and 180 kDa proteins that react with the G1C7 antibody are labelled.

pended in 400 mM KCl, 1.5 mM $MgCl_2$,0.2 mM EDTA, 25 mM Hepes, pH 7.9, 0.5 mM PMSF, 1 mM DTT and 25% glycerol. Nuclear extracts were clarified by centrifugation and then treated with calf intestinal alkaline phosphatase (Gibco BRL, 20 units/oocyte nucleus) for 1 hour at 37°C in 50 mM Tris-HCl, pH 8.5, 0.1 mM EDTA, in the presence or absence of phosphatase inhibitors 0.3 mM Na_3VO_4 and 20 mM NaF. Samples were analysed by SDS-polyacrylamide gel electrophoresis, western blotted and probed with mAb G1C7.

RESULTS

Immunofluorescent staining and western blotting with the monoclonal antibody G1C7

The hybridoma G1C7 was developed by Rabiya Tuma and Mark Roth at the Fred Hutchinson Cancer Centre in Seattle. When mAbG1C7 is used in indirect immunofluorescent staining of oocyte nuclear spreads, intense staining over amplified nucleoli is observed (Fig. 1A). Similarly, indirect immunofluorescent staining of *Xenopus* cells grown in culture shows intense staining over the nucleoli (Fig. 1B). Western blots of extracts from oocyte nuclei with this monoclonal antibody detect proteins of molecular mass 95 kDa and 180 kDa (Fig. 1C). Both 95 kDa and 180 kDa bands appear as doublets.

Initial attempts to screen expression libraries with mAb G1C7 identified a clone that encodes the N-terminal 88 amino acids of *Xenopus* nucleolin (B. McStay, unpublished observation). *Xenopus* oocytes contain two forms of nucleolin, of apparent molecular mass 95 kDa and 90 kDa (Messmer and Dreyer, 1993). Sequence analysis of cDNA clones that encode each of these forms has demonstrated that the difference between them is the number of acidic and basic domains at their amino termini. We therefore conclude that the doublet of bands with apparent mass of 95 and 90 kDa that are detected with mAb G1C7 are the two forms of *Xenopus* nucleolin. This conclusion is further strengthened by the fact that mAb C7 recognises full length recombinant *Xenopus* nucleolin (B. McStay, unpublished observation). Thus we predicted that the 180 kDa protein also recognised by mAb G1C7 would be related in sequence to nucleolin and that this protein would be nucleolar in location, since little staining was observed other than that in nucleoli. This 180 kDa protein is now called xNopp180.

Cloning of a cDNA that encodes xNopp180

In order to characterise xNopp180 we screened a λ ZAP (Stratagene) cDNA expression library that was prepared with RNA isolated from stage 1 and 2 oocytes (Cheng et al.,1993). This

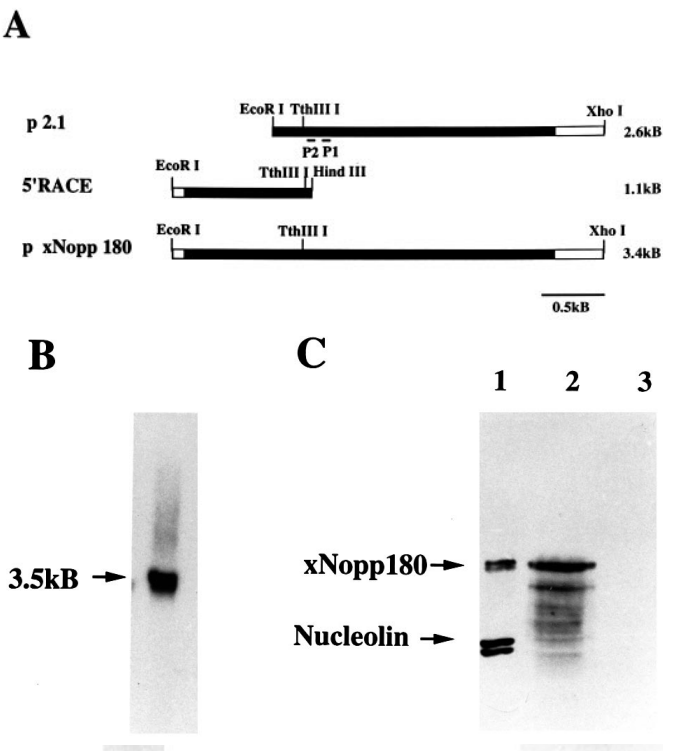

Fig. 2. xNopp180, cloning strategy, northern blot and expression by in vitro transcription/translation. (A) Structures of p2.1, the 5′RACE product and pxNopp180 generated by fusing the 5′RACE product and p2.1. The locations of relevant restriction sites are shown. Filled box represents open reading frame and open box represents untranslated region. (B) A northern blot of total stage 1 and 2 oocyte RNA probed with the insert of p2.1. (C) Western blot of in vitro translated xNopp180 probed with mAb G1C7. Lane 1, oocyte nuclear extract. Lane 2, 2 μl of in vitro translation reaction programmed with pCITExNopp180 RNA. Lane 3, 2 μl of control in vitro translation reaction.

library was chosen because of the abundance of the 180 kDa epitope in stage 1 and 2 oocytes.

Screening of the oocyte library yielded four positive clones. Inserts from these positive clones were rescued into the plasmid vector pBluescript SK+ (Stratagene). One of these plasmids p2.1 contained an insert of 2.6 kb (see Fig. 2A). Northern blots of total oocyte RNA with the p2.1 insert detected a single message of approximately 3.5 kb (Fig. 2B). This suggested that we were missing approximately 1 kb from the 5′ end of this cDNA. Attempts to rescreen the library using a DNA probe from the 5′ end of the p2.1 insert did not yield clones with more extensive 5′ sequence, so we chose to use 5′RACE (Clontech) to obtain the remaining sequences and subsequently construct a full length cDNA clone (see Materials and Methods for details). The structure of the 5′RACE product and the strategy for consructing the full length cDNA are shown in Fig. 2A. The size of the reconstructed full length cDNA is 3.44 kb, which is in good agreement with the size of the message observed by northern blotting. This suggests that we have indeed obtained a full length cDNA.

Within this 3.44 kb cDNA clone there is a continuous open reading frame that encodes a peptide of 990 amino acids. The entire DNA sequence of the 3.44 kb cDNA is shown in Fig. 3. The amino acid sequence of the open reading frame is also shown. The predicted molecular mass of the peptide encoded by this open reading frame is 102 kDa. This is approximately 80 kDa less than observed for xNopp180. This size discrepancy will be discussed below.

In order to prove that we have obtained a cDNA clone that encodes xNopp180, we chose to express this open reading frame by in vitro transcription/translation (Fig. 2C). To facilitate high levels of translation we converted the initiation codon to an *Nco*I restriction site using oligonucleotide directed mutagenesis and then replaced the *Eco*RI to *Nco*I 5′ untranslated region (UTR) with a 596 nucleotide *Eco*RI to *Nco*I restriction fragment from the plasmid pCITE 1 (Novagen). This fragment encodes the internal ribosome entry site (IRES) from encephalomyocarditis virus. The final plasmid is called pCITE xNopp. We have previously demonstrated that this IRES element increases translational efficiency up to 10-fold

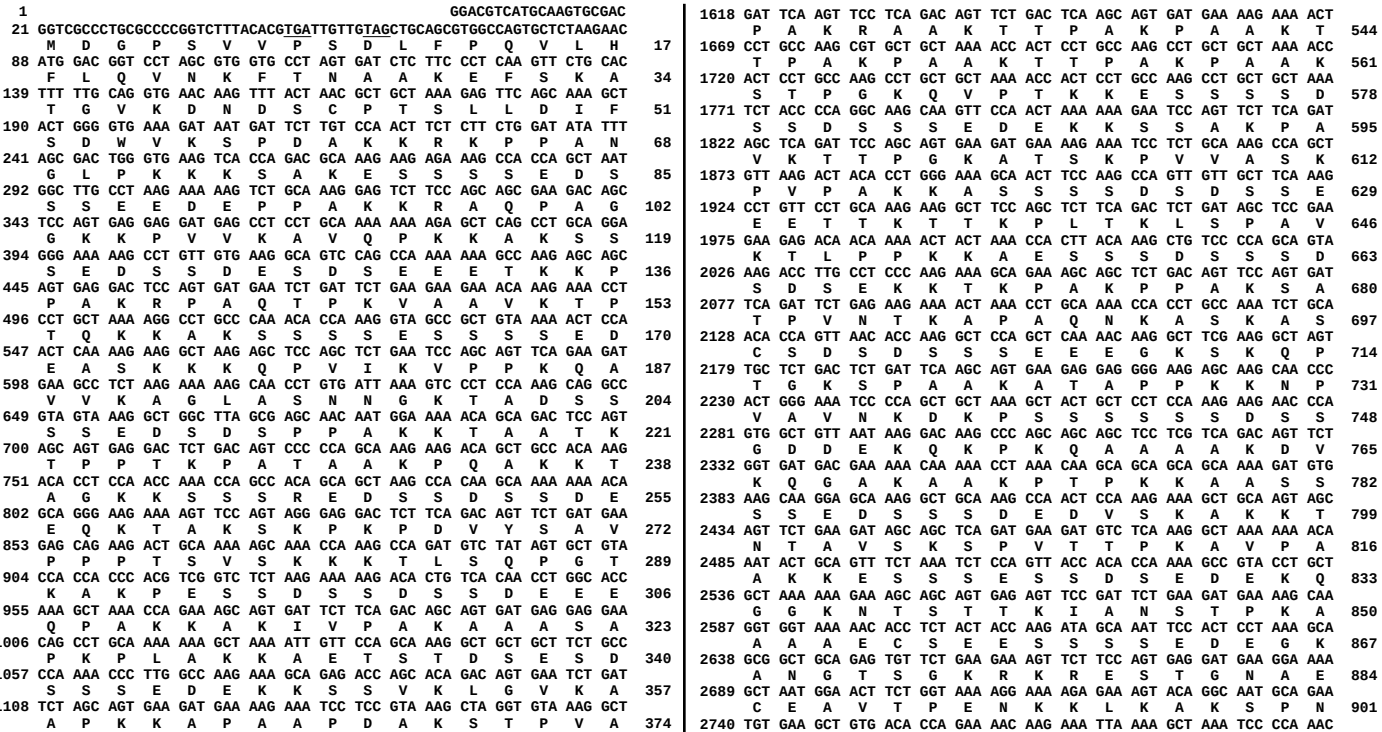

Fig. 3. xNopp180 DNA and peptide sequence. The entire DNA sequence of the insert in pxNopp180 is shown. The amino acid sequence (single letter code) of the xNopp180 open reading frame is shown above the DNA sequence. Numbers on the left refer to the position in the DNA sequence and numbers on the right refer to the position in the peptide sequence. In-frame stop codons in the 5′ UTR are underlined. The cDNA sequence data of xNopp180 have been deposited in the EMBL Data Library under the accession number X88927.

(McStay et al., 1991). pCITExNopp was linearised with the restriction enzyme *Xho*I, transcribed with phage T3 RNA polymerase, and translated in a rabbit reticulocyte lysate.

Aliquots of this and control translations were elecrophoresed on an SDS-polyacrylamide gel alongside an aliquot of oocyte nuclear extract. Western blots of this gel were then probed with mAbG1C7 (Fig. 2C). We observe a band in the in vitro translation reaction that co-migrates with xNopp180 in oocyte nuclear extract. We conclude therfore that we have indeed obtained a full length cDNA that encodes xNopp180.

Two other reasons lead us to conclude that we have identified the in vivo open reading frame for xNopp180. Firstly, this open reading frame utilises the first ATG in the cDNA clone and the 78 nucleotide 5′ UTR contains a number of in-frame stop codons. Secondly, the amino acid sequence at the amino terminus of this open reading frame is homologous to that of a previously characterised rat protein, Nopp140 (see below).

Analysis of the peptide sequence of xNopp180 (Fig. 3) shows that it is a remarkably charged protein: 13% of the amino acid residues are acidic (glutamic acid or aspartic acid) and the basic residue lysine comprises some 18.1% of amino acid residues in the protein. Other amino acids that are disproportionately represented include serine (18.4%), alanine (13.2%), proline (10.1%) and threonine (9.1%).

The charged amino acids in xNopp180 are organised into blocks that are acidic in overall charge, interspersed with blocks that are basic in charge (Fig. 4A).

The acidic blocks are almost entirely composed of glutamic and aspartic acid residues as well as multiple serines, and are on average 13 residues in length (Fig. 4B). Between these acidic blocks are groups of amino acids, ranging from 23 to 40 residues, which are basic in overall charge as a result of their high lysine content. The other feature of these basic domains is that they are remarkably rich in threonine, proline and alanine residues.

xNopp180 is *Xenopus* homolog of rat Nopp140

Approximately 80% of the primary sequence of xNopp180 is composed of alternating acidic and basic domains. This organisation of charged domains is very similar to that of the amino terminus of veretebrate nucleolin. However, xNopp180 is more similar in primary structure to a previously characterised rat nucleolar phosphoprotein of 140 kDa, termed Nopp140 (Meier and Blobel, 1992).

xNopp180 contains 18 blocks of acidic residues alternating with 17 blocks of basic residues (Fig. 4A), whereas rat Nopp140 contains 11 acidic and 10 basic blocks. The similarity between rat Nopp140 and xNopp180 is not confined to these acidic and basic regions. Indeed when the amino acid sequences of xNopp180 and rat Nopp140 are directly compared it is clear that the sequence of both their amino and carboxy termini are very similar; 50% identity between their N termini and and 65% identity between their C termini (Fig. 4A,B).

xNopp 180 is localised to the nucleolus

Immunofluorescent staining of oocyte nuclei (Fig. 1A) and *Xenopus* culture cells (Fig. 1B) demonstrates that the majority of protein recognised by mAB G1C7 is nucleolar. Since mAb recognises nucleolin in addition to xNopp180 we cannot conclude from staining with this antibody alone that xNopp180 is also a nucleolar protein. In order to determine the cellular location of xNopp180 we constructed an epitope tagged version of xNopp 180 in the expression construct pCITExNopp (Fig. 5). Site directed mutagenesis was used to convert the DNA encoding

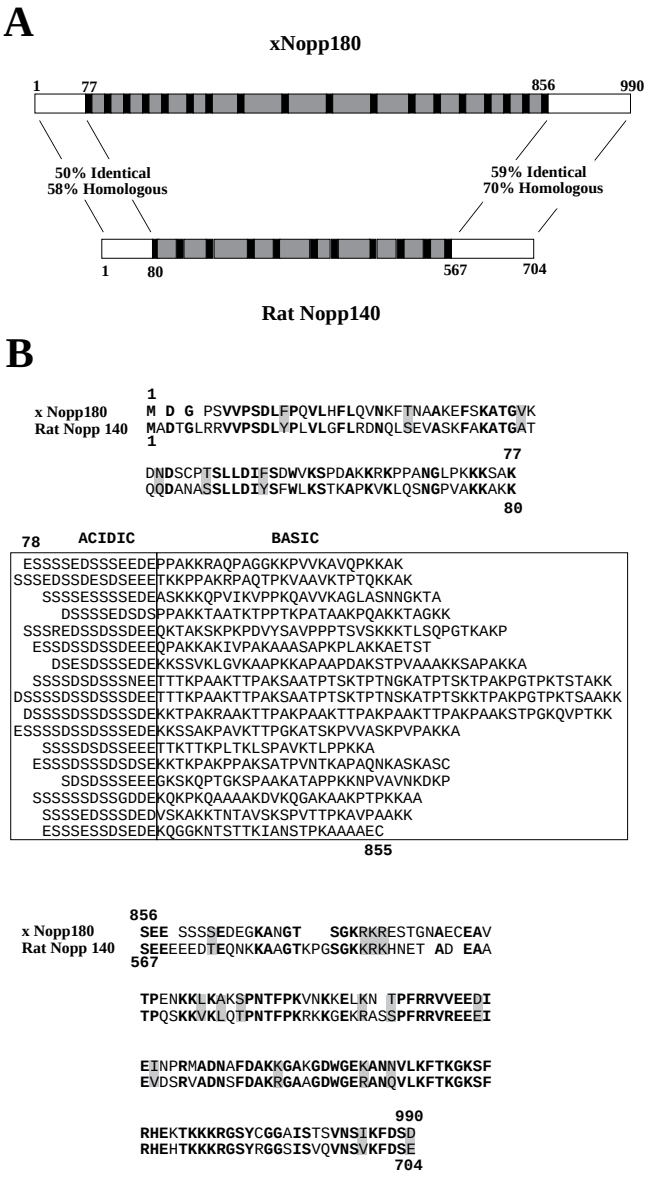

Fig. 4. Comparison of xNopp180 and rat Nopp140 peptide sequences. (A) The structures of xNopp180 and rat Nopp140 are compared in cartoon form. Black boxes are acidic domains, shaded boxes are basic domains and open boxes represent both amino- and carboxy-terminal domains. The % identity and homology between the amino and carboxy termini of both proteins is also shown. The % homology was calculated allowing conservative amino acid sequence changes as follows: (K,R) (E,D) (Q,N) (S,T) (F,Y,W,H) (L,I V,A) (P,G) (M,C). (B) The entire peptide sequence of xNopp180 is presented in a form that highlights its domain structure. The amino-terminal domain of xNopp180 (residues 1-77) is shown above that of rat Nopp140 (residues1-88). Amino acids that are identical are in bold type; those that represent conservative changes are shaded. Similarly the carboxy termini of xNopp180 (residues 856-990) and rat Nopp140 (residues 567-704) are compared. The entire amino acid sequence of the acidic and basic domains of xNopp180 (78-855) is shown in a form that emphasises the blocks of acidic and basic residues.

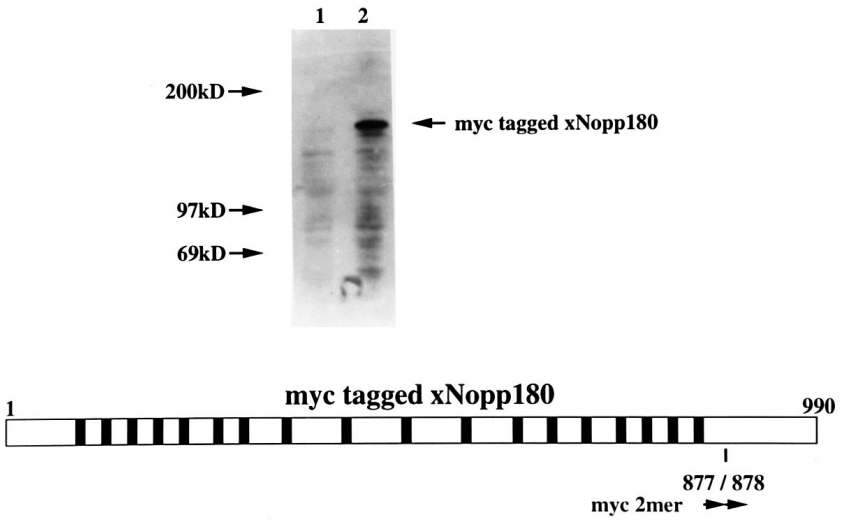

Fig. 5. Epitope tagged xNopp180 translated from injected RNA is imported into oocyte nucleoli. The structure of myc tagged xNopp180 is shown in cartoon form. Two copies of the human c-myc epitope recognised by mAb9E10 were inserted between residues 877 and 878 of a modified xNopp180 encoding plasmid (see Materials and Methods for details). Extracts from nuclei of uninjected oocytes (lane 1) and oocytes injected with T3 RNA polymerase transcripts of myc tagged xNopp 180 (lane 2) were electrophoresed on an SDS-8% polyacrylamide gel, western blotted and probed with the mAB9E10. The positions of molecular mass markers (in kDa) and tagged xNopp180 are shown by arrowheads.

amino acids 877 and 878 into a novel *Bgl*II restriction site. This *Bgl*II restriction site was used as the point of insertion of two copies of an oligoncleotide that encoded the epitope recognised by an anti-human c-myc monoclonal antibody (mAb 9E10; Evan et al., 1985). The resulting plasmid was transcribed with phage T3 RNA polymerase and synthetic transcripts were injected into the cytoplasm of stage 5 and 6 *Xenopus* oocytes. Following incubation for 40 hours, nuclei from injected and uninjected oocytes were isolated and the presence of epitope tagged xNopp180 was determined by probing western blots of nuclear extracts with mAb 9E10 (Fig. 5). In order to visualise nucleoli, spreads of nuclei from both injected and control oocytes were stained with polyclonal antibodies raised against full length recombinant *Xenopus* nucleolin. These antibodies are very specific for nucleolin and do not recognise any other oocyte nuclear protein as judged by western blotting (B. McStay, unpublished observation). Thus the bright spots observed by immunofluorescent staining with anti-nucleolin antibodies (Fig. 6B and E) are nucleoli. These brightly staining spots have also been identified as nucleoli by immunofluorescent staining with antibodies against the RNA polymerase I transcription factor xUBF (B. McStay, unpublished observation). When these same spreads are now probed with mAb 9E10 to detect epitope tagged xNopp180 we observe immunofluorescent staining over nucleoli in injected oocytes but not uninjected oocytes (Fig. 6F and C, respectively).

From this experiment we conclude that the major cellular location of xNopp180 is in nucleoli. This conclusion is further strengthened by the observation that xNopp180 is identical to a previously described 180 kDa nucleolar protein (see below).

xNopp180 is heavily phosphorylated

The consensus site for phosphorylation by CKII is a serine or threonine acceptor site with an acidic residue (D,E) three residues away on the carboxy-terminal side. Rat Nopp140 contains numerous CKII consensus sequences in its acidic domains that are thought to be phosphorylated by CKII (Meier and Blobel, 1992). Furthermore, it has been proposed that once a serine in a CKII site is phosphorylated it mimics an acidic residue and targets the phosphorylation of adjacent serines (Meggio and Pinna, 1988). Thus in the case of rat Nopp140 it has been proposed that the majority of the 82 serine residues found in the acidic domains are phosphorylated by CKII and that this contributes to its aberrant electophoretic mobility (Meier and Blobel, 1992). Dephosphorylation of rat Nopp140

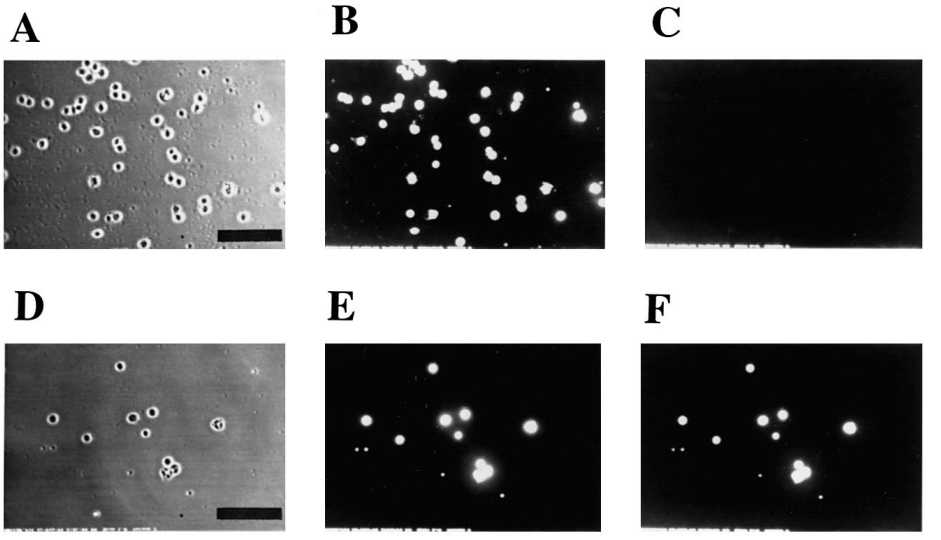

Fig. 6. Indirect immunofluorescent staining of oocytes injected with epitope tagged xNopp180. Spreads of uninjected oocyte nuclei were visualised by phase-contrast (A); by double indirect immunofluorescent staining with anti-nucleolin polyclonal antisera and FITC labelled second antibody (B); and the 9E10 monoclonal antibody with TRITC labelled second antibody (C). (D-F) Spreads of nuclei from oocytes injected with epitope tagged xNopp180 visualised as described above with phase-contrast, anti-nucleolin and 9E10, respectively. Bars, 50 μm.

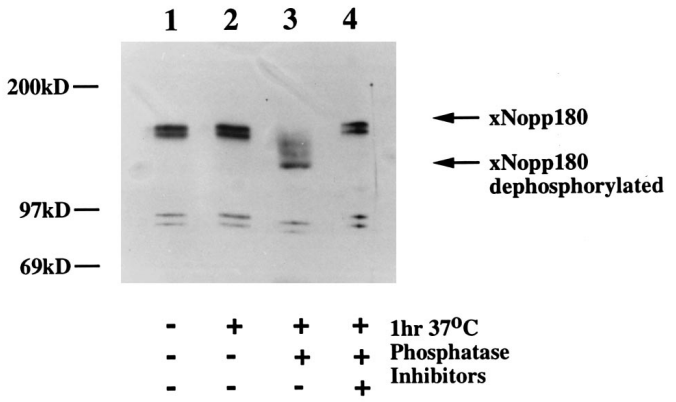

Fig. 7. xNopp180 is heavily phosphorylated. Untreated *Xenopus* oocyte nuclear (lane 1) extract was electrophoresed on an SDS-8% polyacrylamide gel alongside aliquots of extract that had been incubated at 37°C in the absence (lane 2) or presence (lane 3) of calf intestinal alkaline phosphatase, and in the presence of phosphatase plus the inhibitors sodium vanadate and sodium fluoride (lane 4). The gel was western blotted and probed with the G1C7 monoclonal antibody. The positions of molecular mass markers (in kDa), xNopp180 and dephosphorylated xNopp180 are shown by arrowheads.

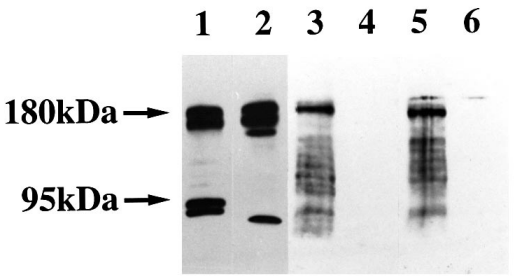

Fig. 8. xNopp180 is recognised by the monoclonal antibody No-114. *Xenopus* oocyte nuclear extract was electrophoresed on an SDS-8% polyacrylamide gel and western blotted with mAb G1C7 and mAb No-114 in parallel (lanes 1 and 2, respectively). Aliquots (2 μl) of in vitro translation reactions programmed with RNA transcribed from pCITE xNopp (lanes 3 and 5) were electrophoresed alongside aliquots of control translations (lanes 4 and 6) on an SDS-8% polyacrylamide gel, and western blotted with mAb G1C7 (lanes 3 and 4) and mAb No114 in parallel (lanes 5 and 6).

with alkaline phosphatase results in a reduction of its apparent molecular mass to 100 kDa.

Recently the human homolog of rat Nopp140, p130, has been identified and cloned. p130 like Nopp140 is very heavily phosphorylated. Dephosphorylation converts p130 into a form that has an apparent molecular mass of 95 kDa. CK II can convert this dephosphorylated form of p130 into a form that again runs on SDS-polyacrylamide gels with an apparent molecular mass of 130 kDa.

It appears that xNopp180 is also heavily phosphorylated. Upon treatment of *Xenopus* oocyte nuclear extracts with alkaline phosphatase we observe that the apparent molecular mass of xNopp180 on SDS-polyacryalmide gels was reduced to approximately 140 kDa (Fig. 7). Whereas incubation of extract without phosphatase, or with phosphatase and inhibitors, showed no shift in mobility. A similar increase in mobility upon phosphatase treatment of in vitro translated xNopp180 is observed (data not shown). We conclude from these experiments that, like rat Nopp140, xNopp180 is heavily phosphorylated and that this high degree of phosphorylation contributes in part to the discrepancy between its predicted and observed molecular mass. As mentioned above, xNopp 180 appears as a doublet. Upon dephosphorylation of xNopp 180 it appears as if this doublet is converted into a single band of increased mobility. This suggests that the doublet arises as a result of differential phosphorylation. Although, it is still possible that the 180 kDa doublet may result from partial proteolysis, or it may arise as a result of their being two genes for xNopp180 as is the case with nucleolin.

xNopp180 is the epitope recognised by the monoclonal antibody No-114

The monoclonal antibody (No-114) reacts with a polypeptide of molecular mass 180 kDa, which is present in *Xenopus* somatic cell nucleoli and in the amplified nucleoli of *Xenopus* oocytes (Schmidt-Zachmann et al., 1984). During mitosis this antigen disassociates from the nucleolar organiser region and is dispersed throughout the cytoplasm. At telophase it reassociates with the re-forming nucleolus (Schmidt-Zachmann et al., 1984). Furthermore, immunoelectron microscopy has demonstrated that this 180 kDa protein is localised to the dense fibrillar component of nucleoli (Schmidt-Zachmann et al., 1984).

In order to determine the relatedness of the No-114 antigen to xNopp180, we performed western blots of oocyte nuclear extracts with mAbs G1C7 and No-114 in parallel (Fig. 8, lanes 1 and 2, respectively). This demonstrated that xNopp180 and the No-114 antigen co-migrate on SDS-polyacrylamide gels. Furthermore, we can state that, in contrast to G1C7, No-114 does not cross-react with nucleolin. The identity of the 85 kDa antigen weakly recognised by mAb No-114 is unknown.

To prove rigorously that mAb No-114 recognises xNopp180, an in vitro translation reaction programmed with synthetic xNopp180 message was probed with mAbs G1C7 and No-114 in parallel. In each case a protein of apparent molecular mass 180 kDa was detected (Fig. 8, lanes 3 and 5), whereas in control translation reactions neither antibody detected a protein of this molecular mass (Fig. 8, lanes 4 and 6). We conclude from this experiment that the major antigen recognised by mAb No-114 is xNopp180.

DISCUSSION

xNopp180 is the *Xenopus* homolog of rat Nopp140

Rat Nopp140, originally termed p140, was first described as a nuclear localisation signal (NLS) binding protein (Meier and Blobel, 1990). Subsequently it was demonstrated that the major cellular location of Nopp140 is in the nucleolus and in coiled bodies (Meier and Blobel, 1992, 1994). It was demonstrated that Nopp140 shuttles between the nucleolus and the cytoplasm along curvilinear tracks that extend from the nucleolus to the edge of the nucleus. Other nucleolar proteins have been demonstrated to shuttle between the nucleolus and cytoplasm, most notably nucleolin (Borer et al., 1989). *Xenopus* Nopp180 is very similar in primary structure to Nopp140. The most notable difference between both proteins

is in the number of acidic and basic domains, 18 and 17, respectively, in xNopp180 and 11 and 10, respectively, in Nopp140. Amino-terminal and carboxy-terminal domains of both proteins are remarkably conserved in primary sequence, suggesting a functional role.

Nopp140 forms a complex with a protein termed NAP57 (Nopp140 associated protein of 57 kDa). The role of NAP57 is unclear; however, it is a remarkably conserved protein with homologs in yeast and *Escherichia coli* (Meier and Blobel, 1994). The fact that NAP57 is conserved in yeast suggests that there is also a xNopp180/Nopp140 homolog in yeast. Bou et al. (1993) have reported the sequence of an open reading frame in yeast (YKR412) that has significant homology to the carboxy-terminal end of rat Nopp140. We have aligned the peptide sequences of human p130, rat Nopp140 and xNopp180, and this yeast open reading frame, and find 50% identity between all four sequences in the carboxy-terminal 50 residues. In addition, this yeast open reading frame contains blocks of acidic residues rich in serine interspersed with blocks of basic residues. Thus it is likely that this open reading frame encodes the yeast homolog of Nopp140.

The role of Nopp140/ xNopp180

The nucleolus can be divided into three ultrastructurally distinct regions, the fibrillar centre (FC), the dense fibrillar component (DFC) and the granular component (GC). These three components are thought to reflect the various stages of ribosome biogenesis. Ribosomal genes are found in the FC and transcribed in association with the DFC. The DFC and the GC are thought to be the sites of the processing of the ribosomal RNA precursor into mature 18 S and 28 S rRNAs and the site of ribosome assembly (for review see Scheer et al., 1993). Nopp 140 and indeed nucleolin are located in the DFC (Meier and Blobel, 1992) and a role in rRNA processing and or ribosome assembly has been inferred.

The identity of xNopp180 with the mAb No-114 antigen allows us now to conclude that xNopp180 also localises to the DFC. As Nopp140 is an NLS binding protein, one role may be the import of ribosomal proteins into the nucleolus (discussed by Xue and Melese, 1994). Alternatively, given the high concentration of charged molecules in the nucleolus (rRNA, small nucleolar RNAs, and ribosomal proteins) it is possible that a highly charged protein with repeated acidic and basic domains may perform a chaperoning or a structural role.

At this point it is worth considering the distribution of Nopp140/xNopp180 throughout the cell cycle. A recent study of p130 (The human Nopp140 homolog) has demonstrated that in metaphase and anaphase p130 is undetectable by immunofluoresence but at telophase p130 appears in granular structures that resemble prenucleolar bodies (Pai et al., 1995). Similarly, we can now reinterpret previous studies with mAb No-114 as showing that xNopp180 dissociates from the NOR during mitosis in *Xenopus* cells (Schmidt-Zachmann et al., 1984). More recent work has shown that when demembranated sperm nuclei are incubated in a *Xenopus* egg extract the pronuclei that are formed contain so-called prenucleolar bodies. Immunofluorescent staining with mAb No-114 shows the presence of what we now know to be xNopp 180 in these bodies (Bell et al., 1992; Bauer et al., 1994). These observations are consistent with a role for Nopp140/xNopp180 in the re-formation of nucleoli after mitosis.

Phosphorylation of Nopp140/ xNopp180

There is no direct evidence that either Nopp140 or xNopp180 is phosphorylated on serines in their acidic domains by CKII in vivo; however, given the high degree of phosphorylation of both proteins and the fact that phosphatase treated p130 can be re-phosphorylated by CK II in vitro, this seems extremely likely. The role of phosphorylation may be to increase the net negative charge in the acidic domain, thus increasing their affinity for oppositely charged species, such as basic ribosomal proteins. Indeed, the observation that an NLS peptide binds preferentially to the phosphorylated Nopp140 supports this notion (Meier and Blobel,1992).

The basic domains of Nopp140 and xNopp180 also contain potential sites of phosphorylation. These include 26 TP motifs, a subset of which forms part of 8 TPAK motifs. TP motifs are the targets sites for threonine phosphorylation by many kinases including mitogen activated protein kinase and cyclin dependent kinases. TPAK motifs are also found in the basic domains at the amino terminus of nucleolin. Indeed we believe that it is these multiple TPAK motifs that are the epitope for mAbG1C7 that is shared between nucleolin and xNopp180. Similar TPAK motifs in chick nucleolin have been demonstrated to be a mitotic substrate for cdc2 kinase in vivo (Peter et al., 1990). Again it has been demonstrated recently that p130 is hyperphosphorylated in metaphase cells and that p130 from interphase cells can be phosphorylated in vitro by mitotic extracts (Pai et al., 1995).

It is tempting to speculate that phosphorylation of both nucleolin and Nopp140/ xNopp180 by cdc2 kinase is associated with the loss of nucleolar structure that is observed at mitosis.

Whatever the role of Nopp140/180, it is clear that identification of the *Xenopus* homolog should allow full exploitation of the *Xenopus* oocytes with their amplified nucleoli in the study of the function of this protein.

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