The EMBO Journal vol.8 no.13 pp.4015-4024, 1989

A yeast nucleolar protein related to mammalian fibrillarin is associated with small nucleolar RNA and is essential for viability

Thomas Schimmang, David Tollervey, Hildegard Kern, Rainer Frank and Eduard C.Hurt

EMBL, Postfach 10.2209, D-6900 Heidelberg, FRG

Communicated by K.Simons

In order to study the structural and functional organization of the eukaryotic nucleolus, we have started to isolate and characterize nucleolar components of the yeast *Saccharomyces cerevisiae*. We have identified a major 38 kd nucleolar protein (NOP1), which is located within nucleolar structures resembling the dense fibrillar region of mammalian nucleoli. This 38 kd protein is conserved in evolution since affinity-purified antibodies against the yeast protein stain the nucleolus of mammalian cells in indirect immunofluorescence microscopy and the yeast protein is decorated by antibodies directed against human fibrillarin. Affinity-purified antibodies against the yeast NOP1 efficiently precipitate at least seven small nuclear RNAs involved in rRNA maturation. We have cloned the gene encoding the yeast NOP1 protein. Haploid cells carrying a disrupted copy of the gene are not viable, showing that NOP1 is essential for cell growth. The gene codes for a 34.5 kd protein which contains glycine/arginine rich sequence repeats at the amino terminus similar to those found in other nucleolar proteins. This suggests that NOP1 is in association with small nucleolar RNAs, required for rRNA processing and likely to be the homologue of the mammalian fibrillarin.

Key words: fibrillarin/nucleolus/ribosome biogenesis/RNA processing/yeast

Introduction

The nucleolus is the structural framework around the chromosomal loci which contains the rRNA gene clusters (for a review see Hadjiolov, 1985). It functions as the site of pre-rRNA transcription and assembly of ribosomal precursor particles, thereby supplying the cytoplasm of the eukaryotic cell with ribosomes (for reviews see Hadjiolov, 1985; Sommerville, 1986). The nucleolus is highly structured (for a review see Goessens, 1984), consisting of distinct subcompartments which have been identified by electron microscopic analysis in combination with immunoelectron microscopy (Scheer and Rose, 1984; Spector *et al.*, 1984; Hügle *et al.*, 1985a,b; Ochs *et al.*, 1985b; Schmidt-Zachmann *et al.*, 1987). The tandemly arranged rRNA genes and RNA polymerase I are found within the fibrillar centres, which are therefore believed to be the sites of rRNA transcription (Scheer and Rose, 1984). These centres are embedded in a dense fibrillar compartment which is considered to contain the primary rRNA transcripts associated with proteins (for a review see Nigg, 1988). Around these fibrillar compartments pre-ribosomal particles at various stages of assembly are found which constitute the granular compartment (Spector *et al.*, 1984; Hügle *et al.*, 1985a,b; Schmidt-Zachmann *et al.*, 1987). Whereas the organization and transcription of the rRNA genes, and the processing pathways which yield mature rRNA species, are comparatively well understood, little is known about the role of nucleolar proteins in ribosomal biogenesis (for reviews see Sommerville, 1986; Nigg, 1988). Among the nucleolar proteins identified so far, two are proposed to be involved in processing of pre-rRNA transcripts. Nucleolin (also referred to as C23 or 100 kd nucleolar protein) has been implicated in regulating RNA polymerase I transcription and binding pre-rRNA (for a review see Jordan, 1987). Fibrillarin, so called because of its localization to the fibrillar compartments of nucleoli, has an apparent mol. wt of 34-36 kd and was originally identified in mammalian cells by autoimmune sera from scleroderma patients (Ochs *et al.*, 1985b; Parker and Steitz, 1987). Based on immunological cross-reactivity this protein appears to be well conserved among various species ranging down to lower eukaryotes (Ochs *et al.*, 1985b; Christensen *et al.*, 1986). Interestingly, affinity-purified antibodies against fibrillarin have been shown to precipitate ribonucleoprotein particles (RNP) containing the small nucleolar RNAs (snoRNAs) U3, U8 and U13 (Lischwe *et al.*, 1985b; Parker and Steitz, 1987; Tyc and Steitz, 1989) for which roles in pre-rRNA processing have been proposed (Steitz *et al.*, 1988).

In the budding yeast *Saccharomyces cerevisiae* the nucleolus is less well defined both morphologically and functionally (for a review see Warner, 1989). So far, its appearance has been described as a 'dense crescent' region when viewed by electron microscopy (Sillevis Smitt *et al.*, 1973), lacking the distinct sub-compartmentation of vertebrate nucleoli (Goessens, 1984; Hadjiolov, 1985). However, there is evidence that the yeast nucleolus fulfils the same functions as in higher eukaryotic cells, since ribosomal precursor RNAs have been found highly enriched within the 'dense crescent' (Sillevis Smitt *et al.*, 1972, 1973). Furthermore, several snoRNAs appear to be involved in ribosomal biogenesis. The essential RNAs U3 (snR17) and snR128 as well as snR3, snR4, snR5, snR8, snR9, snR10 and snR190 have been shown to be associated with the various rRNA precursors (Hughes *et al.*, 1987; Tollervey, 1987; Parker *et al.*, 1988; Zagorski *et al.*, 1988). Of these snoRNAs, snR10 at least is likely to be directly involved in pre-rRNA processing: in snr10-deficient strains, pre-rRNA processing is slowed and occurs predominantly by an aberrant pathway (Tollervey, 1987).

So far only two proteins have been immunologically localized within the yeast nucleolus. (i) SSB1, a 33 kd protein which was initially isolated as a single-stranded DNA binding protein, appears to be involved in RNA metabolism, but it is not essential for cell growth (Jong and Campbell, 1986; Jong *et al.*, 1987); (ii) a major 38 kd protein was previously

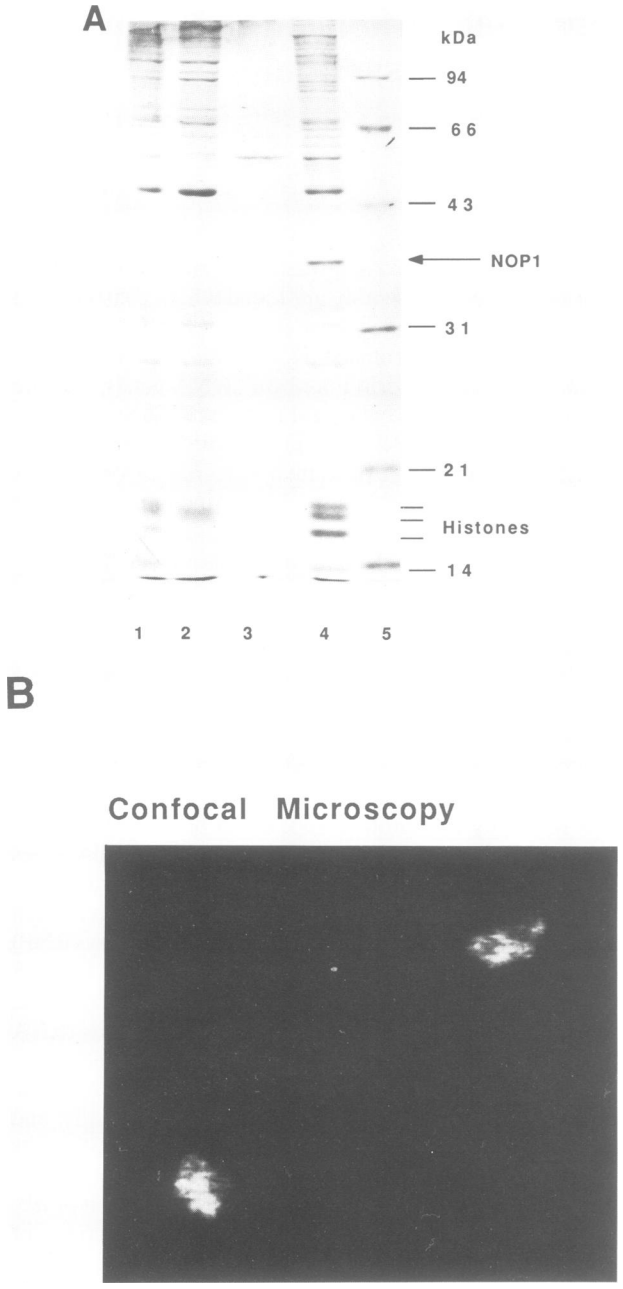

Fig. 1. Subcellular and subnuclear localization of the major 38 kd nucleolar protein in the yeast cell. (**A**) SDS−13% PAGE of purified yeast nuclei and subnuclear fractions. Nuclear subfractionation using 2% Triton X-114 and 150 mM NaCl was done as described earlier (Hurt *et al.*, 1988). Purified nuclei (**lane 1**) and 4-fold equivalents of the soluble (**lane 2**), the membrane (**lane 3**) and the insoluble nuclear fraction (**lane 4**) and a protein standard (**lane 5**) are shown on the Coomassie-stained gel. The positions of the major 38 kd nucleolar protein (NOP1) and the histones in the insoluble fraction are indicated by arrows and lines. (**B**) Indirect immunofluorescence staining of formaldehyde-fixed yeast cells as visualized by confocal microscopy. Indirect immunofluorescence in combination with confocal microscopy was done as described in Materials and methods. The optical section shown had a thickness of 0.5 μm. Structures showing a fibrillar/punctate staining were detected by the affinity-purified antibodies against the 38 kd nucleolar protein. The diameter of the stained nuclear structures of two yeast cells shown in the figure was ~1.5 μm.

identified and characterized in our laboratory (Hurt *et al.*, 1988). This 38 kd nucleolar protein appears to be identical with the nucleolar p38 described by Aris and Blobel (1988) which was shown to share epitopes with rat liver fibrillarin.

We have now cloned the gene of this yeast nucleolar protein and shown that it is essential for the life cycle of *Saccharomyces cerevisiae*. Furthermore, we found that it is associated with snoRNAs and appears to be the homologue of the mammalian protein fibrillarin. This is the first demonstration that a nucleolar RNA-associated protein is essential for cell growth.

Results

The 38 kd NOP1 protein is not homogeneously distributed within the yeast nucleolus

Indirect immunofluorescence and immunoelectron microscopy have shown that NOP1 (standing for nucleolar protein 1), a major nuclear protein with an apparent molecular mass of 38 kd on SDS−polyacrylamide gels, is located within the nucleolus (Hurt *et al.*, 1988). Furthermore, subcellular and subnuclear fractionation had revealed that the 38 kd protein is not significantly released from isolated nuclei by 150 mM NaCl (or 200 mM NaCl) and detergent (Figure 1A), but can be extracted at higher ionic strength (500 mM NaCl) (Hurt *et al.*, 1988), indicating that NOP1 is not tightly bound to nucleolar structures.

The detection of the 38 kd NOP1 protein in whole yeast cells by indirect immunofluorescence microscopy is greatly facilitated by fixing the yeast cells in formaldehyde instead of glutaraldehyde. This observation allowed us to perform confocal microscopy in order to reveal structural details of the subnucleolar localization of NOP1 in yeast cells. In Figure 1(B) the immunostaining of the 38 kd protein is seen to be exclusively nuclear; the cytosol is not detectably stained and is therefore not visible. Moreover, the use of the confocal microscope allows the resolution of the nuclear staining into a clear fibrillar/punctate pattern (Figure 1B). In the electron microscopy of frozen thin sections of fixed yeast cells, electron-dense structures within the nucleus reminiscent of the dense fibrillar compartments of higher eukaryotic nucleoli (Spector *et al.*, 1984; Ochs *et al.*, 1985a,b; Lischwe *et al.*, 1985b; Reimer *et al.*, 1987) were labelled by the affinity-purified antibodies against the 38 kd nucleolar protein (Hurt *et al.*, 1988). It is thus likely that the structures labelled by the anti-NOP1 antibodies in yeast are related to the dense fibrillar compartments of higher eukaryotic nucleoli which contain nucleolar proteins such as fibrillarin and nucleolin (Spector *et al.*, 1984; Ochs *et al.*, 1985b; Reimer *et al.*, 1987; Raska *et al.*, 1989).

Affinity-purified antibodies against the yeast 38 kd nucleolar protein cross-react with mammalian nucleolar proteins

To test whether the yeast NOP1 protein has counterparts in higher eukaryotes, we used affinity-purified antibodies against the yeast 38 kd protein (Hurt *et al.*, 1988) on immunoblots and in indirect immunofluorescence microscopy of mammalian cells. On immunoblots containing total protein extracts from Madin−Darby canine kidney (MDCK) cells, predominantly two proteins of 34 and 100 kd are recognized by the affinity-purified antibodies (Figure 2A). The 34 kd protein could correspond to fibrillarin (Ochs *et al.*, 1985b) and the 100 kd protein to nucleolin (Lapeyre *et al.*, 1985). Both proteins are located in the dense fibrillar compartments of mammalian nucleoli and have at least one

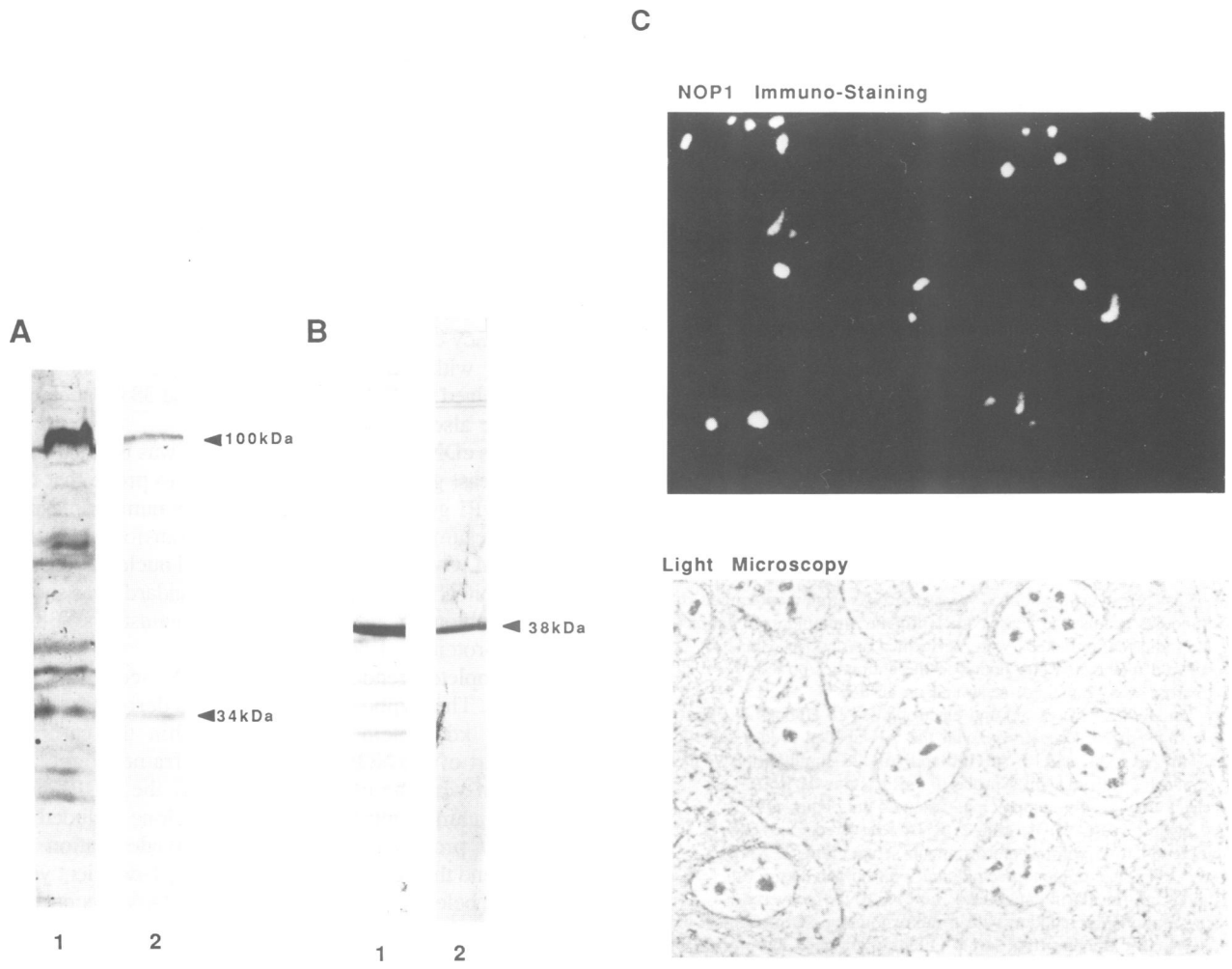

Fig. 2. Cross-reactivity of the 38 kd yeast nucleolar protein with mammalian nucleolar proteins. (**A**) Immunoblot of a MDCK extract reacted with the affinity-purified antibodies against the yeast 38 kd nucleolar protein. Details of the immunoblotting are described in Materials and methods. **Lane 1**, crude immune serum made against the yeast-insoluble nuclear fraction; **lane 2**, affinity-purified antibodies against the NOP1 protein. Equal amounts of a MDCK extract were applied on both lanes. The position of the major immunoreactive bands in **lane 2** are indicated by arrows. (**B**) Immunoblot of the yeast-insoluble nuclear fraction using an autoimmune serum against mammalian fibrillarin. **Lane 1**, immune serum containing antibodies against the yeast 38 kd nucleolar protein (1:1000 dilution); **lane 2**, antibodies against human fibrillarin (1:100 dilution). The position of the 38 kd nucleolar protein is indicated by an arrow. Equal amounts of protein equivalent to 50 μg were analysed by SDS−12% PAGE and immunoblotting. Further experimental details are given in Materials and methods. (**C**) Indirect immunofluorescence microscopy on fixed mammalian MDCK cells using the affinity-purified anti-NOP1 antibody. MDCK cells grown on coverslips were fixed and treated for indirect immunofluorescence as described in Materials and methods. Both the immunostaining, which was nucleolar, and the Nomarski picture (the nucleoli are identified by virtue of their darker appearance) of the corresponding MDCK cells are shown.

common epitope, which is a glycine/arginine-rich repeat sequence motif (Lischwe *et al.*, 1985a,b; Lapeyre *et al.*, 1986). It is possible that our antibodies against the yeast 38 kd nucleolar protein cross-react with the mammalian glycine/arginine-rich sequence which is also present in the yeast 38 kd protein (see below).

Furthermore, an autoimmune patient serum, S4, containing antibodies against human fibrillarin (Ochs *et al.*, 1985; G.Tessars and R.Lührmann, personal communication) reacted with the yeast 38 kd protein on immunoblots containing a nuclear fraction enriched in nucleolar proteins (Figure 2B).

Indirect immunofluorescence microscopy using affinity-purified anti-yeast NOP1 antibodies on mammalian MDCK cells (Figure 2C) and human HeLa cells (data not shown) exclusively stained the nucleoli, but not the rest of the nucleus. In addition, the nucleolar staining caused by the antibodies against yeast NOP1 was cell-cycle dependent and drastically reduced during mitosis (data not shown). This is consistent with the behaviour of nucleolar proteins like fibrillarin, nucleolin and B23 at mitosis when nucleolar disintegration and reformation takes place (Ochs *et al.*, 1983; 1985b; Gas *et al.*, 1985).

Association of the yeast NOP1 protein with small nucleolar RNAs

To test the association of yeast NOP1 protein with snoRNAs, anti-NOP1 antibodies were used to immunoprecipitate RNPs present in cell lysates.

Lysis of cells in 150 mM KCl using glass beads for cellular and organellar disintegration releases a proportion of snoRNAs (25−50%). It is likely that the released snoRNAs represent free RNPs rather than pre-ribosomal complexes. The 150 mM KCl extract was subjected to immunoprecipitation using a crude immune serum made against the insoluble fraction of yeast nuclei which is highly enriched in anti-

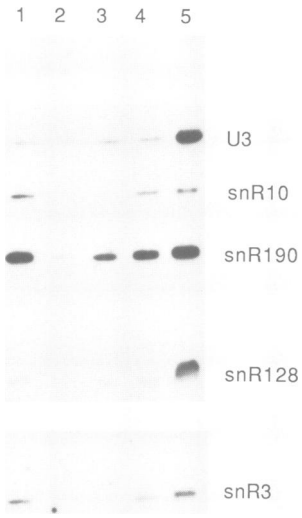

Fig. 3. Northern hybridization of yeast snoRNAs immunoprecipitated with antibodies against the yeast NOP1 protein. Immunoprecipitations using the indicated antibodies were performed as described in Materials and methods. RNA bound to the antibodies/protein A–Sepharose was recovered, separated on an 8% polyacrylamide/8.3 M urea gel and electroblotted to Hybond-N. The filter was hybridized successively with the ^{32}P-labelled probes specific for the snoRNAs U3, snR10, snR190 and snR128 simultaneously, then stripped and hybridized with a probe to snR3. **Lane 1**, RNA recovered following immunoprecipitation with antibodies affinity purified against yeast NOP1; **lane 2**, RNA recovered following immunoprecipitation with a control human serum; **lane 3**, RNA recovered following immunoprecipitation with human serum S4 (containing anti-fibrillarin antibodies); **lane 4**, RNA recovered following immunoprecipitation with a rabbit immune serum against the insoluble nuclear fraction (this serum is highly enriched in anti-NOP1 antibodies); **lane 5**, total RNA extracted from the 150 mM KCl cell lysate. The corresponding autoradiogram and the position of the various snoRNAs are shown.

NOP1 antibodies (Figure 3, lane 4) and affinity-purified anti-NOP1 antibodies (Figure 3, lane 1). The snoRNAs, snR3, snR4, snR8, snR9, snR10, snR189 and snR190 were efficiently immunoprecipitated under these conditions; U3 and snR128 were precipitated only weakly by the anti-NOP1 antibodies, but at a level which appears to be reproducibly above background (shown for snR3, snR10, snR128, snR190 and U3 in Figure 3). None of these snoRNAs were precipitated by a control human serum (Figure 3, lane 2) or by anti-Sm serum Küng, which efficiently precipitated yeast U1, U2 and U4 snRNA from the same lysate (data not shown). Surprisingly, autoimmune serum S4, which recognizes human fibrillarin and yeast NOP1 on Western blots, efficiently precipitated only snR4 and snR190 (Figure 3, lane 3). Following immunoprecipitation of snoRNPs eluted from isolated nuclei with affinity-purified anti-NOP1 antibodies, a small number of proteins, including NOP1, were shown to be present in the immune precipitate (data in preparation). We are currently investigating whether the co-precipitated proteins are components of snoRNPs.

The NOP1 gene codes for a 34.5 kd protein with similarities to nucleolar proteins of higher eukaryotes and is essential for cell growth

In order to obtain protein sequence data for cloning the NOP1 gene, the 38 kd nucleolar protein was partially purified by subcellular and subnuclear fractionation as shown in Materials and methods. Fractions enriched in the 38 kd protein were separated by SDS–PAGE (Figure 1A), the band corresponding to the 38 kd protein was excised from the gel, tryptic fragments were generated and micro-sequencing of isolated peptide fragments was performed (see also Materials and methods). Oligonucleotide probes corresponding to tryptic fragments of the 38 kd nucleolar protein were designed on the basis of the yeast codon usage rules (Davis and Thorner, 1987) and a λ gt11 yeast cDNA library was screened with the oligonucleotide probes under low-stringency conditions. A final λ gt11 clone specifically hybridizing with the oligonucleotide probe was obtained which contained the full-length cDNA of the 38 kd nucleolar protein (see also below).

Using this cDNA clone a genomic clone was isolated from a YEP13 yeast genomic library. In order to prove that this was the NOP1 gene, the YEP13 high copy number plasmid with the presumptive NOP1 gene was transformed into a yeast diploid. Overproduction of the 38 kd nucleolar protein but not of mitochondrial porin (internal standard) was shown by immunoblotting using antibodies against the 38 kd nucleolar protein and porin (Figure 5C).

The complete genomic NOP1 DNA sequence was determined. The tryptic peptide fragments derived from the isolated 38 kd protein were found within the carboxy-terminal part of the NOP1 open reading frame (Figure 4). Comparison with the cDNA showed that the NOP1 gene does not contain an intron. The genomic clone included the complete 5′ promoter region and 3′ polyadenylation site (Figure 4) and thus could complement a nop1-deficient yeast mutant (see below). The complementing DNA sequence of the genomic clone (and also of the cDNA clone) contained a single open reading frame coding for a protein of 327 amino acid residues corresponding to a predicted mol. wt of 34.5 kd. The cDNA of the NOP1 gene was placed under the control of a strong bacteriophage promoter and was expressed by coupled *in vitro* transcription/translation. *In vitro* synthesized NOP1 protein had an apparent mol. wt of 38 kd on SDS–PAGE and exactly co-migrated with the 38 kd nucleolar protein made *in vivo* by the yeast cells (data not shown). This confirmed that the isolated cDNA clone carried the full-length NOP1 gene. The NOP1 protein therefore migrates slightly abnormally on SDS–PAGE.

Northern analysis showed a major transcript of 1.3 kb which is consistent with the length of the cloned cDNA (Figure 5A). In addition, a weak mRNA signal of ~3 kb was seen on the Northern blot which could be the transcript of a NOP1-related gene. The NOP1 open reading frame contains a glycine/arginine-rich sequence at the amino terminus, which is also found in mammalian nucleolar proteins, like fibrillarin and nucleolin (Lischwe *et al.*, 1985a,b).

The NOP1 open reading frame was interrupted by inserting the URA3 gene (present on a 1.1 kb DNA fragment) into the unique *Bgl*II restriction site found in the middle of the NOP1 open reading frame. This interrupted NOP1 gene was inserted into the genome of a diploid yeast strain by homologous recombination. Integration at the homologous NOP1 locus was verified by Southern analysis (Figure 5A). Diploid yeast cells heterozygous for NOP1 were viable. Haploid progeny containing only the disrupted NOP1 gene were non-viable, but could be complemented

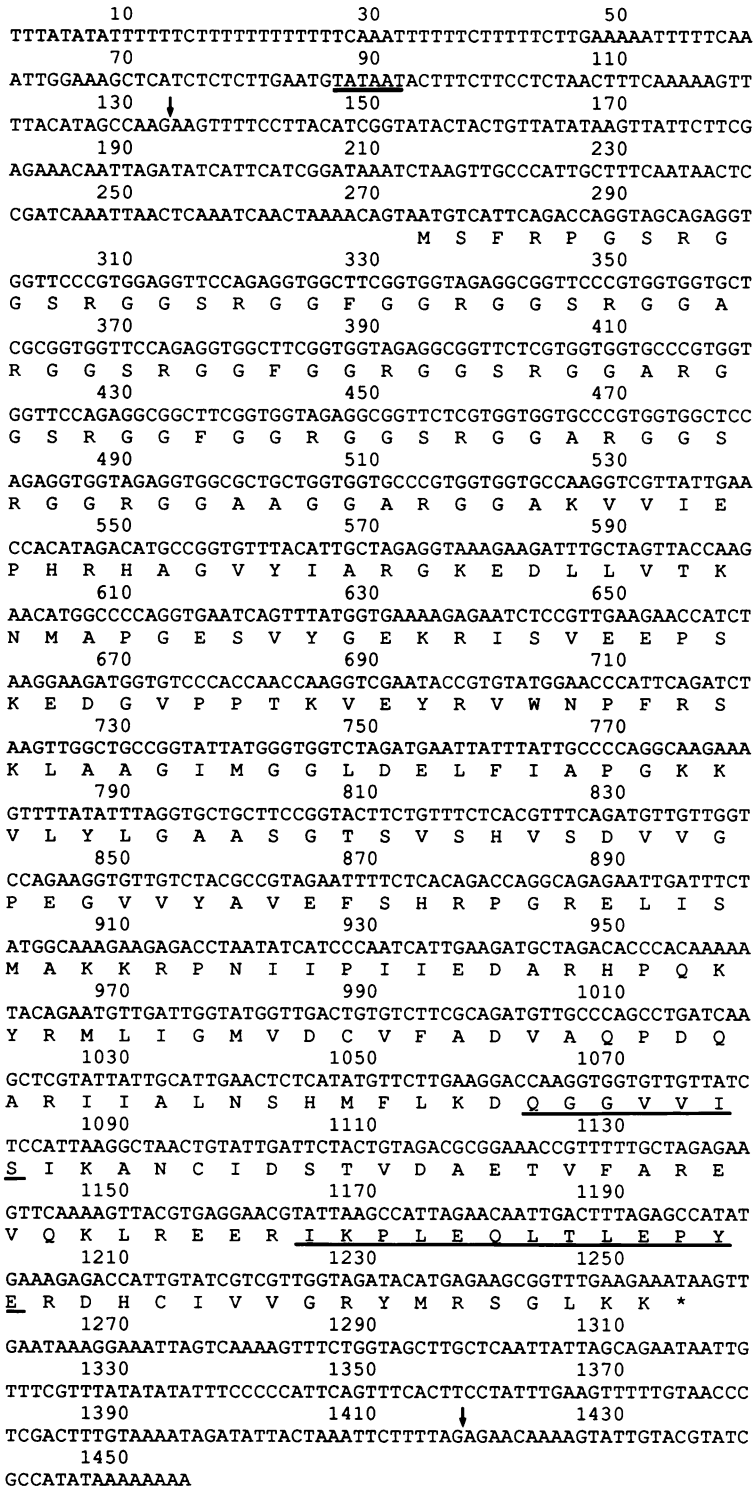

Fig. 4. Sequence analysis of the NOP1 gene. The DNA sequence of the NOP1 gene isolated from a yeast genomic DNA library was determined as described in Materials and methods. The DNA sequence for the cDNA of NOP1 was determined only for 5′ and 3′ end of the clone (~200 bases were sequenced from both ends). As deduced from the genomic DNA sequence, a long open reading frame preceded by an ATG start codon was found which can code for a protein of 327 amino acids. A putative TATA box is underlined in the 5′ promoter region of the NOP1 gene. The putative start of transcription and the start of polyadenylation (as deduced from comparing the cDNA and genomic NOP1 clone) are indicated by arrows in the 5′ and 3′ non-coding region of the NOP1 gene respectively. Two peptide sequences which were obtained by microsequencing of tryptic peptides of the isolated 38 kd protein were found in the NOP1 open reading frame and are underlined.

by plasmids carrying the cloned NOP1 gene (Figure 5B). Microscopic inspection of the spores containing the disrupted NOP1 gene showed that the spores germinated, but stopped cell growth predominantly in a two-cell stage (data not shown). These results show that NOP1 is a single-copy gene in yeast and is essential for cell growth.

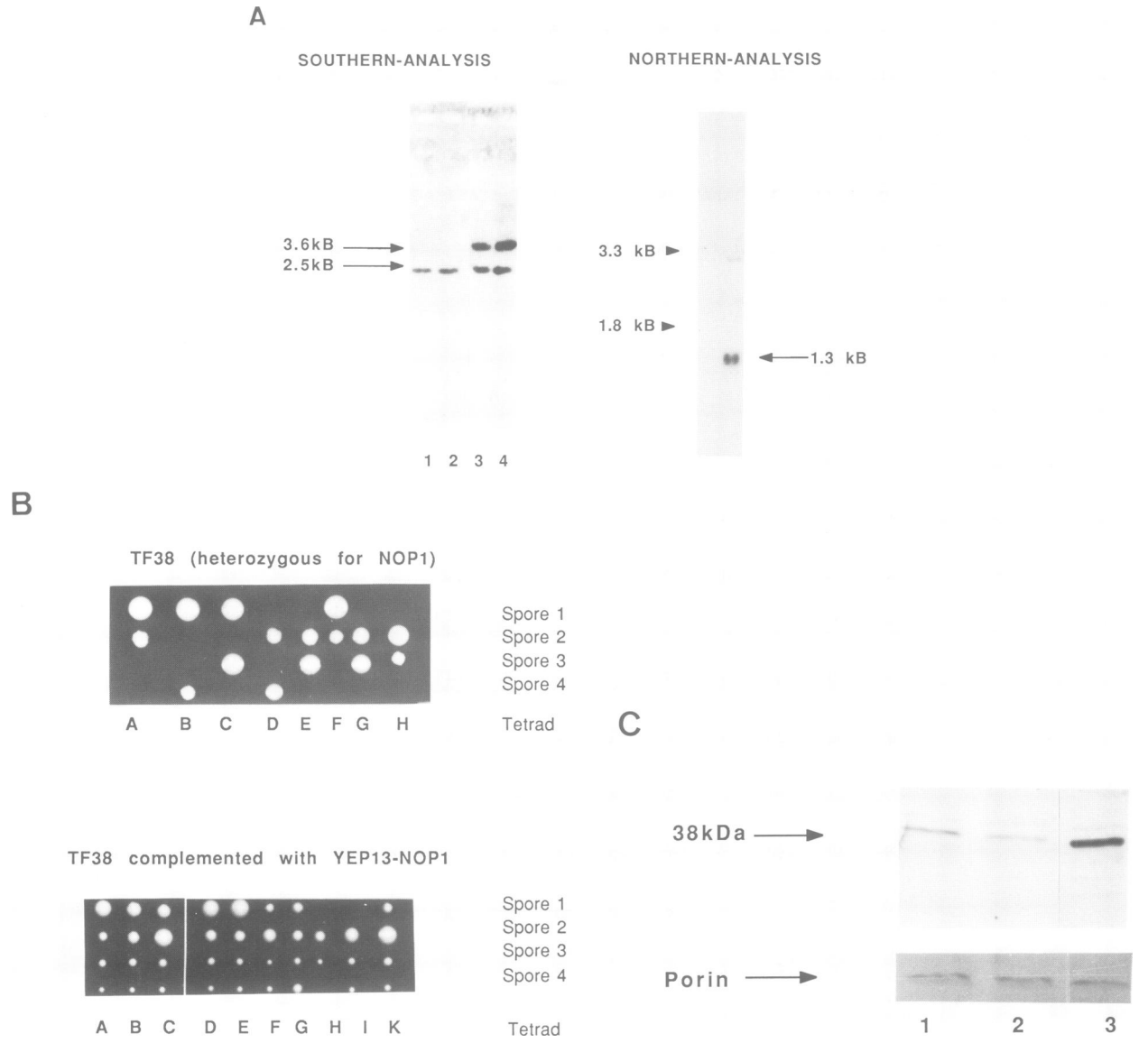

Fig. 5. The NOP1 gene is a single copy gene essential for cell growth. (**A**) Southern and Northern blot analysis of the NOP1 gene. Southern and Northern hybridization was performed as described in Materials and methods. For the Southern analysis genomic DNA was digested with the restriction endonuclease *Eco*RI. **Lanes 1** and **2**, DNA from the parental diploid strain JR26-19B × JU4-2; **lanes 3** and **4**, DNA from two different diploid transformants heterozygous for the NOP1 allele (TF38). The position of the 2.5 kb *Eco*RI fragment containing the NOP1 gene and of a 3.6 kb *Eco*RI fragment containing the NOP1 gene interrupted by the 1.1 kb URA3 gene are indicated by arrows. For the Northern analysis, total yeast RNA was used. The position of the 1.3 kb NOP1 transcript and of the 25S (3.3 kb) and 18S (1.8 kb) rRNA are indicated by arrows. As a probe, the ^{32}P-labelled, 1.4 kb *Eco*RI fragment of the NOP1 cDNA clone was used for both Southern and Northern analysis. (**B**) Tetrad analysis of TF38 (a yeast mutant which is heterozygous of the NOP1 allele) and complementation of haploid nop1-deficient mutants by the cloned NOP1 gene present on a plasmid. Construction of strain TF38, tetrad analysis and yeast transformation with plasmid YEP13-NOP1 was done as described in Materials and methods. The four spores from individual tetrads were placed at the indicated positions of YPD plates and it was grown for 4 (TF38) and 3 days (TF38/YEP13-NOP1) at 30°C. A 2:2 segregation was found for TF38 which is heterozygous for NOP1 and the two viable spores were of ura$^-$ genotype (data not shown), showing that the URA$^+$ genotype co-segregates with cell lethality. A 4:0 segregation was found for TF38 if it was transformed with plasmid YEP13-NOP1 (high copy number plasmid containing the full-length NOP1 gene) prior to tetrad dissection. Here, two out of the four tetrad spores were always URA$^+$. (**C**) Overproduction of the 38 kd nucleolar protein in yeast transformed with a high copy number plasmid containing the NOP1 gene. Yeast cells were grown in minimal medium (SD medium) and immediately after harvest resuspended in hot SDS−sample buffer. Extracts were separated on a SDS−12% polyacrylamide gel, blotted onto nitrocellulose and probed with the indicated antibodies. **Lane 1**, parental yeast strain JR26-19B × JU4-2; **lane 2**, strain TF38 which is heterozygous for the NOP1 gene; **lane 3**, strain TF38 transformed with plasmid YEP13-NOP1 (high copy number plasmid containing the full-length gene of the 38 kd nucleolar protein). The position of the 38 kd nucleolar protein and of mitochondrial porin (internal standard) are indicated by arrows. Only the representative area of the immunoblot is shown.

Discussion

Yeast NOP1 is the homologue of vertebrate fibrillarin, a nucleolar protein implicated in rRNA processing (Nigg, 1988). NOP1 has 72% sequence identity and 83% sequence similarity to *Xenopus* fibrillarin (Lapeyre, *et al.*, 1980) and contains a glycine/arginine-rich amino-terminal repetitive domain (Figure 6A) that shows homology to the amino terminus of rat fibrillarin, the only region sequenced to date (Lischwe *et al.*, 1985b), and to the putative homologue B36 from the lower eukaryote *Physarum* (Christensen and Fuxa, 1988; Pierron *et al.*, 1989). In addition, by virtue of this

A

```
7 SRGGSRGGSRGGFGGRGG
  SRGGARGGSRGGFGGRGG
  SRGGARGGSRGGFGGRGG
  SRGGARGGSRGGRGGAAG 78
```

```
NOP1   SRGGSRGGSRGGFGGRGGSRGGARGGSRGGFGGRGGSRGG
        ::: ::  :::: :::: ::: ::: :::: :::. ::        74 % identity
SSB1   QRGGFRG--RGGFRGRGGFRGGFRGGYRGGFRGRGNFRGR

NOP1   GGSRGGSRGGFGGRGGSRGGARGG----SRGGFGGRGGSRGGARGGSRGGFG
        :.::::.:::::::::::::: :::    .:::::::::: ::: :::. :.:    72 % identity
NUCR   FGGRGGGRGGFGGRGGGRGG-RGGFGGRGRGGFGGRGGFRGG-RGGG-GDFK

NOP1   GGFGGRGGSRGGARGGSRGGFGGRGGSRGGARGGS
        ::. :::. ::      ::::::.::: :::.:::    64 % identity
FIBH   PGFSPRGGGFGG-----RGGFGDRGG-RGGGRGGR
```

B

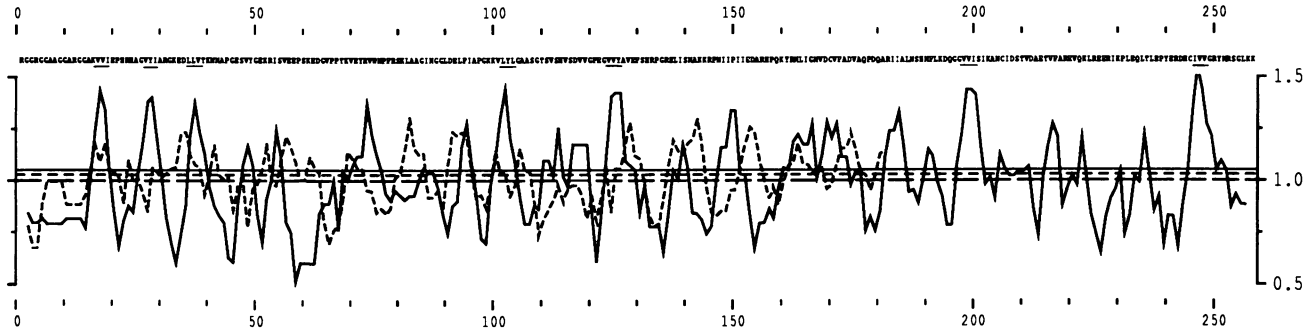

Fig. 6. The yeast NOP1 protein shares sequence homology with yeast and higher eukaryotic nucleolar proteins such as SSB1, nucleolin and fibrillarin. (**A**) A repetitive sequence motif rich in glycins and arginines found at the amino terminus of NOP1 (**upper panel**; the four repeat units were aligned and range from residues 7 to 78) and its similarity to different nucleolar proteins (**lower panel**). The percentage of identity for the overlap is given. For comparison, the corresponding yeast SSB1 (SSB1; Jong *et al.*, 1987), rat nucleolin (NUCR; Bourbon *et al.*, 1988) and rat fibrillarin [FIBH (hepatoma); Lischwe *et al.*, 1985b] sequence were used. (**B**) Secondary structure analysis of the NOP1 protein using the algorithm of Chou and Fasman (1978). The analysis was done using the program PEPPLOT (see also Materials and methods) and the corresponding plot is shown (the plot lacks the structural analysis of the amino-terminal glycine/arginine-rich domain); α-helical structures are indicated in dashed lines, β-structures are shown in solid lines and the amino acid sequence is given above the graph. Clusters of three hydrophobic amino acids which are often found within the peaks of β-stranded structures were underlined.

sequence, yeast NOP1 is related to nucleolin and other proteins, such as yeast SSB1 (Figure 6A) and heterogeneous nuclear ribonucleoprotein A1 from rat, which also contain stretches of glycine/arginine-rich sequence repeats (Lischwe *et al.*, 1985a; Cobianchi *et al.*, 1986; Jong *et al.*, 1987; Christensen and Fuxa, 1988). The role of the glycine/arginine-rich sequence, which is post-translationally modified in different nucleolar proteins by dimethylation of arginine residues, is not known, but they may be involved in RNA binding (Lischwe *et al.*, 1985a; Lapeyre *et al.*, 1986; Christensen and Fuxa, 1988), or alternatively be a structural domain required for integration into the fibrillar compartments of the nucleolus. These possible functions are now testable *in vivo* by genetic manipulation of the NOP1 gene.

The conservation of the amino acid sequence of fibrillarin between yeast and *Xenopus* is strikingly high. This degree of conservation is only found in a few 'housekeeping' proteins such as tubulin, actin or ubiquitin, which play essential roles in universal eukaryotic cell functions. This suggests that the basic function of fibrillarin is extremely conserved in evolution and may be identical from yeast to man.

What is the exact role of the yeast NOP1 and fibrillarin in general? Clues for understanding their function within the nucleolus come from immunoelectron microscopy and immunoprecipitation studies. Within higher eukaryotic cells fibrillarin is located at sites in the nucleolus where transcription and initial processing of rRNA take place and is associated with snoRNA such as U3 (Lischwe *et al.*, 1985b;

Parker and Steitz, 1987). This is consistent with the present study where we find the NOP1 protein located in electron-dense structures within the yeast nucleus resembling the dense fibrillar regions of higher eukaryotic nucleoli. Furthermore, NOP1 is, like its counterpart in mammalian cells, associated with snoRNA. These findings support the model in which fibrillarin functions in early steps of rRNA maturation or pre-ribosome assembly and it may do so by binding to RNA (Nigg, 1988).

In yeast, nine snRNAs (snR3, snR4, snR5, snR8, snR9, snR10, snR128, snR190 and U3) have been reported to be hydrogen bonded to pre-rRNA in the nucleolus (Tollervey, 1987). Strikingly, seven of these species (snR3, snR4, snR5, snR8, snR9, snR10 and snR190) are strongly immunoprecipitated by antibodies affinity purified against NOP1, while the remaining two species (snR128 and U3) are precipitated weakly, but at a level above background. It is thus likely that NOP1 is a component of many different snoRNPs. We speculate that this association is required for correct assembly of the snoRNPs into pre-ribosomal particles and/or is required for nucleolar localization. Association with the Sm proteins common to many nucleoplasmic snRNAs is required for nuclear localization of these snRNAs (Mattaj and De Robertis, 1985). The association of NOP1 with snoRNAs appears to be rather labile; immunoprecipitation from purified yeast nuclei is substantially reduced by relatively mild sonication or incubation at 500 mM KCl (D.Tollervey, unpublished results). Human fibrillarin can also be dissociated from snoRNPs by sonication or incubation at elevated salt concentrations (Parker and Steitz, 1987; Tyc and Steitz, 1989). Moreover, precipitation of human U3 is lost at lower salt concentration than that of other human snoRNAs, indicating a more labile association with fibrillarin. This suggests an explanation for the low levels of precipitation of snR128 and U3; these species may be associated with NOP1 *in vivo* but dissociate during nuclear elution. Alternatively, NOP1 may be a component of the isolated snR128 and U3 snRNPs, but epitopes recognized by anti-NOP1 antibodies may be masked by the structures of the snoRNPs. That this can happen is strongly supported by the behaviour of the autoimmune serum S4. This serum recognizes human fibrillarin and yeast NOP1 on Western blots and efficiently immunoprecipitates snR4 and snR190. However, it fails to precipitate other yeast snRNPs including snR10, the species most efficiently precipitated by anti-NOP1 antibodies. It is very likely that this snoRNP contains NOP1 but the epitope recognized by the S4 immune serum is not accessible for immunoprecipitation.

An RNA binding domain has been identified in many, but not all snRNP and hnRNP proteins (Dreyfuss *et al.*, 1988; Query *et al.*, 1989). Although direct binding of fibrillarin to RNA has not yet been demonstrated, we looked for RNA consensus binding sequences within the yeast NOP1 protein. We find similarities between the consensus sequence domains I, II and IV of RNA binding proteins (Query *et al.*, 1989) within a stretch of ~70 amino acids of the NOP1 protein ranging from residues 95 to 168 (data not shown). It has yet to be shown whether this sequence in NOP1 indeed mediates binding to small nucleolar RNA. Interestingly, secondary structure analysis of the NOP1 protein according to Chou and Fasman (1978) predicts several distinct β-structured sequences scattered along the polypeptide chain and these β-strands generally peak at positions where three hydrophobic amino acids (mainly valines, leucines, isoleucines and tyrosines) cluster (Figure 6B). Pairs of anti-parallel β-polypeptide chain segments (Carter and Kraut, 1974) and hydrophobic residues (Chase and Williams, 1986) were already proposed to be involved in RNA binding.

Our future analysis on the yeast 38 kd nucleolar protein will concentrate on a mutational analysis to define the functionally important domains within NOP1 by *in vivo* complementation and *in vitro* assays. We will also generate temperature-sensitive (ts) mutants of the NOP1 allele which should be helpful in finding extragenic suppressors which may turn out to be components interacting with the 38 kd nucleolar protein. We are currently expressing the NOP1 gene under the regulatory GAL10 promoter which will enable us to shut off specifically NOP1 expression. The primary defect of this mutant should give insight into the overall function of this nucleolar protein in ribosomal biogenesis.

Materials and methods

Strains, media and microbiological methods

The haploid strain D273-10B (ATTC 25657; a; see also Hurt *et al.*, 1988) and the diploid strain JR26-19B × JU4-2 (*α/a, ade 2-1/ade 2-1, ade 8/ADE 8, can 1-100/can 1-100, his 4/HIS 4, his 3/HIS 3, leu 2-3/leu 2-3, lys 1-1/lys 1-1, ura 3-52/ura 3-52*; kindly provided by Dr O.Fasano, EMBL, Heidelberg), the diploid transformant TF38 obtained by transforming JR26-19B × JU4-2 with the URA 3 interrupted NOP1 gene (*α/a, ade 2-1/ade 2-1, ade 8/ADE 8, can 1-100/can 1-100, his 4/HIS 5, his 3/HIS 3, leu 2-3/leu 2-3, lys 1-1/lys 1-1, ura 3-52/URA 3, nop1/NOP1*) were used. Strains were grown in YPD liquid medium/plates containing 1% yeast extract, 2% bactopeptone, 2% glucose and selective medium/plates complemented with the appropriate nutrients (SD-medium: 2% glucose, 0.7% yeast nitrogen base plus nutrients; Sherman *et al.*, 1986). Yeast diploids were sporulated by growing the cells on YPA plates (1% yeast extract, 2% bactopeptone, 1% potassium acetate, 2% agar). It was grown for 3 days at 30°C. Tetrad analysis was performed by incubating sporulated diploids for 10 min with cytohelicase and dissecting the ascus into the four tetrad spores on YPD plates. Yeast transformation was performed by the lithium acetate method (Itoh *et al.*, 1983).

Recombinant DNA work

Standard procedures were used for isolation of plasmid DNA from transformed *E.coli* cells (Birnboim and Doly, 1979), DNA manipulations involving restriction endonucleases, the large fragment of *E.coli* DNA polymerase, polynucleotide kinase and T4 DNA ligase, electrophoresis in agarose gels and recovery of DNA fragments from agarose gels (Maniatis *et al.*, 1982).

Purification of antibodies against the 38 kd nucleolar protein and indirect immunofluorescence

Immune sera made against the insoluble fraction of yeast nuclei (see also Hurt *et al.*, 1988) were generated in rabbits and antibodies against the 38 kd nucleolar protein were affinity purified from this immune serum as described earlier (Hurt *et al.*, 1988) with the following modifications: nuclei were purified from yeast cells and further extracted by 2% Triton X-114 and 200 mM NaCl (Hurt *et al.*, 1988). The insoluble nuclear pellet was resuspended in SDS−sample buffer and boiled at 96°C for 5 min. The sample was applied on a SDS−12% polyacrylamide gel. Separated proteins were blotted on nitrocellulose, the filter was saturated with 2% non-fat milk powder in PBS and incubated with the immune serum in 1/50 dilution in milk/PBS. Antibodies bound to the 38 kd nucleolar protein were re-eluted from the corresponding washed strips by two consecutive elutions in pH 2.8 and pH 2.2 buffer (for details see Hurt *et al.*, 1988). The neutralized eluates were concentrated by ultrafiltration (Amicon; PM 30 filter) to a volume of ~100−200 μl and stored at 4°C. These affinity-purified antibodies against the NOP1 protein were used for immunoblotting, indirect immunofluorescence microscopy and immuno-electron microscopy.

Indirect immunofluorescence microscopy on formaldehyde-fixed yeast cells was performed after Kilmartin *et al.* (1982) with the following modifications: the diploid strain JR26-19B × JU4-2 was grown in YPD medium to OD_{600} ~1. Cells were then fixed in 3% formaldehyde for 1 h at room

temperature, centrifuged and washed in water, incubated for 3 min in 0.1 M Tris−HCl, pH 9.4/10 mM dithiothreitol, washed in 1.2 M sorbitol, 20 mM KP_i, pH 7.4, and converted into spheroplasts using 60 mg zymolyase 20T/g wet weight of cells dissolved in 1.2 M sorbitol, 20 mM KP_i, pH 7.4. Spheroplasts were finally resuspended in a small volume of 1.2 M sorbitol, 20 mM KP_i, pH 7.4, plus 3% formaldehyde, attached to poly-L-lysine-coated coverslips and processed for indirect immunofluorescence microscopy by incubation for 15 min with the affinity-purified antibodies against the NOP1 protein (1/10 dilution). The second antibody was Texas Red-labelled goat anti-rabbit IgG used in a 1:100 dilution. For DNA staining, 0.5 μg/ml Hoechst 33258 (bisbenzimide) was used.

Indirect immunofluorescence on MDCK cells grown on coverslips was done as described by Bre *et al.* (1987). MDCK cells on coverslips were extracted with Triton X-100 and fixed at −20°C in methanol and acetone prior to the incubation with affinity-purified NOP1 antibodies. The second antibody was Texas Red-labelled goat anti-rabbit IgG used in a 1:200 dilution. To stain the nucleus and chromosomes, cells were incubated in a 0.5 μg/ml Hoechst 33258 solution. To prepare MDCK protein extracts, MDCK II cells were grown as described (Bre *et al.*, 1987) and directly dissolved in hot Laemmli's SDS−sample buffer. One hundred micrograms of this extract was run on a SDS−12% polyacrylamide gel, blotted on nitrocellulose and reacted with the affinity-purified antibodies against NOP1 in a 1:100 dilution. Cross-reactive protein bands were visualized using a second antibody coupled to alkaline phosphatase.

Confocal microscopy

Samples to be inspected in the confocal laser scanning microscope were prepared as for indirect immunofluorescence microscopy on yeast spheroplasts described above. The affinity-purified antibodies against NOP1 were used in a 1:100 dilution and the second antibody was Texas Red-labelled goat anti-rabbit IgG used in a 1:100 dilution. The cells were inspected by means of the confocal microscope developed at the EMBL (Stelzer *et al.*, 1989). The images were photographed using a black-and-white monitor (Knott electronik) and a pre-focused camera and recorded on Tmax ASA 100 film.

Isolation of the 38 kd nucleolar protein, generation and microsequencing of tryptic fragments and cloning of the NOP1 gene from a λ gt11 yeast cDNA library

The 38 kd nucleolar protein was isolated from SDS−polyacrylamide gels. To enrich for the protein, yeast nuclei were isolated and extraced with Triton X-114 and 200 mM NaCl as described earlier (Hurt *et al.*, 1988) and the insoluble nuclear pellet was separated on a SDS−12% polyacrylamide gel. The band corresponding to the 38 kd nucleolar protein (see also Figure 1A) was excised from the gel and incubated in water with frequent changes for 16 h. The washed band was cut in pieces and ~90% of the water content was removed by lyophilization. The gel pieces were rehydrated in 100 mM ammonium hydrogen carbonate, pH 8.5, containing trypsin at an enzyme-to-protein ratio of ~1:5. Following incubation at 37°C for 8 h, the generated protein fragments were eluted from the gel by shaking the pieces twice for 3 h with an equal volume of 0.1% trifluoroacetic acid (TFA) in water. Residual water was extracted from the gel matrix by treatment with acetonitrile. The concentrated eluates were extracted twice with a 1:4 mixture of isoamyl alcohol/heptane (Bosserhof *et al.*, 1989) to remove traces of SDS. Separation of the tryptic peptides was performed by reversed-phase HPLC on a Vydac 218TP5 column (1.6 × 250 mm). The peptides were sequenced in a gas-phase sequencer according to Gausepohl *et al.* (1986). A 13 amino acid long peptide was chosen and accordingly a defined oligonucleotide was generated following the yeast codon usage rules (Davis and Thorner, 1987). The oligonucleotide was end-labelled with [γ-^{32}P]ATP and polynucleotide kinase and used to screen a λ gt11 yeast cDNA library (obtained from M.Douglas, Dallas, TX) under low-stringency conditions (hybridization at 42°C, 6 × SSC; washes at 42°C, 2 × SSC). Hybridizing clones were purified and λ DNA was isolated according to standard isolation procedures. The 1.4 kb *Eco*RI insert of the positive λ clone was subcloned into pUC19 and double-stranded dideoxy DNA sequencing after Sanger *et al.* (1977) was performed.

Isolation of the genomic NOP1 gene

A yeast genomic library generated in YEp131 (yeast DNA partially digested with *Sau*3A and inserted into the *Bam*HI site of YEp13; generous gift from O.Fasano, EMBL, FRG) was screened by colony hybridization (Gruenstein and Hogness, 1975) using the ^{32}P-labelled 1.4 kb *Eco*RI fragment from the positive λ gt11 clone under high-stringency conditions (hybridization conditions were as described for genomic Southern blots). Hybridizing clones were purified and plasmid DNA was isolated. Hybridizing plasmids containing inserts covering the whole coding region of the NOP1 gene were used for complementation of the nop1-deficient mutant (see below). The nucleotide sequence of a complementing plasmid covering the entire NOP1 gene including the 5′ promoter sequence and the 3′ polyadenylation site (YEP13-NOP1) was determined for both strands by the dideoxy method (Sanger *et al.*, 1977) using the Sequenase™ system (United States Biochemical Corporation, Cleveland, OH).

Southern and Northern analysis

Genomic yeast DNA and total RNA was isolated as described by Sherman *et al.* (1986). Ten micrograms of yeast DNA (isolated from JR26-19B × JU4-2 and TF38) was digested with *Eco*RI, separated on a 1% agarose gel and blotted onto nitrocellulose. Southern analysis was done according to Maniatis *et al.* (1982) under high-stringency conditions at 68°C in 6 × SSC (1 × SSC is 15 mM sodium citrate plus 150 mM NaCl), 10 mM EDTA, 5 × Denhardt's solution, 0.5% SDS, 200 μg/ml denatured salmon sperm DNA and 1−2 × 10^6 c.p.m. of ^{32}P-labelled 1.4 kb *Eco*RI fragment isolated from the positive λ gt11 clone; the fragment was labelled by nick-translation to a specific activity of ~1 × 10^8/μg DNA using the nick-translation kit of Boehringer (FRG). Twenty micrograms of total yeast RNA denatured in formaldehyde/formamide was separated on a 1.3% agarose gel containing formaldehyde; Northern blotting was done essentially after Maniatis *et al.* (1982). Pre-hybridization and hybridization was performed as described for the Southern analysis (see above) at 68°C in 6 × SSC overnight. The transcript size was estimated using the position of the 25S (3.3 kb) and 18S (1.7 kb) rRNA as a standard.

Gene disruption

The single-step gene disruption was done after Rothstein (1983). The 1.4 kb *Eco*RI fragment from the positive λ gt11 clone was cut at the unique *Bgl*II restriction site which corresponds roughly to the middle of the NOP1 open reading frame (see also Figure 4). 5′ protruding ends were filled in by the large fragment of *E.coli* DNA polymerase and a 1.1 kb blunt-ended *Hin*dIII fragment containing the URA3 gene was inserted. Ten micrograms of the isolated, linear 2.5 kb *Eco*RI fragment containing the URA3 gene (a selectable marker) flanked by coding sequences of the NOP1 gene was used to transform the diploid ura$^-$ strain JR26-19B × JU4-2. Approximately 200 transformants of URA$^+$ genotype were obtained. Integration of the linear, interrupted gene into the homologous NOP1 locus was verified by Southern analysis. One of the transformants (TF38), which contained one wild-type copy and one interrupted copy of the NOP1 gene, was used for tetrad dissection.

Immunoprecipitations and RNA analysis

For preparation of yeast cell lysates, exponentially growing cells were harvested at OD_{600} 1, washed and resuspended at 1% of culture volume in buffer A [150 mM KCl, 20 mM Tris−HCl, pH 8.0, 5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 mM vanadyl ribonucleoside complex (BRL), 0.01% Triton X-100]. Cells were lysed by vigorous vortexing for 5 min at 4°C with 0.45−0.5 mm glass beads. The lysate was cleared by centrifugation for 5 min in an Eppendorf centrifuge and stored at −70°C. Immunoprecipitation was performed as described by Tollervey and Mattaj (1987) using 10 μl lysate, equivalent to 2 × 10^7 cells, per reaction.

Hybridization probes for snRNAs were as follows: snR3, 1.4 kb *Hin*dIII fragment of pDJ3 (Tollervey *et al.*, 1983) containing the SNR3 gene; snR4: 600 bp *Eco*RI−*Hin*dIII fragment of YEpR4 (Wise *et al.*, 1983) containing the SNR4 gene; snR5, 250 bp *Bam*HI−*Xho*I fragment of pES3 (Parker *et al.*, 1988) containing the SNR5 gene; snR8, 500 bp *Sal*I−*Eco*RV fragment of YEpR8 (Parker *et al.*, 1988) containing the SNR8 gene; snR9, oligo (AGTATGTCTGAAGGACT) complimentary to the 3′ region of snR9 (Parker *et al.*, 1988); snR10, *Bgl*II−*Bst*EII fragment of pr109.1 (Tollervey and Guthrie, 1985) containing the 5′ 200 nucleotides of SNR10 (Tollervey and Guthrie, 1985); snR128 and snR190, 1 kb *Cla*I fragment of pDJ14 (Zagorski *et al.*, 1988) containing the SNR128 and SNR190 genes; U3, plasmid pR3 containing the gene SNR17A (Hughes *et al.*, 1987). Hybridization probes were prepared from purified DNA fragments by randomly primed labelling with six nucleotide primers (Pharmacia). Probes for U1, U2 and U4 were the SNU1, SNU2 and SNU4 genes of *Schizosaccharomyces pombe* cloned in Bluescript and transcribed with T3 RNA polymerase.

Computer analysis

The nucleotide sequence of the NOP1 gene and its predicted amino acid sequence were analysed by the programs of the University of Wisconsin Genetics Computer Group (UWGCG). The mol. wt and amino acid composition was analysed by PEPTIDESORT. Secondary structure was predicted by PEPPLOT using the algorithms of Chou and Fasman (1978). LP/PIR/FASTP/FASTA (EMBL data banks) were used to detect sequence

homologies between the predicted NOP1 amino acid sequence and known proteins.

Miscellaneous

Additional experimental procedures are described or cited in Hurt *et al.* (1988) and Hurt (1988) including growing yeast cells, subcellular fractionation and isolation of nuclei, antibody generation in rabbits, SDS−PAGE and immunoblotting using the systems of the alkaline phosphatase coupled to a second antibody or protein A coupled to horseradish peroxidase.

Acknowledgements

We would like to thank Dr M.Douglas (Dallas, TX) for giving the λ gt11 yeast cDNA library, Dr G.Schatz (Basel) for the anti-porin antibody, Dr B.Lapeyre (Toulouse) for communicating the amino acid sequence of *Xenopus* fibrillarin prior to publication, Dr J.Aris and Dr G.Blobel (New York) for sequence data exchange on the NOP1 DNA sequence, Dr R.Lührmann (Marburg) for the autoimmune serum S4, Dr B.Buendia (EMBL, Heidelberg) for her help in performing confocal microscopy, Hanna Lehtonen for excellent technical assistance and Dr I.W.Mattaj for critical reading of the manuscript.

References

Aris,J.P. and Blobel,G. (1988) *J. Cell Biol.*, **107**, 17−31.
Birnboim,H.C. and Doly,J.F. (1979) *Nucleic Acids Res.*, **7**, 1513−1523.
Bre,M.-H., Kreis,T.E. and Karsenti,E. (1987) *J. Cell Biol.*, **105**, 1283−1296.
Bosserhof,A., Wallach,J. and Frank,R. (1989) *J. Chromatogr.*, **473**, 71−77.
Bourbon,H.M., Lapeyre,B. and Amalric,F. (1988) *J. Mol. Biol.*, **200**, 627−638.
Carter,C.W. and Kraut,J. (1974) *Proc. Natl. Acad. Sci. USA*, **71**, 283−287.
Chase,J.W. and Williams,K.R. (1986) *Annu. Rev. Biochem.*, **55**, 103−136.
Chou,P.W. and Fasman,G.D. (1978) *Annu. Rev. Biochem.*, **47**, 251−276.
Christensen,M.E., Moloo,J., Swischuk,J.L. and Schelling,M.E. (1986) *Exp. Cell Res.*, **166**, 77−93.
Christensen,M.E. and Fuxa,K.P. (1988) *Biochem. Biophys. Res. Commun.*, **155**, 1278−12783.
Cobianchi,F., SenGupta,D.N., Zmudzka,B.Z. and Wilson,S.H. (1986) *J. Biol. Chem.*, **261**, 3536−3543.
Davis,T.N. and Thorner,J. (1987) *Methods Enzymol.*, **139**, 248−262.
Dreyfuss,G., Swanson,M. and Pinol-Roma,S. (1988) *Trends Biochem.*, **13**, 86−91.
Gas,N., Escande,M.-L. and Stevens,B.J. (1985) *Biol. Cell*, **53**, 209−218.
Gausepohl,H., Trosin,M. and Frank,R. (1986) In Wittmann-Liebold,B., Salnikow,J. and Erdmann,V.A. (eds), *Advanced Methods in Protein Microsequence Analysis*. Springer-Verlag, Berlin, pp. 149−160.
Goessens,G. (1984) *Int. Rev. Cytol.*, **87**, 107−158.
Gruenstein,M. and Hogness,D.S. (1975) *Proc. Natl. Acad. Sci. USA*, **72**, 3961−3965.
Hadjiolov,A.A. (1985) In Alfert,M., Bermann,W., Goldstein,L., Porter,K.R. and Site,P. (eds), *The Nucleolus and Ribosome Biogenesis*. Springer-Verlag, Wien, Vol. 12, pp. 111−132.
Hügle,B., Hazan,R., Scheer,U. and Franke,W.W. (1985a) *J. Cell Biol.*, **100**, 873−886.
Hügle,B., Scheer,U. and Franke,W.W. (1985b) *Cell*, **41**, 615−627.
Hughes,J.M.X., Konings,D.A.M. and Cesareni,G. (1987) *EMBO J.*, **6**, 2145−2155.
Hurt,E.C. (1988) *EMBO J.*, **7**, 4323−4334.
Hurt,E.C., McDowall,A. and Schimmang,T. (1988) *Eur. J. Cell Biol.*, **46**, 554−563.
Itoh,H., Fukada,Y., Murata,K. and Kimura,A. (1983) *J. Bacteriol.*, **153**, 163−168.
Jong,A.Y.S. and Campbell,J.L. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 877−881.
Jong,A.Y.S., Clark,M.W., Gilbert,M., Oehm,A. and Campbell,J.L. (1987) *Mol. Cell. Biol.*, **7**, 2947−2955.
Jordan,G. (1987) *Nature*, **329**, 489−490.
Kilmartin,J.V., Wright,B. and Milstein,C. (1982) *J. Cell Biol.*, **93**, 576−582.
Lapeyre,B., Caizergues-Ferrer,M., Bouche,G. and Amalric,F. (1985) *Nucleic Acids Res.*, **13**, 5805−5816.
Lapeyre,B., Amalric,F., Ghaffari,S.H., Rao,S.V.V., Dumbar,T.S. and Olson,M.O.J. (1986) *J. Biol. Chem.*, **261**, 9167−9173.
Lapeyre,B., Mariotini,P., Mathieu,C., Ferrer,P., Amaldi,F., Amalric,F. and Caizergues-Ferrer,M. (1990) *Mol. Cell. Biol.*, in press.
Lischwe,M.A., Cook,R.G., Ahn,Y.S., Yeoman,L.C. and Busch,H. (1985a) *Biochemistry*, **24**, 6025−6028.
Lischwe,M.A., Ochs,R.L., Reddy,R., Cook,R.G., Yeoman,L.C., Tan,E.M., Reichlin,M. and Busch,H. (1985b) *J. Biol. Chem.*, **260**, 14304−14310.
Maniatis,T., Fritsch,E.T. and Sambrook,J. (1982) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Mattaj,I.W. and De Robertis,E.M. (1985) *Cell*, **40**, 111−118.
Nigg,E.A. (1988) *Int. Rev. Cytol.*, **110**, 27−92.
Ochs,R., Lischwe,M., O'Leary,P. and Busch,H. (1983) *Exp. Cell Res.*, **146**, 139−149.
Ochs,R.L., Lischwe,M.A., Shen,E., Carrol,R.E. and Busch,H. (1985a) *Chromosoma*, **92**, 330−336.
Ochs,R.L., Lischwe,M.A., Spohn,W.H. and Busch,H. (1985b) *Biol. Cell*, **54**, 123−134.
Parker,K.A. and Steitz,J.A. (1987) *Mol. Cell. Biol.*, **7**, 2899−2913.
Parker,R., Simmons,T., Shuster,E.O., Siliciano,P.G. and Guthrie,C. (1988) *Mol. Cell. Biol.*, **8**, 3150−3159.
Pierron,G., Pedron,J., Schelling,M. and Christensen,M. (1989) *Biol. Cell*, **65**, 119−126.
Query,C.C., Bentley,R.C. and Keene,J.D. (1989) *Cell*, **57**, 89−101.
Raska,I., Reimer,G., Jarnik,M., Kostrouch,Z. and Raska,K. (1989) *Biol. Cell*, **65**, 79−82.
Reimer,G., Raska,I., Tang,E.M. and Scheer,U. (1987) *Virchow's Arch. B*, **54**, 131−143.
Rothstein,R. (1983) *Methods Enzymol.*, **101**, 202−211.
Sanger,F., Nicklen,S. and Coulson,A.R. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 5463−5467.
Scheer,U. and Rose,K.M. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 1431−1435.
Schmidt-Zachmann,M., Hügle-Dörr,B. and Franke,W.W. (1987) *EMBO J.*, **6**, 1881−1890.
Sherman,F., Fink,G.R. and Hicks,J.B.N. (1986) *Methods in Yeast Genetics. A Laboratory Course Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Sillevis Smitt,W.W., Vermeulen,C.A., Vlak,J.M., Rozijn,T.H. and Molenaar,I. (1972) *Exp. Cell Res.*, **70**, 140−144.
Sillevis Smitt,W.W., Vlak,J.M., Molenaar,I. and Rozijn,T.H. (1973) *Exp. Cell Res.*, **80**, 313−321.
Sommerville,J. (1986) *Trends Biochem. Sci.*, **11**, 438−442.
Spector,D.L., Ochs,R.L. and Busch,H. (1984) *Chromosoma*, **90**, 139−148.
Stelzer,E.H.K., Stricker,R., Pick,R., Storz,C. and Hamminer,P. (1989) In Wilson,T. (ed.), *Scanning Imagings, SPIE Proceedings, Confocal Fluorescence Microscopes for Biological Research*. pp. 146−151.
Steitz,J.A., Black,D.L., Gerke,V., Parker,K.A., Kramer,A., Frendewey,D. and Keller,W. (1988) In Birnstiel,M.L. (ed.), *Structure and functions of Major and Minor Small Nuclear Ribonucleoprotein Particles*. Springer-Verlag, Berlin, pp. 115−154.
Tollervey,D. (1987) *EMBO J.*, **6**, 4169−4175.
Tollervey,D. and Guthrie,C. (1985) *EMBO J.*, **4**, 3873−3878.
Tollervey,D. and Mattaj,I.W. (1987) *EMBO J.*, **6**, 469−476.
Tollervey,D., Wise,J.A. and Guthrie,C. (1983) *Cell*, **35**, 753−762.
Tyc,K. and Steitz,J.A. (1989) *EMBO J.*, **8**, 3113−3119.
Warner,J.R. (1989) *Microbiol. Rev.*, **53**, 256−271.
Wise,J.A., Tollervey,D., Maloney,D., Swerdlow,H. Dunn,E.J. and Guthrie,C. (1983) *Cell*, **35**, 743−751.
Zagorski,J., Tollervey,D. and Fournier,M. (1988) *Mol. Cell. Biol.*, **8**, 3282−3290.

Received on September 14, 1989; revised on October 9, 1989