Evolutionary Conservation of the Human Nucleolar Protein Fibrillarin and its Functional Expression in Yeast

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Abstract. NOP1 is an essential nucleolar protein in yeast that is associated with small nucleolar RNA and required for ribosome biogenesis. We have cloned the human nucleolar protein, fibrillarin, from a HeLa cDNA library. Human fibrillarin is 70% identical to yeast NOP1 and is also the functional homologue since either human or *Xenopus* fibrillarin can complement a yeast nop1$^-$ mutant. Human fibrillarin is localized in the yeast nucleolus and associates with yeast small nucleolar RNAs. This shows that the signals within eucaryotic fibrillarin required for nucleolar association and nucleolar function are conserved from yeast to man. However, human fibrillarin only partially complements in yeast resulting in a temperature-sensitive growth, concomitantly altered rRNA processing and aberrant nuclear morphology. A suppressor of the human fibrillarin ts-mutant was isolated and found to map intragenically at a single amino acid position of the human nucleolar protein. The growth rate of yeast nop1$^-$ strains expressing *Xenopus* or human fibrillarin or the human fibrillarin suppressor correlates closely with their ability to efficiently and correctly process pre-rRNA. These findings demonstrate for the first time that vertebrate fibrillarin functions in ribosomal RNA processing in vivo.

THE nucleolus is the site of transcription of the rRNA genes, processing of precursors to cytoplasmic rRNA molecules, and assembly of ribosomal particles (for review see Hadjiolov, 1985; Scheer and Benavente, 1990). The mechanisms underlying these processes are largely unknown, but some of these biochemical pathways have been localized to specific subnucleolar compartments identified by immunocytochemical and electron microscopy approaches. The transcription of the rRNA genes by RNA-polymerase I takes place in the fibrillar centers (FC)[1] of the nucleolus, the subsequent preribosome formation in the dense fibrillar component (DFC) surrounding the fibrillar centers. Further assembly and maturation steps of the preribosomal particles finally occur outside the FC-region in the granular compartment (GC) (for review see Hadjiolov, 1985; Scheer and Benavente, 1990).

It is assumed that this complex structural organization of the nucleolus is a requirement for the coordinate assembly of ribosomes (for review see Hadjiolov, 1985). Comparatively little is known about the role of nucleolar proteins in ribosome biogenesis. Several nucleolar proteins have been identified (for review see Nigg, 1988; Scheer and Benavente, 1990) which have been localized to the different subnucleolar compartments. Among these nucleolar proteins a 34–36-kD protein called fibrillarin, because of its location to the DFC, appears to be one key component in ribosomal biogenesis. Fibrillarin was initially identified in *Physarum* (Christensen et al., 1977) and later in mammalian cells with the help of autoimmune sera from patients with scleroderma (Ochs et al., 1985).

Anti-fibrillarin antibodies precipitate RNPs that contain the small nucleolar RNAs (snoRNA) U3, U8, and U13 (Tyc and Steitz, 1989). This physical association between fibrillarin and snoRNAs implied a role of the protein in processing of rRNA precursors within the dense fibrillar component and direct evidence for this recently came from an in vitro processing system using antibodies against fibrillarin (Kass et al., 1990). In the budding yeast *Saccharomyces cerevisiae*, a 38-kD nucleolar protein named NOP1 has been identified that is immunologically related to vertebrate fibrillarin (Aris and Blobel, 1988; Hurt et al., 1988; Schimmang et al., 1989). NOP1 has been cloned and sequenced (Schimmang et al., 1989; Henriquez et al., 1990) and its glycine/arginine-rich amino-terminal domain was shown to have sequence homology to other nucleolar proteins, such as yeast SSB1 (Jong et al., 1987), nucleolin (Lapeyre et al., 1987), as well as rat fibrillarin (Lischwe et al., 1985). Yeast NOP1 is an abundant nucleolar protein that is essential for growth (Schimmang et al., 1989) and required for the modification and processing of pre-rRNA (Tollervey et al., 1991), suggesting that it may be one of the key components in nucleolar structure and function. Furthermore, NOP1 is associated with snoRNAs impli-

cated in rRNA maturation (Schimmang et al., 1989) including U3, snR10, snR190, and U14 (previously snR128) (Zagorski et al., 1988; Tollervey, 1987; Li et al., 1990). *Xenopus* fibrillarin has also been cloned (Lapeyre et al., 1990) and, as deduced from the DNA-sequence, its amino acid sequence is highly homologous to yeast NOP1 (Schimmang et al., 1989). These data suggested that yeast NOP1 and vertebrate fibrillarin may perform a conserved function in the eucaryotic nucleolus.

To analyze the general role of eucaryotic fibrillarin for nucleolar structure and ribosome biogenesis, we have isolated from a HeLa cDNA library the gene coding for human fibrillarin. When expressed in yeast, human fibrillarin complements a yeast mutant defective in NOP1. This shows that the basic function of fibrillarin is conserved from yeast to man.

Materials and Methods

Growth of Yeast Strains and Microbiological Methods

The diploid yeast strain JU4-2xJR26-19B (a/α, ade2-1/ade2-1, ade8/ADE8, can1-100/can1-100, his4/HIS4, his3/HIS3, leu2-3/leu2-3, lys1-1/lys1-1, ura3-52/ura3-52) and the diploid transformant TF38NULL heterozygous for the NOP1 alleles (a/α, ade2-1/ade2-1, ade8/ADE8, can 1-100/can1-100, his4/HIS4, his3/HIS3, leu2-3/leu2-3, lys1-1/lys1-1, ura3-52/URA3, nop1/NOP1) were used. Strains were grown in rich glucose-containing medium (YPD-medium) or minimal glucose-containing medium with the appropriate nutrients as described earlier (Schimmang et al., 1989). Yeast transformation was done by the lithium acetate method (Sherman et al., 1986). Diploid yeast strains were sporulated on YPA plates (1% yeast extract, 2% bactopeptone, 1% potassium acetate, 2% agar); 3 d later, sporulated asci were treated with cytohelicase and then dissected to isolate four tetrad spores which were germinated and grown on YPD plates at 23°C.

Gene Disruption

One copy of the NOP1 gene was completely evicted from the diploid strain JU4-2xJR26-19B according to Rothstein (1983). The genomic NOP1 gene located on a 2.5-kb EcoRI restriction fragment (see also, Schimmang et al., 1989) was inserted into the single EcoRI restriction site of plasmid pUC19. It was cut with restriction enzymes EcoRV and SnabI, which released from the plasmid the complete coding region of the NOP1 gene. A 1.1-kb blunt-ended HindIII restriction fragment containing the URA3 gene was inserted into this plasmid thereby attaching to the URA3 gene 5′ and 3′ noncoding sequences of the NOP1 gene. This construct composed of the URA3-gene plus adjacent 5′ and 3′ noncoding NOP1 DNA was excised from the plasmid by EcoRI and ~10 μg of this linear DNA fragment was used to transform the diploid yeast strain JU4-2xJR26-19B. URA$^+$ transformants were obtained which carried the NOP1 gene disruption at the homologous locus as demonstrated by Southern analysis (data not shown). One of these transformants (TF38NULL) heterozygous for NOP1 was transformed with the human fibrillarin gene present on a 2μ yeast plasmid and tetrad analysis was performed.

Recombinant DNA Work, Southern and Northern Analysis, Immunoprecipitation, and Isolation of the Human Fibrillarin cDNA by PCR

DNA recombinant work was performed as outlined by Maniatis et al. (1982). DNA restriction fragments were purified from agarose gels using the "Gene Clean" kit (Bio101, La Jolla, CA). Southern and Northern analysis was performed under high stringency conditions as described by Schimmang et al. (1989). Immunoprecipitation was performed as described by Schimmang et al. (1989). A HeLa cDNA library inserted into plasmid pUEX1 (kindly provided by Dr. G. Banting, Bristol, England) was used to clone the human fibrillarin gene. The cDNA library present in *Escherichia coli* was amplified in liquid L-Broth medium containing 25 μg/ml ampicillin at 30°C. Plasmid DNA was isolated and used for PCR amplification of the human fibrillarin gene. Oligonucleotide primers corresponding to 5′- and 3′- conserved regions of the yeast NOP1 (Schimmang et al., 1989) and *Xenopus* fibrillarin (Lapeyre et al., 1990) were designed according to Moreman (1989). The forward and reverse primer which correspond to cytosine-610 and thymidine-1084 of the NOP1 gene (see also, Schimmang et al., 1989) were:

5′-CCIGGIGAG/AIIIGTITAC/$_T$GGIGAA/$_G$AAG-3′
5′-A/$_G$TCIATA/$_G$CAA/$_G$TTIGCT/$_C$TTIAT-3′.

A typical PCR amplification assay contained in a 50-μl reaction volume: 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris, pH 8.3, 0.05% gelatine, 0.2 mM dNTPs, 10% DMSO, 75 pM forward and backward primers (corresponding to the 5′ and 3′ part of the NOP1 gene), 50 ng plasmid DNA with inserted HeLa cDNA and 2.5 U Taq-polymerase from Perkin-Elmer Corp. (Norwalk, CT). The samples were placed in an automated heating/cooling block which was programmed for a temperature step cycle of 94°C (1 min), 50°C (2 min), and 72°C (2 min). 30 cycles were run and the products were then analyzed by 1.5% agarose gel electrophoresis. The amplified band of expected size (470 nucleotides) was isolated, labeled with ^{32}P-dCTP using the "multi-prime DNA labeling" kit from Amersham Corp. (Arlington Heights, IL) and a full-length cDNA clone of human fibrillarin was isolated from the same cDNA library in pUEX1 by colony hybridization under high stringency conditions (Gruenstein and Hogness, 1975; see also Schimmang et al., 1989). Double-stranded dideoxy sequencing of the alkaline denatured cDNA probe (a total length of 1,090 nucleotides) from plasmid pUEX1 was performed for both strands (Sanger et al., 1977).

Construction of the Human Fibrillarin Gene under the Alcohol Dehydrogenase Promoter

The cloned human fibrillarin cDNA present in plasmid pUEX1 contains in the 5′ noncoding region an extra ATG-codon which is not in frame with the long open reading frame of human fibrillarin (see also Fig. 1 *A*). To maximize expression in yeast, this upstream ATG was removed by digesting the cDNA with restriction enzyme BanII which cuts four nucleotides upstream of the fibrillarin ATG-start codon (see also Fig. 1 *A*). A blunt-ended BanII restriction fragment containing the entire human fibrillarin gene was then inserted into the blunt-ended restriction site EcoRI of plasmid pBPHI which contained 5′ to this EcoRI site the alcohol dehydrogenase I (ADHI) promoter (see also Hurt et al., 1985). pBPHI is a YCp50 derivative which carries the ADHI-promoter (McKnight and McConaughy, 1983) and a pUC19-polylinker with multiple cloning sites downstream of the promoter.

From this constructed plasmid, the human fibrillarin gene under ADHI promoter control was excised as a 1.7-kb EcoRV/HindIII restriction fragment and inserted into the 2μ yeast plasmid YEP13 which had been previously cut with restriction enzymes PvuII/HindIII. YEP13 contains as a selectable marker the LEU2 gene (Broach et al., 1979). Plasmid YEP13-ADHI-humFib was then used to transform the diploid strain TF38NULL heterozygous for the NOP1 allele (see above). LEU$^+$/URA$^+$-transformants were sporulated and haploid progeny containing the evicted NOP1 gene and plasmid YEP13-ADHI-humFib were further characterized. For control, the human fibrillarin gene was inserted also in opposite orientation downstream of the ADH-promoter to block its expression in yeast. Furthermore, the authentic NOP1 gene (a genomic 2.5-kb EcoRI restriction fragment) was inserted into the ARS/CEN plasmid pSB32 (kindly provided by R. Serrano, EMBL, Heidelberg) and introduced in TF38NULL by transformation and selection for LEU$^+$ transformants. Haploid progeny carrying the disrupted genomic NOP1 gene and plasmid pSB32-NOP1 or YEP13-ADHI-humFib were compared.

To obtain the expression of *Xenopus* fibrillarin in *Saccharomyces cerevisiae*, the cDNA from pXomfib (Lapeyre et al., 1990) was cloned as a SalI-BamHI fragment into the SalI-BamHI sites of YEp51 (Broach et al., 1983) to generate pXenfib. The vector contains the 2μ origin of replication and the LEU2 gene, permitting its replication and selection in yeast. The promoter region of the GAL10 gene is located 5′ to the SalI cloning site; transcription of the inserted cDNA is therefore induced during growth on galactose medium and strongly repressed by growth in the presence of glucose.

Isolation of Human Fibrillarin ts-Suppressors

A haploid yeast strain carrying the disrupted genomic NOP1 gene and plasmid YEP13-ADHI-humFib (called A2), which is temperature-sensitive for growth at elevated temperatures, was plated on YPD-plates and incubated at 37°C for 5 d. Spontaneous suppressors arose which after purification showed efficient growth at 30°C and still could grow at the restrictive temperature of 35°C. Plasmid DNA was recovered from these suppressors by isolation of total DNA (Sherman et al., 1986) followed by transformation

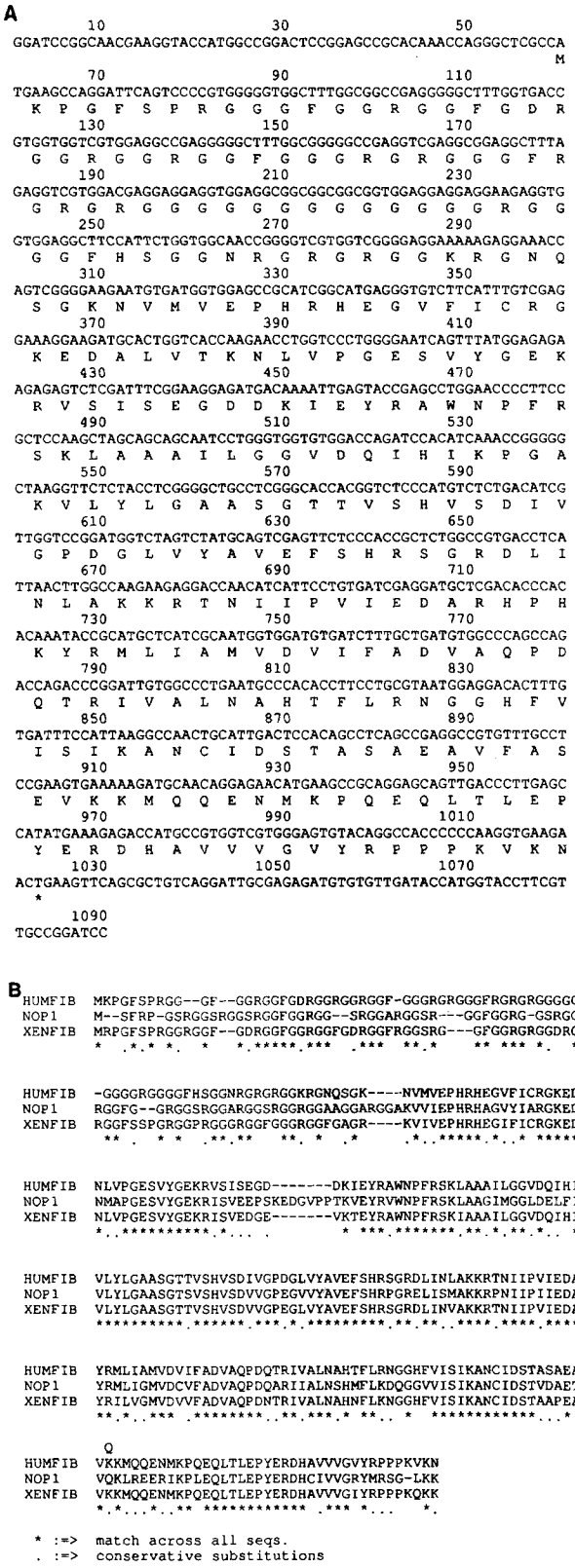


Figure 1. Human fibrillarin and yeast NOP1 are highly homologous. (*A*) Sequence analysis of the human fibrillarin cDNA. Cloning and sequencing of the human fibrillarin cDNA is described in Materials and Methods. The full-length cDNA clone with a length of 1,090 nucleotides contains a long open reading frame preceded by an ATG-start codon coding for a protein of 321 amino acids. These sequence data are available from EMBL/GenBank/DDBJ under accession number 56597. (*B*) Amino acid sequence comparison between human and *Xenopus* fibrillarin and yeast NOP1. The amino acid sequence of yeast NOP1 (*middle* sequence, NOP1), of human fibrillarin (*top* sequence, HUMFIB), and *Xenopus* fibrillarin (*bottom* sequence, XENFIB) were compared by computer analysis. Identical and structurally related amino acids are marked. The amino acid (K → Q) altered in the temperature resistant mutant obtained for human fibrillarin is indicated above the HUMFIB sequence.

of *E. coli* and selection for ampicillin-resistant clones. Reisolated plasmids YEP13-ADHI-humFib were used to transform a haploid yeast strain whose authentic NOP1 gene was replaced by a modified NOP1 gene under the control of the regulatory GAL10 promoter (Tollervey et al., 1991). If grown on glucose, this strain was completely dependent on the functional expression of the human fibrillarin. Reisolated plasmids YEP13-ADHI-humFib that supported growth at 35°C in the GAL10-NOP1 strain were assumed to contain an intragenic mutation suppressing the temperature-sensitive growth. Accordingly, the DNA sequence of the coding region of these human fibrillarin genes was determined by the dideoxy sequencing method (Sanger et al., 1977).

Pulse-Chase Labeling of rRNA

For pulse-chase labeling of pre-rRNA, 3 ml of cells growing in glucose minimal medium at OD_{600} ~0.3, were labeled with 100 μCi of either [^{3}H-methyl]methionine or [^{3}H]uracil for 2 min at 30°C. Unlabeled methionine was added to a final concentration of 5 mM, unlabeled uracil was added to a final concentration of 240 $\mu g/ml^{-1}$. Samples (1 ml) were taken, transferred to 1.5-ml centrifuge tubes at room temperature and centrifuged for 8 s at full speed in an Eppendorf 5414 centrifuge (Brinkman Instruments Co., Westbury, NY) at room temperature. Cell pellets were immediately frozen in a dry ice/ethanol bath and stored at −80°C until used. The time for harvesting and freezing of the cells (1 min) is included in the chase times. RNA extraction, agarose/formaldehyde gels, gel transfer and fluorography were as previously described (Tollervey, 1987).

Yeast Indirect Immunofluorescence and Confocal Microscopy

Indirect immunofluorescence on yeast cells complemented by human fibrillarin was done essentially as previously described (Schimmang et al., 1989). Double indirect immunofluorescence was done using affinity-purified anti-NSP1 antibodies (anti-rabbit) and a monoclonal antibody against yeast NOP1 and cross-reactive with human fibrillarin (anti-mouse; mAb A66, kindly provided by Dr. J. Aris, Rockefeller University, New York) followed by second antibodies, Texas red goat anti-rabbit and fluorescein goat anti-mouse IgGs, diluted 1:50. Cells stained for NSP1 and NOP1 were inspected in the confocal microscope developed at the EMBL (Stelzer et al., 1989).

HeLa Cell Culture, Fractionation, and Immunocytochemistry

Suspension HeLa cells (S3) were grown to a density of 1–4 × 10^5 cells/ml in Joklik's modified minimum essential medium supplemented with 5% newborn calf serum and containing penicillin and streptomycin.

Nuclei were prepared according to Ohtsubo et al. (1989). Briefly, the cells were homogenized with a Dounce homogenizer in ice-cold hypotonic buffer (10 mM Hepes, pH 8.0, 5 mM KCl, 2 mM $MgCl_2$) containing 0.5% NP-40 and 1 mM PMSF. Nuclei were pelleted at 1,000 *g* for 5 min.

Immunofluorescence microscopy was performed using isolated HeLa nuclei attached to poly-L-lysine-coated glass coverslips. The nuclei were fixed in 3.7% paraformaldehyde and sequentially incubated with anti-human fibrillarin affinity-purified antibodies (diluted 1:10) and Texas red–conjugated goat anti-rabbit IgG (diluted 1:100). Immunofluorescence was carried out with a Zeiss Axiophot Photomicroscope.

Monolayer cultures of HeLa cells were grown in minimum essential medium supplemented with 10% fetal calf serum and nonessential amino acids and containing penicillin and streptomycin. The cells were fixed with 8% paraformaldehyde in 200 mM Hepes (pH 7.4), prepared for cryosectioning and labeled with antibodies and protein A-gold as described (Hurt et al., 1988).

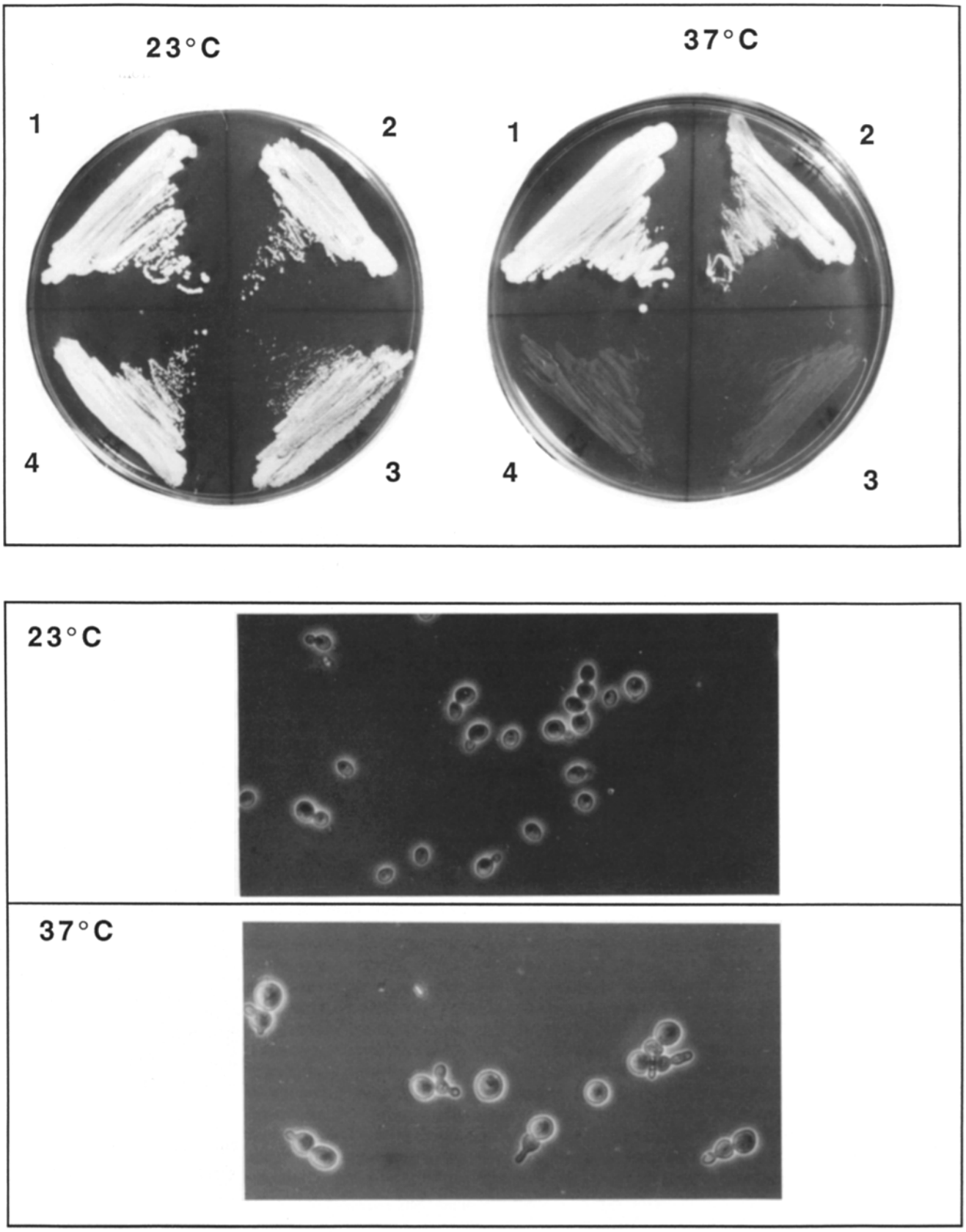


Figure 2. Human fibrillarin can complement a yeast mutant lacking the NOP1 gene, but are temperature sensitive for growth. Transformation of TF38NULL heterozygous for NOP1 with plasmid YEP13-ADHI-humFib and tetrad analysis of such a transformant was done as described in Materials and Methods. (*Top*) Four tetrad spores (*1–4*) were germinated and then grown on YPD plates at 23°C (5 d) and 37°C (3 d). *1* and *2*, haploid progeny containing genomic NOP1 and plasmid YEP13-ADHI-humFib (ura3$^-$/LEU2$^+$); *3* and *4*, haploid progeny with evicted genomic NOP1 gene and plasmid YEP13-ADHI-humFib (URA3$^+$/LEU2$^+$). Strains *3* and *4* are temperature sensitive

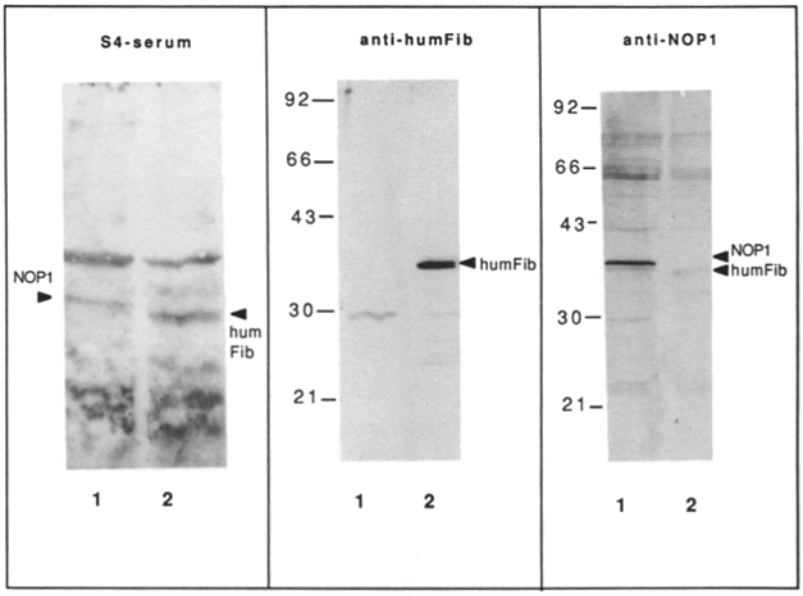


Figure 3. Synthesis of human fibrillarin in yeast. Lane *1*, yeast cell extract from the nopl⁻ yeast strain complemented by cloned NOP1; lane *2*, yeast cell extract from the nopl⁻ strain complemented by human fibrillarin (YEP13-ADHI-humFib). Immunoblots were prepared from total cell extracts. Three identical immunoblots were decorated with S4-autoimmune serum (*left*), anti-human fibrillarin affinity-purified antibodies (*center*), and an immune serum made against isolated, SDS-denatured yeast NOP1 (*right*). The position of NOP1 and human fibrillarin is indicated by arrows and molecular mass markers (in kilodaltons) are shown. The migration difference between NOP1 and human fibrillarin is due to a difference in molecular mass. A protein band of ~30 kD is weakly cross-reactive with anti-human fibrillarin antibodies in a total yeast homogenate (*center*, lane *1*), but this protein is nonnuclear and exclusively found in the post-nuclear supernatant (data not shown).

Propidium Iodide Staining of Yeast Cells and Confocal Microscopy

Haploid yeast cells with evicted NOP1 gene and complemented by YEP13-ADHI-humFib were grown in YPD medium to OD_{600} ~0.5 at 23°C. Half of the culture was shifted for 15 h to 37°C. Cells from a 20-ml culture grown at 23°C and 37°C were harvested by centrifugation, washed in PBS and fixed in 1 ml ethanol for 30 min at 0°C. Cells were centrifuged, washed in PBS, and incubated in 1 ml of 1 mg/ml RNAse A for 90 min at 37°C. After a wash in PBS, cells were stained in a volume of 0.3 ml PBS with 50 μg/ml propidium iodide for 20 min at 0°C. Cells were finally washed twice in PBS, mounted in 80% glycerol on a coverslip and inspected in the fluorescence confocal microscope developed at the EMBL (Stelzer et al., 1989).

Generation of Anti-Fibrillarin Peptide Antibodies

A peptide corresponding to the carboxy-terminal end of human fibrillarin (YERDHAVVVGVYRPPPKVKN) was coupled to hemocyanin using glutaraldehyde as cross-linker and injected into rabbits. Antibodies against human fibrillarin were affinity-purified from this immune serum using a fusion protein between *E. coli* β-galactosidase and human fibrillarin expressed in *E. coli* as an affinity ligand.

Computer Analysis

The DNA sequence of human fibrillarin cDNA and its predicted amino acid sequence was analyzed by the programs of the University of Wisconsin Genetics Computer Group (UWGCG). The mol wt and amino acid composition was obtained by PEPTIDESORT. The amino acid sequence comparison between yeast NOP1 and human fibrillarin was done using the program BESTFIT.

Miscellaneous

Preparation of total protein extracts from yeast cells, SDS-PAGE, immunoblotting, affinity purification of antibodies, subcellular fractionation of yeast cells, and purification of yeast nuclei was done as described by Schimmang et al. (1989).

Results

Human Fibrillarin Is 70% Identical to Yeast NOP1

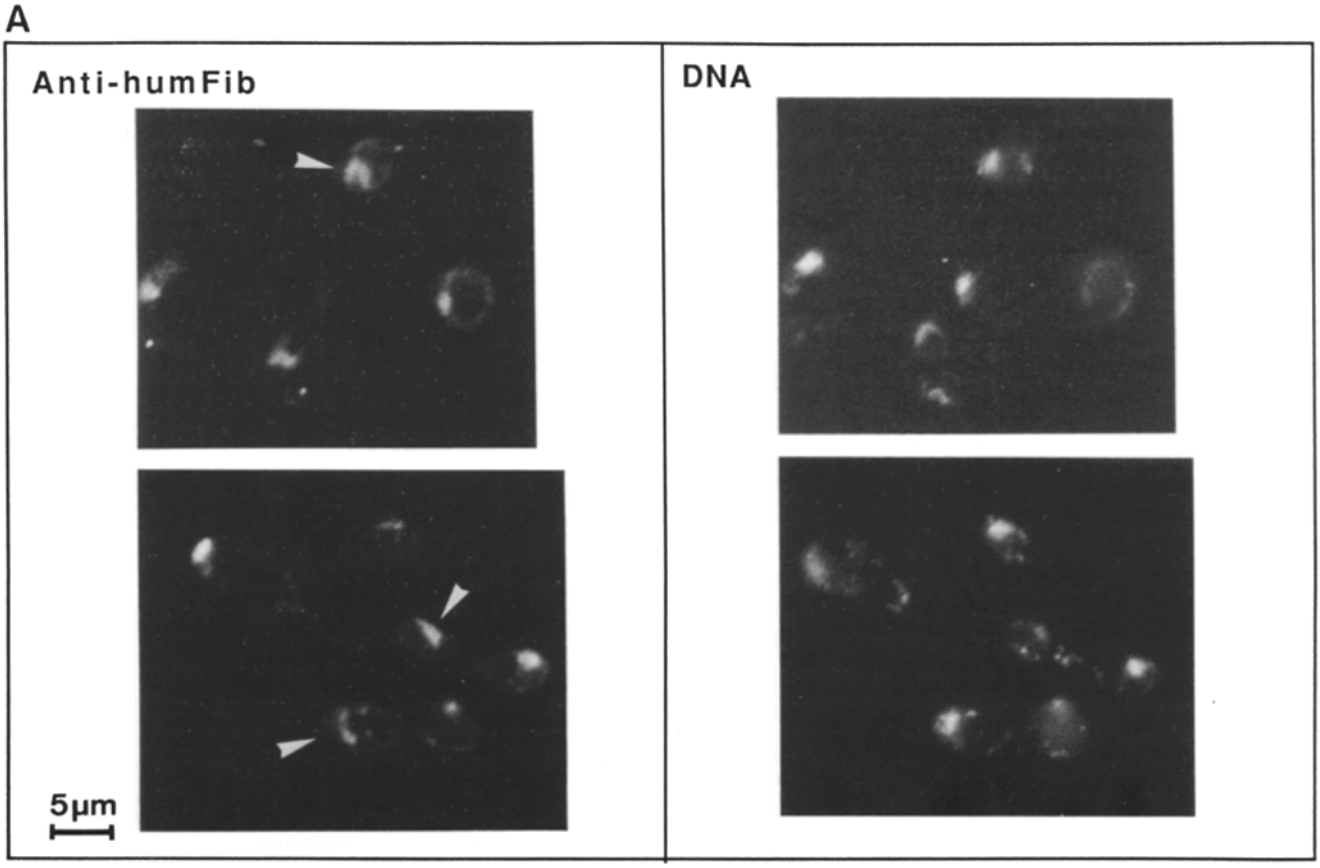
We have isolated the gene coding for the human homologue of NOP1 and tested its in vivo function in *Saccharomyces cerevisiae*, which is amenable to genetic manipulation. By PCR using oligonucleotides corresponding to the 5′ and 3′ regions of the yeast NOP1 gene, a DNA-fragment of 470 nucleotides was amplified from a HeLa cDNA library. This was subsequently used to isolate the putative full-length cDNA clone (1,090 nucleotides) by colony hybridization. On Northern blots, the isolated cDNA predominantly hybridized to a human polyA⁺ RNA species of ~1.1 kb, but no hybridization was detectable to yeast RNA (data not shown). The DNA sequence of the full-length HeLa cDNA clone contains a single open reading frame, potentially encoding a protein of 321 residues corresponding to 34 kD (Fig. 1 *A*). The calculated isoelectric point of 10.98 points to a basic protein. A salient feature of the open reading frame is a glycine/arginine-rich sequence at the amino terminus found also in several other nucleolar proteins.

Comparison of yeast NOP1 with the deduced amino acid of the cloned human gene and *Xenopus* fibrillarin (Lapeyre et al., 1990) shows that yeast NOP1 and human fibrillarin are 70% identical and 80% similar if conserved amino acid exchanges are also included, while NOP1 and *Xenopus* fibrillarin are 72% identical and 83% similar (Fig. 1 *B*). Human and *Xenopus* fibrillarin are 81% identical.

Human Fibrillarin Can Complement a Yeast Mutant Lacking the Nucleolar Protein NOP1

The high degree of structural similarity between NOP1 and

for growth at 37°C. (*Bottom*) Haploid nopl⁻ yeasts complemented by human fibrillarin (YEP13-ADHI-humFib) were grown at 23 or 37°C for 15 h and inspected in the light microscope. Cells grown at 23°C reveal normal cell morphology, in contrast to cells arrested at 37°C that have increased cell size and daughter cells show long bud projections.



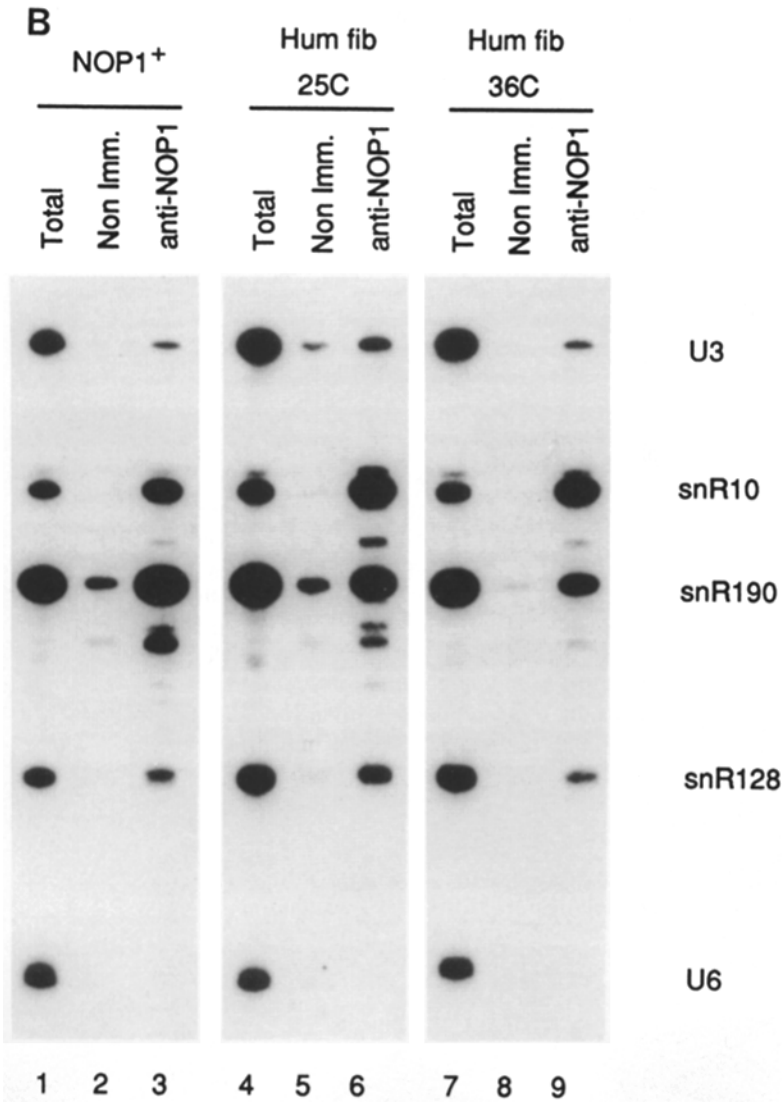


Figure 4. Human fibrillarin expressed in yeast is localized to the nucleolus and associated with small nucleolar RNA. (*A*) Indirect immunofluorescence microscopy using affinity-purified anti-human fibrillarin antibodies. The yeast strain lacking the genomic NOP1 gene and complemented by human fibrillarin was grown at 23°C in YPD-medium, fixed in 3% formaldehyde, and processed for immunofluorescence microscopy. The immunostaining using affinity-purified anti-fibrillarin antibodies (*left*) and DNA staining using 5 μg/ml Hoechst 33258 (*right*) is shown. Cells with a crescent-shaped nucleolar staining are indicated by arrows. (*B*) Immunoprecipitation of yeast small nucleolar RNAs from yeast strains expressing human fibrillarin. Lane *1*, total RNA from a NOP1$^+$ strain grown at 25°C; lane *2*, RNA immunoprecipitated from a NOP1$^+$ strain grown at 25°C with nonimmune human serum; lane *3*, RNA immunoprecipitated from a NOP1$^+$ strain grown at 25°C with affinity-purified anti-NOP1 antibody; lane *4*, total RNA from a nop1$^-$ strain expressing human fibrillarin and grown at 25°C; lane *5*, RNA immunoprecipitated from a nop1$^-$ strain expressing human fibrillarin and grown at 25°C with nonimmune human serum; lane *6*, RNA immunoprecipitated from a nop1$^-$ strain expressing human fibrillarin and grown at 25°C with affinity-purified anti-NOP1 antibody; lane *7*, total RNA from a nop1$^-$ strain expressing human fibrillarin and grown at 36°C; lane *8*, RNA immunoprecipitated from a nop1$^-$ strain expressing human fibrillarin and grown at 36°C with nonimmune human serum; lane *9*, RNA immunoprecipitated from a nop1$^-$ strain expressing human fibrillarin and grown at 36°C with affinity-purified anti-NOP1 antibody (derived from immune serum EC1-1). After immunoprecipitation, RNA was recovered and analyzed by Northern hybridization using probes to the snoRNAs U3, snR10, snR190, snR128, and the snRNA U6.

human or *Xenopus* fibrillarin suggested that these proteins may be functionally equivalent. To test this, the human fibrillarin gene was placed under the control of the constitutively active alcohol dehydrogenase I (ADHI) promoter on a 2μ yeast plasmid. In the absence of a functional NOP1 gene, haploid strains of yeast are not viable (Schimmang et al., 1989). To rescue the mutant, the plasmid carrying the human fibrillarin gene was introduced into a diploid strain of yeast, heterozygous for nop1::URA3 (one NOP1 allele has been destroyed by gene replacement). Diploid transformants containing the plasmid were sporulated and asci with tetrad spores were dissected. From strains expressing human fibrillarin, all four tetrad spores could be recovered (4:0 segregation) and two of them always contained both the URA3 marker (indicative of the disrupted NOP1 gene) as well as the plasmid carrying the human fibrillarin gene (Fig. 2, *top*, 23°C). From strains transformed with the same vector lacking the human fibrillarin cDNA, or with the cDNA inserted downstream of the ADHI promoter but in the opposite orientation, a 2:2 segregation for viability was found. This demonstrates that human fibrillarin is functional in yeast and can complement an otherwise nonviable yeast mutant lacking authentic NOP1.

To obtain the expression of *Xenopus* fibrillarin in *Saccharomyces cerevisiae*, the cDNA (Lapeyre et al., 1990) was cloned under the control of the GAL10 promoter in YEp51 (Broach et al., 1983) to generate pXenFib. Transcription of the inserted cDNA is induced during growth on galactose medium and strongly repressed by growth in the presence of glucose. To test the ability of *Xenopus* fibrillarin to function in yeast, pXenFib was transformed into a diploid strain in which one chromosomal copy of NOP1 is destroyed by replacement with a disrupted nop1::URA3 allele. When the diploid transformed with pXenFib is sporulated and the spores germinated on galactose medium, four viable spores can be recovered from tetrads, with 2:2 segregation for NOP1$^+$ and nop1::URA3. Spore outgrowth on glucose medium results in 2:2 segregation of viability and, as with the parent diploid (Schimmang et al., 1989), the nop1::URA3 marker is not recovered. Haploids shown genetically to carry nop1::URA3 and pXenFib are able to grow on galactose but not glucose medium (data not shown), showing them to be dependent on expression of *Xenopus* fibrillarin.

Synthesis of human fibrillarin in yeast was demonstrated by immunoblotting (Fig. 3). An autoimmune serum containing anti-human fibrillarin antibodies (serum S4; kindly provided by Dr. R. Lührmann, Marburg, FRG) reacted on immunoblots with proteins corresponding to the expected position of yeast NOP1 and human fibrillarin, but additional bands were also observed (Fig. 3, *left*). Specific antibodies were generated by raising an immune serum against a peptide present in the less well-conserved carboxy-terminal region of human fibrillarin (see also Fig. 1 *B*). Affinity-purified antibodies derived from this serum reacted on immunoblots with human fibrillarin expressed in yeast, but not with authentic NOP1 (Fig. 3, *center*). Conversely, antibodies raised against purified, SDS-denatured yeast NOP1 only weakly cross-reacted with human fibrillarin (Fig. 3, *right*). These experiments show that the complemented haploid strains contain human fibrillarin but do not contain NOP1.

Human Fibrillarin Expressed in Yeast Is Localized to the Nucleolus and Can Associate with Small Nucleolar RNA

To locate the human protein in yeast, subcellular fractionation of yeast cells expressing HeLa fibrillarin was performed. A crude nuclear pellet was applied on a sucrose gradient to separate nuclei from other organelles (see also, Hurt et al., 1988). Human fibrillarin cofractionated on this gradient within histone H2B and was mostly recovered in fraction IV containing the purified nuclei (data not shown).

The nuclear location of human fibrillarin in yeast was confirmed by indirect immunofluorescence microscopy using affinity-purified anti-fibrillarin antibodies (Fig. 4 *A*). The immunolabeling was mostly restricted to the nucleus, but did not exactly colocalize with the DNA staining. Frequently, the immunolabeling was crescent shaped (Fig. 4 *A*, *arrows*) which is typical for the appearance of the yeast nucleolus in the light microscope (see also, Sillevis Smitt et al., 1973; Hurt et al., 1988).

The anti-peptide antibody against the cloned human fibrillarin also recognizes a single band ~36 kD on immunoblots containing a total HeLa cell extract (data not shown). The electrophoretic mobility on SDS-PAGE of the HeLa cell protein is identical to that of human fibrillarin expressed in yeast. Furthermore, immunocytochemistry using the anti-fibrillarin peptide antibody showed specific labeling of the nucleolar DFC region on HeLa cells and isolated nuclei (data not shown). This observation is consistent with previous data describing fibrillarin as a nucleolar protein strictly located at the DFC.

To determine whether human fibrillarin can associate in vivo with yeast snoRNAs, the pattern of immunoprecipitation was compared from lysates of cells expressing NOP1 or human fibrillarin. Anti-NOP1 antibodies that cross-react with human fibrillarin (Aris and Blobel, 1989; Schimmang et al., 1989) immunoprecipitate yeast snoRNAs with differing efficiencies (Fig. 4 *B*; see also, Schimmang et al., 1989); snR10 and snR190 are more efficiently precipitated than U3 and snR128. From the strain expressing human fibrillarin, snR10, snR128 and U3 are immunoprecipitated by anti-NOP1 antibodies with an efficiency similar to that from NOP1$^+$ strains (see also, Schimmang et al., 1989), while snR190 is precipitated with slightly reduced efficiency. The non-nucleolar snRNA U6 is not immunoprecipitated by anti-NOP1 antibodies (Fig. 4 *B*). The immunoprecipitation of the snoRNAs is very likely to be due to the presence of human fibrillarin in the yeast snoRNPs. We have placed NOP1 under the control of an inducible promoter; from strains depleted of NOP1 these snoRNAs are very weakly immunoprecipitated by anti-NOP1 antibodies (Tollervey et al., 1991). An antibody directed against a peptide present in the less well-conserved carboxy-terminal region of human fibrillarin, gives a weak precipitation of U3, snR10, snR190, and snR128 from the strain expressing human fibrillarin (data not shown). The carboxy terminus of human fibrillarin may be not exposed and thus be less accessible for immunoprecipitation. The impaired growth of strains expressing human fibrillarin (see below) is probably not due to defective interactions with the snoRNAs, since immunoprecipitation with anti-NOP1 antibodies is equally efficient from lysates of cells grown at 25 or 36°C (Fig. 4 *B*).

Yeast Cells Dependent on Human Fibrillarin Are Temperature Sensitive for Growth and Arrest at 37°C with an Aberrant Nuclear Morphology

Complementation by the human NOP1-homologue is not complete, perhaps due to a less efficient interaction of human fibrillarin with the corresponding nucleolar structures in yeast. At 23°C the doubling time for growth in liquid me-

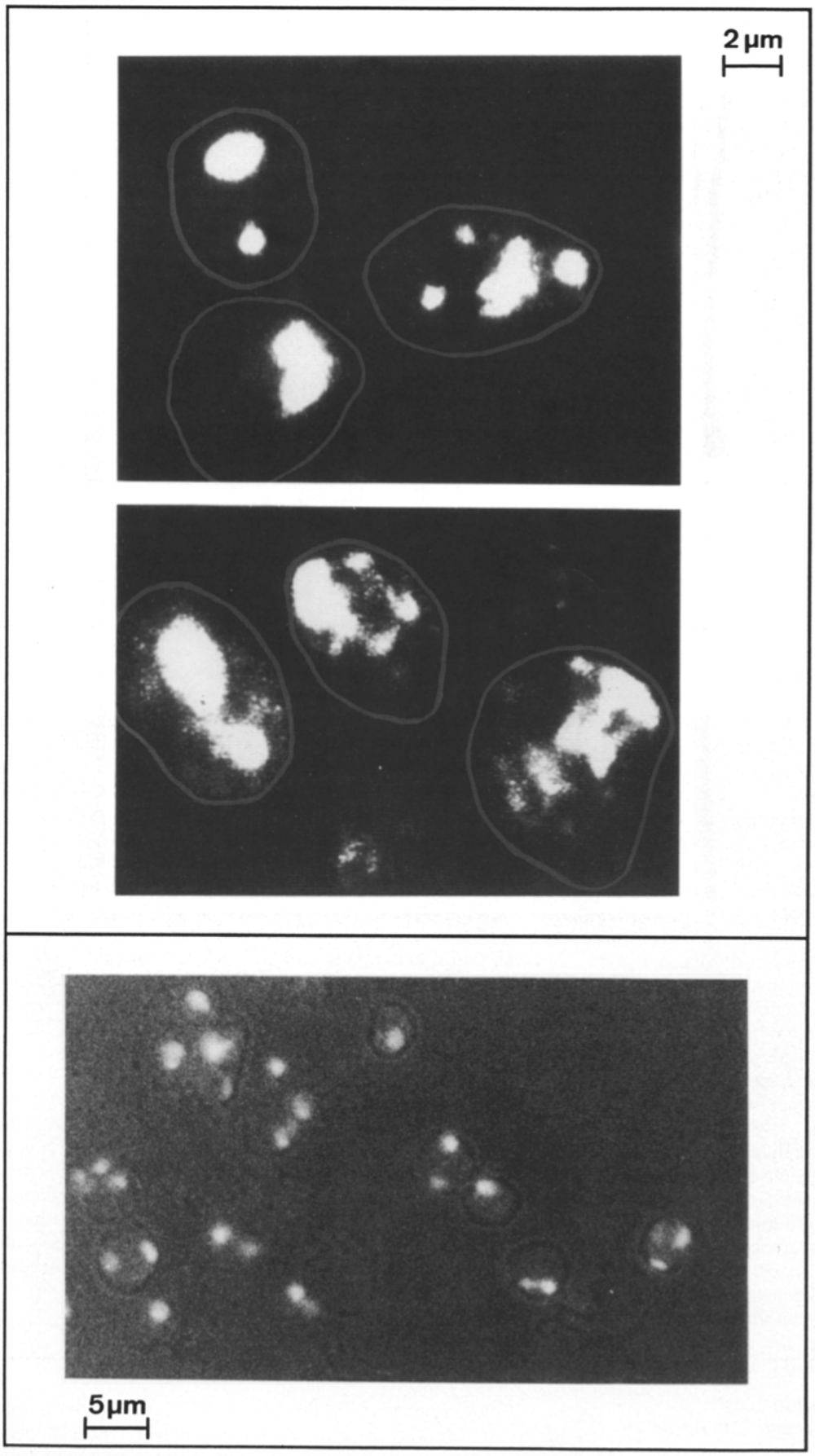


Figure 5. Yeast cells complemented by human fibrillarin arrest at 37°C with aberrant nuclear morphology. Expression of human fibrillarin in a yeast nopl⁻ mutant at 23 and 37°C, nuclear staining of cells with propidium iodide, confocal microscopy, indirect immunofluorescence and electron microscopy is described in Materials and Methods. (*A*) Confocal microscopy of propidium iodide stained nuclei of nopl⁻ cells expressing human fibrillarin and arrested for 15 h at 37°C (upper panel) and indirect immunofluorescence microscopy using affinity-purified anti-NOP1 antibodies (*bottom*). In many cells, irregular DNA-staining and multinucleate structures are observed. The cell boundary is indicated in the top panel. In the bottom panel, the immunofluorescence signal was superimposed with the light microscopic picture in order to visualize both nuclei and the cell boundary. (*B*) Anti-NSP1/anti-NOP1 double immunofluorescence using affinity-purified anti-NSP1 antibodies and a monoclonal antibody against yeast NOP1 (kindly provided by Dr. J. Aris, Rockefeller University, New York) cross-reactive with human fibrillarin followed by confocal microscopy. A yeast cell complemented by human fibrillarin and arrested at 37°C is shown. Two nuclear rings (NSP1 staining) are evident in this single cell, but only one nucleus reveals fibrillarin staining. (*C*) Electron microscopy of fixed and Epon-embedded cells. Yeast cells with a defective genomic NOP1 gene and expressing either authentic NOP1 on a plasmid (*NOP1*) or human fibrillarin (*humFib*) were shifted for 15 h to 37°C before fixation and embedding. Control cells (*NOP1*) contain a single nucleus, whereas mutant cells (*humFib*) often reveal more than one nucleus. Nuclei are marked (*N*).

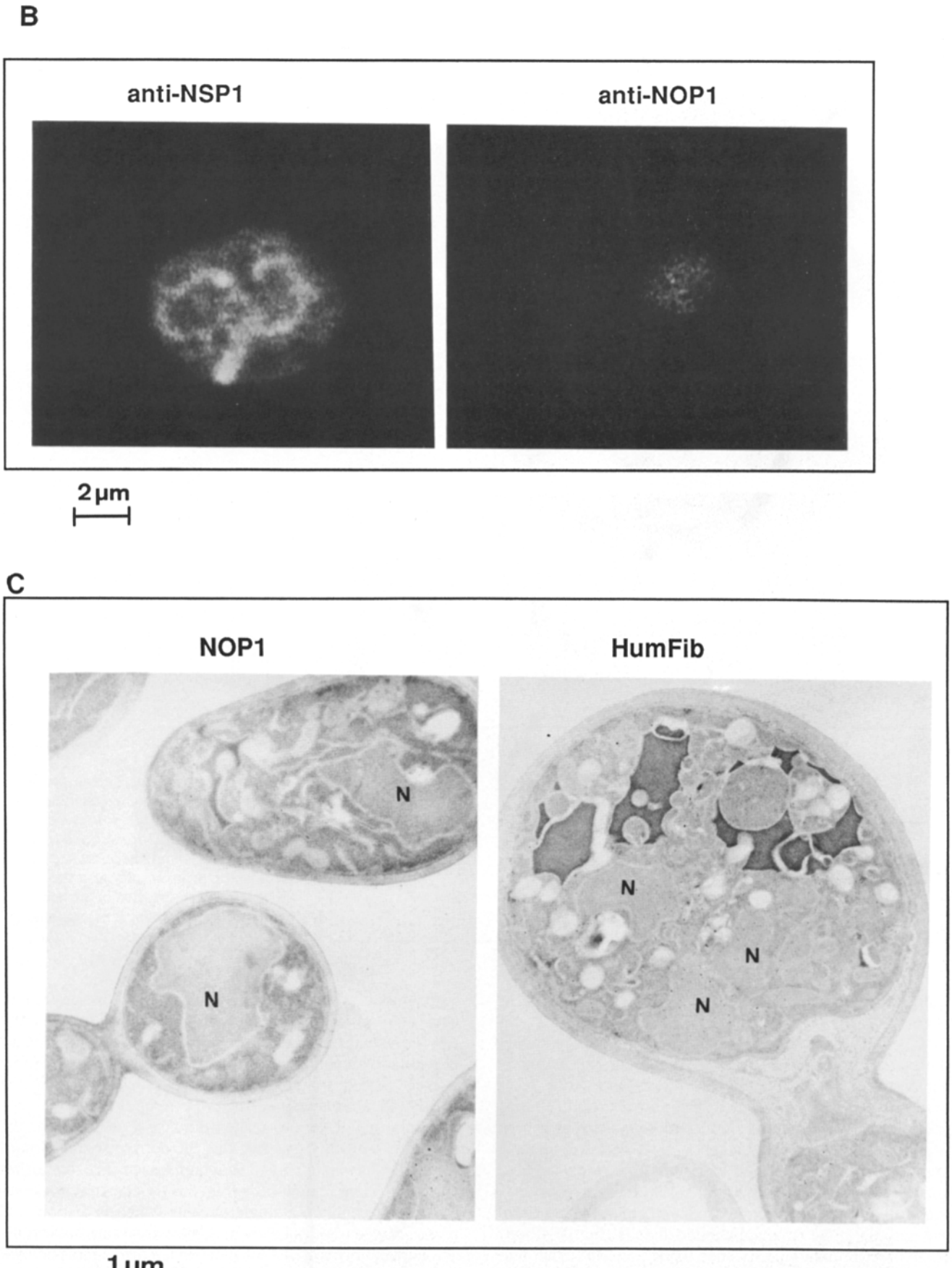
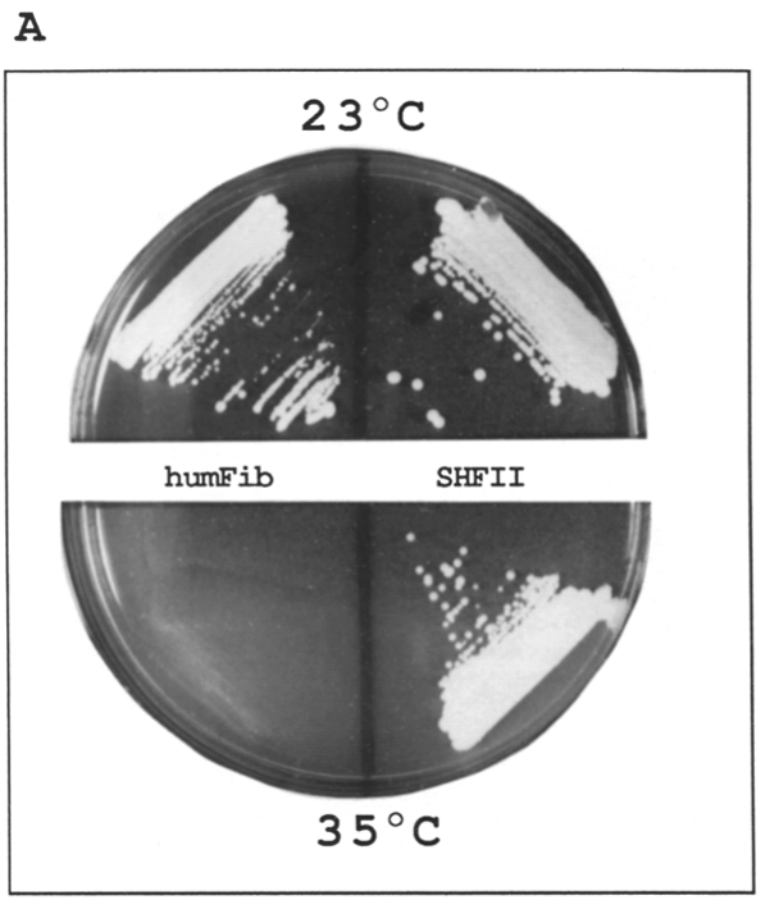


Figure 5.



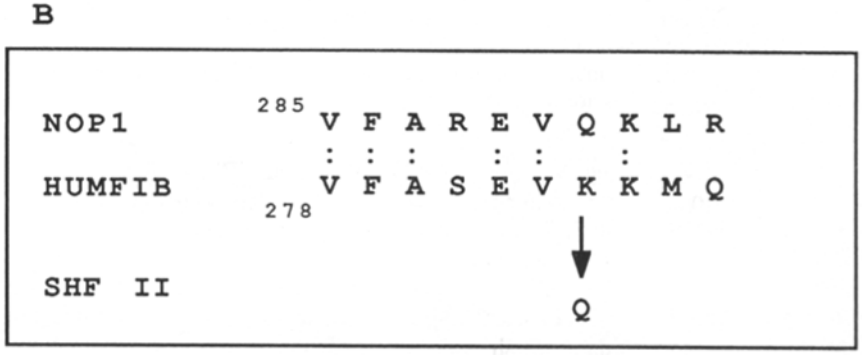


Figure 6. An intragenic suppressor allele of human fibrillarin. (*A*) Growth of a nopl:: URA3 strain expressing human fibrillarin (*humFib*) or a derived intragenic suppressor of human fibrillarin (*SHFII*) on YPD medium at 23 and 35°C for 4 d. (*B*) Amino acid sequence comparison between yeast NOP1 and human fibrillarin in the region where intragenic reversion of the human fibrillarin occurred. Lysine (284) was changed into a glutamine (see also Fig. 1 *B*).

dium is ~6 h for strains expressing only human fibrillarin, as compared to ~2 h for control cells (see also Fig. 2, *top*, 23°C). This growth rate difference is even more prominent at elevated temperatures. At 37°C, yeast cells expressing the human fibrillarin completely stop cell growth within 10–15 h (Fig. 2, *top*) and arrest with a strikingly aberrant cell morphology (Fig. 2, *bottom*). Unbudded cells were in general larger than normal. When daughter cells were still attached to mother cells, they carried long bud projections. Intracellularly, the nucleus was irregularly shaped in arrested cells and propidium iodide staining frequently revealed more than one nucleus in the enlarged unbudded cells (Fig. 5 *A*, *top*). By indirect immunofluorescence using anti-NOP1 antibodies, two clearly separated nucleoli can often be seen in a single arrested cell (Fig. 5 *A*, *bottom*). If mutant cells are stained with antibodies against NSP1, a nuclear envelope protein located at the nuclear pores (Nehrbass et al., 1990), frequently two individual rings corresponding to the nuclear periphery of two individual nuclei are seen (Fig. 5 *B*). By performing double indirect immunofluorescence using anti-NSP1 and anti-NOP1 antibodies, we see with a significant frequency that only one of the two nuclei in a single cell contains the nucleolar antigen suggesting that the correct segregation of the nucleolus in mother and daughter nucleus may be further affected in these mutant cells (Fig. 5 *B*). Cells grown at room temperature contain a single nucleus which is distinct and round-shaped (data not shown). Cells expressing both NOP1 and human fibrillarin do not show an abnormal morphology.

On the ultrastructural level, strains expressing human fibrillarin and arrested at 37°C revealed altered nuclear mor-

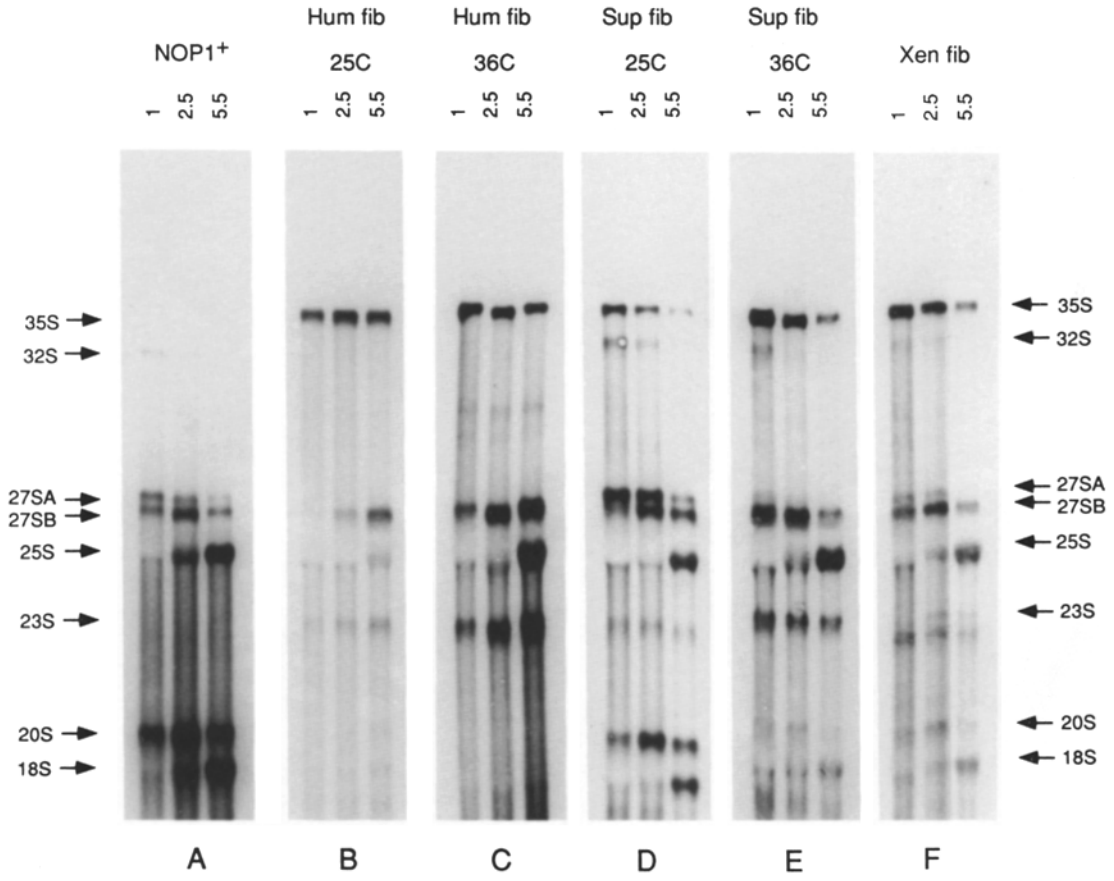


Figure 7. Pulse-chase labeling of pre-rRNA with [^{3}H]uracil. (*A*)NOP1$^+$ strain, (*B*) nopl::URA3 strain complemented by human fibrillarin, grown at 25°C, (*C*) nopl::URA3 strain complemented by human fibrillarin, grown for 5 h at 36°C, (*D*) nopl::URA 3 strain complemented by human fibrillarin containing the suppressor mutation, grown at 25°C, (*E*) nopl::URA3 strain complemented by human fibrillarin containing the suppressor mutation, grown at 36°C, (*F*) nopl::URA3 strain complemented by *Xenopus* fibrillarin. Pre-rRNA was pulse labeled with [^{3}H]uracil for 2.5 min at 25 or 36°C and chased with a large excess of unlabeled uracil for 1, 2.5, or 5 min as indicated. After gel separation, RNAs were transferred to Genescreen + membrane and visualized by fluorography. The positions of 25S and 18S rRNAs and 35S, 32S, 27SA, 27SB, 23S, 22S, and 20S pre-rRNAs are indicated.

phology and enlarged cells often had more than one nucleus (Fig. 5 *C*). These nuclei were surrounded by a continuous nuclear membrane (Fig. 5 *C*, *right*). In comparison, control cells expressing authentic NOP1 and grown at 37°C contained a single nucleus (Fig. 5 *C*, *left*).

Cells complemented by *Xenopus* fibrillarin grow better than cells complemented by human fibrillarin. At 25°C in YPGal medium the doubling times are 2.8 h for NOP1$^+$ cells and 3.5 h for nopl::URA3 cells complemented by *Xenopus* fibrillarin. At 30°C the doubling times are 2.2 h for NOP1$^+$ and 3 h for the complemented strain. At 36°C the relative growth rate of the complemented strain is reduced (doubling times are 2.2 h for NOP1$^+$ and 5 h for the complemented strain). Therefore the strain complemented by *Xenopus* fibrillarin does not show the tight TS lethality of yeasts complemented by human fibrillarin. However, *Xenopus* fibrillarin was expressed in yeast using the strong GAL10 promoter, whereas human fibrillarin was under the control of the less strong ADHI promoter. Interestingly, if *Xenopus* fibrillarin is expressed in a nopl$^-$ strain using the ADHI promoter, it still can complement the mutant at the permissive temperature (i.e., 23°C), but not at 37°C. Expression levels of both human and *Xenopus* fibrillarin using the ADHI promoter were similar in yeast as shown by immunoblot analysis (Jansen, R., unpublished results). Thus, TS lethality of yeast cells complemented by *Xenopus* fibrillarin depends on the level of fibrillarin expression. We have not checked whether the same is true for human fibrillarin, but by completely derepressing the ADHI promoter (growth on a nonfermentable carbon source) yeast cells are still temperature sensitive for growth (data not shown).

A Critical Amino Acid Residue within Human Fibrillarin Possibly Involved in Interaction with Yeast Nucleolar Components

The tight TS-lethal phenotype of nopl::URA3 strains complemented by human fibrillarin enabled us to select for suppressor mutations which allow this strain to grow at the nonpermissive temperature. Among these, intragenic mutations within the human fibrillarin gene were found to be able to rescue the complemented strain at the restrictive temperature (Fig. 6 *A*). When the suppressor human fibrillarin gene (SHF II) was sequenced, only 1 bp within the entire coding region was changed (Fig. 6 *B*). As deduced from the DNA sequence, a single amino acid was altered: lysine (284) to glutamine. The amino acid change has the consequence that the suppressor human fibrillarin becomes more similar to yeast NOP1.

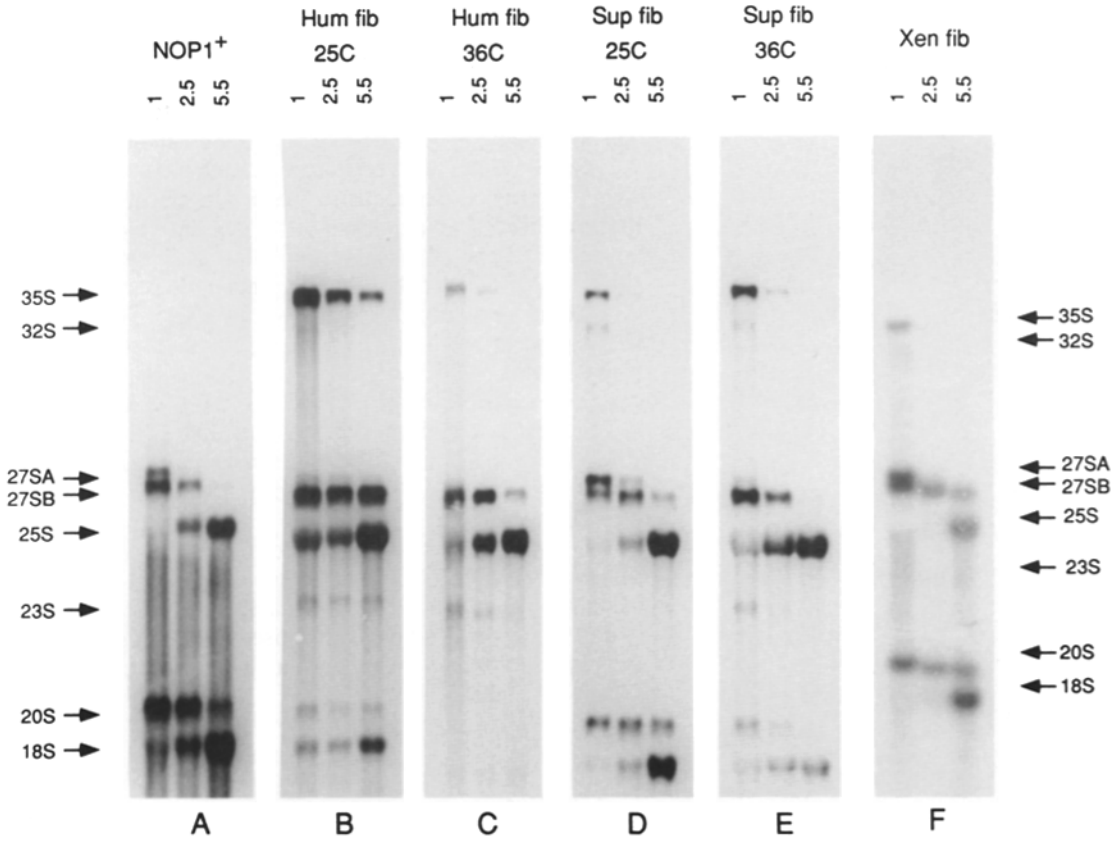


Figure 8. Pulse-chase labeling of pre-rRNA with [^{3}H-methyl]methionine. (*A*) NOP1$^+$strain, (*B*) nopl::URA3 strain complemented by human fibrillarin, grown at 25°C, (*C*) nopl::URA3 strain complemented by human fibrillarin, grown for 5 h at 36°C, (*D*) nopl::URA3 strain complemented by human fibrillarin containing the suppressor mutation, grown at 25°C, (*E*) nopl::URA3 strain complemented by human fibrillarin containing the suppressor mutation, grown at 36°C, (*F*) nopl::URA3 strain complemented by *Xenopus* fibrillarin. Pre-rRNA was pulse labeled with [^{3}H-methyl]methionine for 2.5 min at 25 or 36°C and chased with a large excess of unlabeled methionine for 1, 2.5, or 5 min as indicated. After gel separation, RNAs were transferred to Genescreen+ membrane and visualized by fluorography. The positions of 25S and 18S rRNAs and 35S, 32S, 27SA, 27SB, 23S, 22S, and 20S pre-rRNAs are indicated.

Processing of pre-rRNA in nopl::URA3 Strains Complemented by Xenopus or Human Fibrillarin

To test the ability of human and *Xenopus* fibrillarin to support pre-rRNA processing in yeast, pulse-chase labeling experiments were performed using [^{3}H]uracil or [^{3}H-methyl]-methionine (Figs. 7 and 8). For both isotopes, labeling was for 2.5 min at 25°C, with 1-, 2.5-, or 5.5-min chase. These two isotopes show different aspects of pre-rRNA processing. [^{3}H]Uracil labeling shows the fate of the RNA backbone, [^{3}H-methyl]methionine labels the methyl groups which are added to newly synthesized pre-rRNAs, generally in 35S, and shows the degree of methylation of the pre-rRNA. In NOP1$^+$ strains (Figs. 7 *A* and 8 *A*) the processing of 35S pre-rRNA is very rapid, and even after 1 min of chase most has been processed to 27SA and 20S pre-rRNA, and after 5.5-min chase almost all has been matured to 25S and 18S rRNA.

In nopl::URA3 strains complemented by human fibrillarin, the processing of 35S RNA is greatly slowed, even at 25°C (Figs. 7 *B* and 8 *B*). The 32S and 27SA pre-rRNAs are absent and 20S pre-rRNA is greatly reduced, and the processing of 27SB is also slowed. 18S rRNA is synthesized in lower yield than 25S rRNA. At 36°C, processing of pre-rRNA is even more severely impaired (Figs. 7 *C* and 8 *C*). The most striking effect is the loss of detectable synthesis of 18S rRNA, and the immediate precursor to 18S rRNA, 20S pre-rRNA, is also absent. A faint band below 18S is visible in Figs. 7 *C* and 8 *C*; this is also visible in other samples and does not comigrate with 18S rRNA. The processing of 35S and 27SB pre-rRNAs is substantially slowed, 32S and 27SA are not detected and a 23S species accumulates strongly. Mature 25S rRNA is labeled by [^{3}H-methly]methionine, even in the earliest time point (Fig. 8 *B*), indicating that methyl groups which in the wild type are added to 35S RNA, are added to later processing products, perhaps 25S rRNA itself, in the human fibrillarin complemented strain. Moreover, 35S and 23S pre-rRNA which accumulate at 36°C are detected by uracil labeling but not by labeling with methionine (compare Fig. 7 *C* with 8 *C*), indicating that the unprocessed pre-rRNAs are not methylated. The accumulation of unmethylated pre-rRNAs is also observed in strains depleted of NOP1 (Tollervey et al., 1991).

In strains complemented by human fibrillarin containing the suppressor mutation, processing of pre-rRNA is dramatically improved. At 25°C the efficiency of processing is restored to a level close to that of NOP1$^+$ strains (compare Fig. 7, *B* with *D* and Fig. 8, *B* with *D*), although some delay

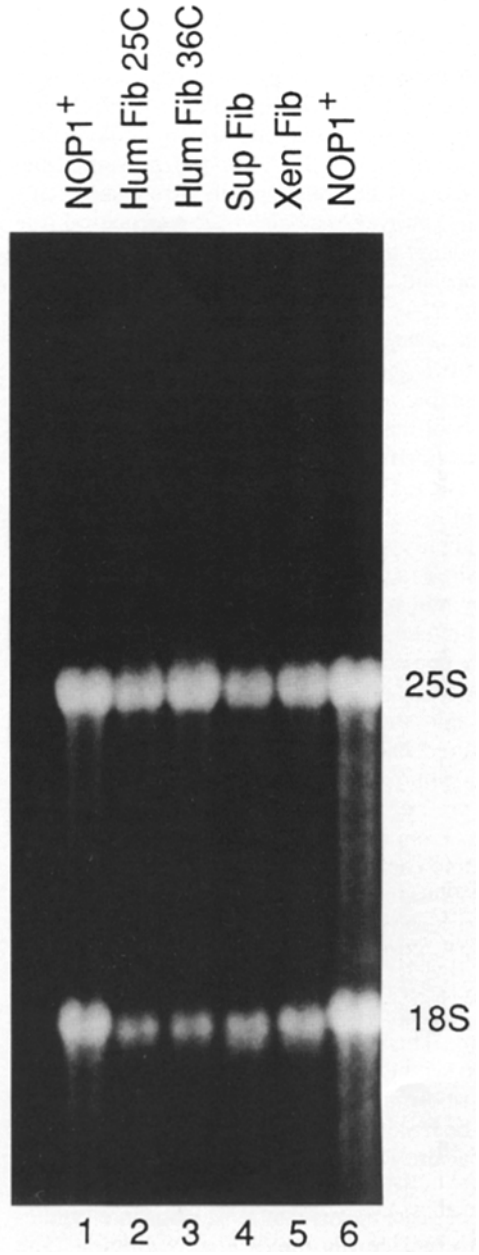


Figure 9. Steady-state levels of ribosomal RNA in nop1⁻ strains complemented by vertebrate fibrillarins. Lane *1*, NOP1⁺ strain; lane *2*, nop1⁻ strain complemented by human fibrillarin grown at 25°C; lane *3*, nop1⁻ strain complemented by human fibrillarin grown at 36°C; lane *4*, nop1⁻ strain complemented by suppressor human fibrillarin; lane *5*, nop1⁻ strain complemented by *Xenopus* fibrillarin grown at 25°C; lane *6*, nop1⁻ strain complemented by NOP1⁺ on a high copy number plasmid. Where not stated, cells were grown at 30°C. RNA equivalent to 10^7 cells was separated by electrophoresis on an agarose/formaldehyde gel and is visualized by ethidium staining.

in processing of 35S pre-rRNA is detected. At 36°C, processing is less efficient (Figs. 7 *E* and 8 *E*), although much better than in strains complemented by the wild-type human fibrillarin (compare Fig. 7, *C* with *E* and Fig. 8, *C* with *E*). As well as restoring efficient processing of pre-rRNA, the human fibrillarin mutation also reduces the aberrant methylation observed in strains complemented by wild-type human fibrillarin (compare Figs. 8, *B* and *D* with 8, *C* and *E*).

In nop1::URA3 strains complemented by *Xenopus* fibrillarin (under GAL10 promoter control) (Figs. 7 *F* and 8 *F*), the processing of pre-rRNA is less defective than in strains complemented by human fibrillarin, but is detectably impaired. The processing of 35S pre-rRNA is slowed and the levels of 32S and 27SA are reduced, although both are detected. 18S rRNA clearly accumulates in the 5-min chase sample although a number of abnormal pre-rRNA species are visible between 25S and 18S rRNA. Depletion of *Xenopus* fibrillarin by repression of the GAL promoter during growth in glucose minimal medium for 30 h results in a block in pre-rRNA processing closely resembling that seen in strains depleted of yeast NOP1 by glucose repression of a GAL::nop1 control mutant (data not shown) (Tollervey et al., 1991).

The steady-state levels of the 18S and 25S ribosomal RNAs were compared for nop1⁻ strains complemented by human fibrillarin at 25°C or following growth for 5 h at 35°C (Fig. 9, lanes *2* and *3*), by the suppressor human fibrillarin (Fig. 9, lane *4*) or by *Xenopus* fibrillarin (Fig. 9, lane *5*). Comparison to the NOP1⁺ strain (Fig. 9, lane *1*) or a nop1⁻ strain carrying the NOP1 gene on a high copy number plasmid (Fig. 9, lane *6*) shows that those strains complemented by vertebrate fibrillarins all have a similar, significantly reduced level of 18S rRNA.

Discussion

Recently, an essential nucleolar protein, NOP1, has been cloned in yeast, which is related to vertebrate fibrillarin (Schimmang et al., 1989; Henriquez et al., 1990). To study the conserved role of eucaryotic fibrillarin for nucleolar structure and function in an organism that is amenable to genetic approaches, we cloned the human homologue of NOP1, fibrillarin, and obtained its functional expression in yeast. On the amino acid level, yeast NOP1 and human fibrillarin are 70% identical and 80% similar if conserved amino acids exchanges are included. This high degree of conservation is only found in a few eucaryotic "house keeping" proteins such as ubiquitin (96%), actin (89%), and β-tubulin (75%), which play key roles in universal eucaryotic cell functions (Botstein and Fink, 1988). A salient feature of NOP1 and fibrillarin is an amino-terminal sequence of ~80 amino acids, consisting of a repeated motif rich in glycines and arginines, which is also found in other nucleolar proteins such as nucleolin (Lapeyre et al., 1987) and yeast SSB1 (Jong et al., 1987). In fibrillarin, this sequence is posttranslationally modified by dimethylation of arginine residues (Christensen et al., 1977; Lischwe et al., 1985). The role of the glycine/arginine-rich repeat domain in nucleolar proteins is not clear, but its conservation in evolution and presence in several different nucleolar proteins argues for an important function.

The high degree of structural conservation of eucaryotic fibrillarin is matched by functional interchangeability. Fibrillarin from human (or *Xenopus*), despite more than 1,000 million years of evolution, can still functionally replace yeast NOP1. This demonstrates that the basic role of fibrillarin is identical from yeast to man and that the human protein can interact with the cognate nucleolar components in yeast. Consistent with this, human fibrillarin can bind to yeast snoRNAs and is correctly localized to the nucleolus. Com-

plementation by human (or *Xenopus* fibrillarin) is not complete, however, at 25°C the growth of the complemented strain is about three times slower than that of the wild type, and at 36°C cell growth stops. A human protein is unlikely to be destabilized at 36°C, and this suggests that vertebrate fibrillarin interacts less strongly with yeast components than does authentic NOP1. Consistent with this, high level expression of *Xenopus* fibrillarin from the strong GAL10 promoter on a multicopy plasmid suppresses the TS lethality. Thus TS lethality also depends on the expression level of the vertebrate fibrillarin.

The temperature-sensitive phenotype of the yeast mutant expressing human fibrillarin was exploited to isolate suppressor mutations. In this way we hope to obtain a detailed genetic analysis of the functionally important domains of fibrillarin (intragenic suppression) and the molecules with which it interacts (extragenic suppression). An initial intragenic suppressor mutation of the human fibrillarin which allows growth at the restrictive temperature has been characterized. The alteration of a single amino acid in the carboxy-terminal part of human fibrillarin to the corresponding amino acid within the NOP1 protein is sufficient for suppression at 35°C. This amino acid and the region surrounding it, may be critical for interaction with other nucleolar components; alternatively the changed amino acid residue may cause a conformational change within the suppressor human fibrillarin sufficient for complementation at elevated temperatures. In *Xenopus* fibrillarin a lysine residue is found at the corresponding amino acid position (see Fig. 1 *B*). *Xenopus* fibrillarin does not complement at 35°C if expressed in similar quantities as compared to human fibrillarin (under the control of the ADH promoter), but allows slow growth at elevated temperatures if strongly overexpressed with the help of the GAL10 promoter. The further biochemical, immunological, and genetic characterization of this region in human fibrillarin and NOP1 thus could allow us to identify further components of the eucaryotic nucleolus involved in ribosome biogenesis.

Concomitant with the growth arrest at 37°C, nuclear morphology is changed in the strain expressing human fibrillarin and many cells have two nuclei. Frequently, only one nucleus in such cells contains the nucleolar antigen. The aberrant nuclear and nucleolar organization might reflect ongoing nuclear division, without a block in segregation into mother and daughter cells. This indicates that fibrillarin may play an important role in organizing nucleolar structure and, as a consequence, the overall nuclear structure. It is interesting to note that a nonrandom positioning of the spindle pole body and the nucleolus has been found in yeast (Yang et al., 1989), both structures being associated with the nuclear membrane and preferentially localized at opposite poles of the nucleus. This suggests the existence of communication between these structures that may be lost in the mutant cells with distorted nucleolar structure. As a consequence, nuclear division, nuclear, and/or nucleolar segregation could be impaired. In yeast, the nucleolus is continuously associated with the nuclear envelope during mitosis spanning in a highly elongated form mother and daughter nucleus (Aris and Blobel, 1988; E. Hurt, unpublished results). Thus, the nucleolus could further provide a structural axis inside the nucleus along which correct nuclear division and segregation proceeds.

How could alterations in fibrillarin affect both nucleolar structure and function? As shown for human fibrillarin and yeast NOP1, both proteins are in physical interaction with different small nucleolar RNAs (Tyc and Steitz, 1989; Schimmang et al., 1989). Because of this interaction, a role in processing of precursor ribosomal RNA has been presumed and indeed had been recently demonstrated in vitro (Kass et al., 1990). Fibrillarin, however, may also have a structural role in organizing the dense fibrillar component and thereby providing the appropriate structural support for correct and efficient ribosomal RNA processing. Our data on altered nuclear structure in yeast cells expressing human fibrillarin support such a role.

Pulse-chase labeling of pre-rRNA indicates that the impaired growth of nop1-deleted yeast strains complemented by human or *Xenopus* fibrillarin is likely to be due to defective pre-rRNA processing. The strain complemented by high level expression of *Xenopus* fibrillarin at 25°C has a growth rate that is not greatly slower than NOP1$^+$ strains, and is only mildly impaired in pre-rRNA processing. In the strain complemented by human fibrillarin, processing is substantially impaired at either 25 or 36°C, however, at 25°C a reduced but clearly detectable level of 18S rRNA is synthesized, whereas, at 36°C no 18S rRNA synthesis is detected. The inability of this strain to synthesize 18S rRNA may therefore be the direct cause of the TS-lethality. At 25°C the human fibrillarin suppressor mutation restores growth, and the efficiency of pre-rRNA processing, comes close to that of NOP1$^+$ strains. Examination of the steady-state levels of rRNA in the strains complemented by *Xenopus* fibrillarin, human fibrillarin or the suppressor human fibrillarin, shows that all of these strains have a reduced level of 18S rRNA, corresponding to ~30% of the level in NOP1$^+$ strains. It appears that there is a minimum, but fairly high level of 18S which is either required for growth or maintained by a regulatory system. There are obvious reasons why a cell which is unable to synthesize ribosomes for whatever reason should not let its ribosome pool fall to low levels, since such a position would be irrecoverable if ribosome synthesis then became possible again. Ribosomes are needed to make new r-proteins and there is essentially no free r-protein pool. The high growth rate of yeast means that most ribosome synthesis goes simply to replace ribosomes lost by dilution. The growth rate of the mutants therefore declines until the rate of 18S synthesis of which they are capable allows them to maintain this "basal" 18S level.

Strains depleted of the essential small nuclear RNA U14 (snR128) are also limited for growth by their ability to synthesize 18S rRNA (Zagorski et al., 1988; Li et al., 1990) as are cells depleted of NOP1 by growth of the GAL::nop1 strain on medium containing glucose (Tollervey et al., 1991). In snR128 or NOP1 depleted strains, the precursors to 18S rRNA are very rapidly degraded, whereas in the human fibrillarin complemented strain the unmethylated 23S pre-rRNA accumulates, although no accumulation of methylated pre-rRNA is detected. Unmethylated 35S pre-rRNA also accumulates in the human fibrillarin complemented strain. In this mutant, as in strains depleted of NOP1 (Tollervey et al., 1991), there appear to be two populations of pre-rRNA; unmethylated pre-rRNA accumulates, while methylated pre-rRNA is processed relatively more efficiently.

As well as allowing its functional analysis, the cloning of

human fibrillarin will allow the study of the molecular basis of the autoimmune antigenicity of this protein in patients suffering from scleroderma (Tan, 1989).

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