An Abundant Nucleolar Phosphoprotein Is Associated with Ribosomal DNA in *Tetrahymena* Macronuclei

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Submitted July 30, 1996; Accepted October 1, 1996
Monitoring Editor: Joseph Gall

An abundant 52-kDa phosphoprotein was identified and characterized from macronuclei of the ciliated protozoan *Tetrahymena thermophila*. Immunoblot analyses combined with light and electron microscopic immunocytochemistry demonstrate that this polypeptide, termed Nopp52, is enriched in the nucleoli of transcriptionally active macronuclei and missing altogether from transcriptionally inert micronuclei. The cDNA sequence encoding Nopp52 predicts a polypeptide whose amino-terminal half consists of multiple acidic/serine-rich regions alternating with basic/proline-rich regions. Multiple serines located in these acidic stretches lie within casein kinase II consensus motifs, and Nopp52 is an excellent substrate for casein kinase II in vitro. The carboxyl-terminal half of Nopp52 contains two RNA recognition motifs and an extreme carboxyl-terminal domain rich in glycine, arginine, and phenylalanine, motifs common in many RNA processing proteins. A similar combination and order of motifs is found in vertebrate nucleolin and yeast NSR1, suggesting that Nopp52 is a member of a family of related nucleolar proteins. NSR1 and nucleolin have been implicated in transcriptional regulation of rDNA and rRNA processing. Consistent with a role in ribosomal gene metabolism, rDNA and Nopp52 colocalize in situ, as well as by cross-linking and immunoprecipitation experiments, demonstrating an association between Nopp52 and rDNA in vivo.

INTRODUCTION

In eukaryotes, nucleoli are highly specialized subnuclear organelles that are the sites of production of preribosomal subunits. Transcription of rRNA genes (rDNA), processing of the primary transcripts into mature rRNAs, and the addition of polypeptides to form nascent preribosomes occur within nucleoli. In addition, nucleoli harbor protein and RNA components that are not part of mature cytoplasmic ribosomes. These include proteins that are transiently associated with preribosomes and nucleocytoplasmic shuttling proteins as well as proteins associated with rDNA chromatin and proteins that regulate transcription. In recent years many nucleolar components have been identified and pathways of rDNA metabolism and ribosome biogenesis are beginning to be eluci-

dated by biochemical and genetic approaches (for review, Scheer and Weisenberger, 1994).

Several aspects of the biology of the ciliated protozoan *Tetrahymena thermophila* afford an opportunity to gain insights into nucleolar structure and function. First, each vegetative cell contains two specialized nuclei. A larger polyploid macronucleus is the sole source of transcription during most stages of the life cycle. In contrast, a smaller micronucleus is transcriptionally inert but functions as the germ-line nucleus during the sexual phase of the life cycle. One manifestation of transcription taking place in macronuclei, but not in micronuclei, is the existence of multiple nucleoli localized just within the macronuclear nuclear envelope (Gorovsky, 1973). Nucleoli are not found in micronuclei. Second, the ribosomal gene of *Tetrahymena* has been well studied. It is transcriptionally active and highly amplified in macronuclei. Each rDNA molecule exists as a 21-kb linear extrachromosomal palindrome containing two copies of the rRNA gene. Considerable

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information has been collected on this rRNA gene including its complete sequence (Engberg and Nielsen, 1990) and the identification of *cis*-acting control elements that function in its replication and transcription (for review, Prescott, 1994). The amplified and extrachromosomal nature of rDNA in *Tetrahymena* allows for the formation of free nucleoli, a situation distinct from most other eukaryotes. Finally, transformation techniques have been developed that allow simple modification of all copies of the rDNA gene (Yu *et al.*, 1988; Yao and Yao, 1991; Gaertig and Gorovsky, 1992).

Little is known about the protein components that regulate rDNA metabolism or influence overall nucleolar structure and function in *Tetrahymena* or other organisms. In this study, we report the identification and characterization of a prominent macronuclear-specific phosphoprotein enriched in *Tetrahymena* nucleoli. The cDNA sequence encoding this 52-kDa polypeptide (termed Nopp52) predicts a domain structure similar to other nucleolar proteins, especially vertebrate nucleolin and yeast NSR1 (Lapeyre *et al.*, 1987; Lee *et al.*, 1991). In situ hybridization to rDNA and immunocytochemical localization of Nopp52 indicates these components are colocalized in nucleoli. By using cross-linking and immunoprecipitation with antibodies to Nopp52, our results also indicate an association of Nopp52 and rDNA in vivo.

MATERIALS AND METHODS

Cell Culture and Labeling

Tetrahymena thermophila were grown axenically in enriched proteose peptone (Gorovsky *et al.*, 1975). Strain Cu428 was used for all experiments, except for creation of the cDNA library that was derived from strain SB281. Logarithmic phase cells were harvested at $1\text{--}3 \times 10^5$ cells/ml; cells were starved in 10 mM Tris-HCl (pH 7.4) for 18 to 20 h at a density of 2×10^5 cells/ml. Cells were labeled with ^{32}P by inclusion of 10 $\mu\text{Ci}/\text{ml}$ [^{32}P]orthophosphate into the growth medium; labeled starved cells were obtained from cells labeled during growth before being placed under starvation conditions. For immunoprecipitation experiments, cells were labeled during growth with 15 $\mu\text{Ci}/\text{ml}$ [*methyl*- ^3H]thymidine in medium minus yeast extract.

Nuclear Protein Isolation and Gel Electrophoresis

Isolation of macronuclei and micronuclei as well as extraction and precipitation of acid-soluble proteins were performed as described by Schulman *et al.* (1987), except that nuclei were purified by sedimentation at unit gravity according to Allis and Dennison (1982). *p*-Chloromercuriphenylsulfonic acid (0.1 mM; Sigma, St. Louis, MO) was added to the nuclear isolation buffers to control against protease and phosphatase activities. Proteins were electrophoresed on one-dimensional SDS gels or two-dimensional acid-urea followed by SDS gels and stained with Coomassie blue.

High-Pressure Liquid Chromatography (HPLC) Purification and Peptide Sequencing of Nopp52

Acid-soluble macronuclear proteins were separated by reverse-phase HPLC using a C_8 analytical column (Aquapore Octyl-RP300;

Brownlee Labs, Santa Clara, CA) with a linear gradient of 0–60% acetonitrile and 0.1% trifluoroacetic acid over a period of 30 min. Trypsin or cyanogen bromide (CNBr) peptides of Nopp52 were generated and sequenced as described in Roth *et al.* (1988).

In Vitro Kinase Assays

HPLC-purified Nopp52 (1 mg/ml) was labeled with 1 μCi of [$\gamma\text{-}^{32}\text{P}$]ATP at 35 Ci/mmol and either *Drosophila* casein kinase-II (CKII; kindly provided by C. Glover, University of Georgia, Athens, GA), HeLa p34^{cdc2} kinase (kindly provided by D. Beach, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), or the catalytic subunit of bovine cAMP-dependent protein kinase (kindly provided by David Brautigan, University of Virginia, Charlottesville, VA). Reaction conditions for CKII were 1.5 $\mu\text{g}/\text{ml}$ kinase in 50 mM Tris (pH 8.5), 100 mM NaCl, and 10 mM MgCl_2 at a final volume of 28 μl at 30°C for 15 min. Reaction conditions for p34^{cdc2} and cAMP-dependent protein kinase were 1.0 $\mu\text{g}/\text{ml}$ kinase in 10 mM Tris (pH 8.0), 100 mM MgCl_2 , 10 mM dithiothreitol, and 10 mM ATP at a final volume of 50 μl at 30°C for 15 min. In vivo- and in vitro-labeled phosphotryptic peptide digestion products were resolved on high-percentage alkaline polyacrylamide gels as described by Dadd *et al.* (1993) and examined by autoradiography.

Generation, Affinity Purification, and Characterization of Polyclonal Antibodies Recognizing Nopp52

A rabbit was immunized with a total of approximately 50 μg of HPLC-purified Nopp52 in two injections given 2 weeks apart. Affinity-purified antibodies were isolated by eluting antibodies bound to the Nopp52 region of several preparative SDS gel immunoblots by using the method of Olmsted (1981), except that 5 M NaI was used as eluting agent. Eluted antibodies were immediately dialyzed into 10% goat serum and 0.1% sodium azide in Tris-buffered saline (TBS) and stored at 4°C. Preimmune serum was collected from the same animal before immunization. Polypeptides separated on SDS gels were transferred to nitrocellulose and immunoblot analyses were performed by using standard procedures. Crude serum was used at a 1:20,000 dilution and affinity-purified IgG was at a 1:100 dilution. Immunoreactivity was detected by alkaline phosphatase-conjugated secondary antibodies or by enhanced chemiluminescence (ECL) and autoradiography as indicated. Polyclonal antiserum against macronuclear-specific H1 and an H2A variant, hv1, were used as described (Stargell *et al.*, 1993).

cDNA Library Construction and Nopp52 cDNA Isolation

cDNA was synthesized from growing cells' RNA by using a poly(dT)/*Xho*I primer and Superscript reverse transcriptase (Life Technologies, Gaithersburg, MD) according to the manufacturer's instructions, except that 40 U of RNasin (Promega, Madison, WI) were added. After ligation of *Eco*RI adapters (New England Biolabs, Beverly, MA) and digestion with *Xho*I, electrophoresis was performed on a 1% agarose gel and cDNA ranging from 1 to 2.4 kb was eluted. This cDNA was cloned into a λ phage vector, UniZap XR (Stratagene, La Jolla, CA), packaged with Gigapack II Gold (Stratagene, La Jolla, CA), and plated, and plaques were transferred to nitrocellulose filters and screened according to Stratagene's instructions.

A homologous probe for Nopp52 was obtained by rapid amplification of 3' cDNA ends according to the method of Frohman *et al.* (1988) with a degenerate primer designed from the amino acid sequence of an internal CNBr peptide of Nopp52 (corresponding to amino acids 340–348, see underlined protein sequence in Figure 2A).

The cDNA clones were sequenced using the Sanger method with Sequenase (United States Biochemical, Cleveland, OH) according to

the manufacturer's instructions. Final cDNA sequence was compiled from both strands or from independent clones.

Immunocytochemistry and Electron Microscopy

Cells ($\sim 2 \times 10^6$ cells) were collected by centrifugation, washed with 40 mM *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid) (HEPES) buffer (pH 7.5) and fixed overnight at 4°C. Samples used for immunocytochemistry were fixed in 4% paraformaldehyde and 0.15% glutaraldehyde, dehydrated, embedded in Lowicryl K4 M, and UV light-polymerized for 24 h at -20°C followed by 12–24 h at room temperature. Thin sections were mounted on nickel grids and incubated with 0.5 M glycine for 20 min followed by a 15-min incubation with blocking solution (5% heat-inactivated goat serum) made in incubation buffer (1.5% acetylated bovine serum albumin [Auri, Electron Microscopy Sciences, Fort Washington, PA] and 1.3 $\mu\text{g}/\text{ml}$ sodium azide in TBS, pH 7.6). Samples were incubated for 1 h in primary antisera followed by six 5-min washes in incubation buffer, 1 hr secondary antibody conjugated to 30 nm gold beads, six 5-min washes in incubation buffer, two 5-min washes in TBS, 10 min in 2% glutaraldehyde (postfixing), and two 5-min washes in distilled H_2O . Sections were stained for 5 min in aqueous uranyl acetate and 1.5 min in Reynolds' lead citrate prior to examination with a Siemens 101 transmission electron microscope operated at 80 kV.

Simultaneous Detection of rDNA and Nopp52 In Situ

Cells were fixed and processed for indirect immunofluorescence as described previously (Madireddi *et al.*, 1994), except that cells were exposed briefly (15 s) to a 3:1 mixture of ethanol:acetic acid before being dropped onto slides and allowed to air dry. Cells were then processed for in situ hybridization as follows: Cells were acetylated (0.1 M triethanolamine, 0.25% acetic anhydride, pH 8.0) for 10 min; denatured in 70% formamide and $2\times$ SSC at 70°C for 4 min; and dehydrated sequentially in 70%, 95%, and 100% ethanol. A DNA probe was prepared from the micronuclear copy of the *Tetrahymena* rDNA gene (kindly provided by M.-C. Yao, Fred Hutchinson Cancer Research Center, Seattle, WA) by nick translation using biotin-dUTP (Boehringer Mannheim, Indianapolis, IN). Denatured probe was hybridized to the processed cells in $2\times$ SSC, 10% dextran sulfate, 40% formamide, at 30°C in a moist chamber for 24 to 36 h. The slides were washed with gentle agitation in $2\times$ SSC and 40% formamide at 30°C twice, followed by $1\times$ SSC at room temperature. After hybridization, immunofluorescence was performed as described (Wenkert and Allis, 1984), except that only primary antibody was applied. The cells were washed in TBS and then exposed to two secondary reagents simultaneously, avidin-fluorescein isothiocyanate (Boehringer Mannheim) and rhodamine-conjugated secondary antibody (Pierce, Rockford, IL) at 30°C in a dark moist chamber, mounted in 10% glycerol containing 0.1% *o*-phenylenediamine (Sigma). Samples were examined at $400\times$ using a Zeiss microscope equipped for epifluorescence photomicroscopy.

Cross-Linking and Immunoprecipitation

Cells were labeled in vivo with [^3H]thymidine, and the specific activity of the DNA was determined by using ^3H incorporation and cell number. This allowed quantitation of DNA throughout each experiment by ^3H counts. Formaldehyde cross-linking and immunoprecipitation were performed essentially as described previously (Dedon *et al.*, 1991b), except that the sonicate was layered onto 20% sucrose in sonication buffer plus 120 mM NaCl and centrifuged in a TLS-55 rotor (Beckman Instruments, Fullerton, CA) for 14 h at $135,000 \times g$ at 17°C . The pellet was collected and sonicated three more times, and some of this material was reserved as "total" sample for later DNA isolation. For NaOH controls, the starting material was treated for 5 h at 0.1 N, which eliminated trichloro-

acetic acid-precipitable counts if cells were first in vivo-labeled with uridine. After immunoprecipitation (0.2–1 μg of DNA/ml, 10 μl of antisera/1 μg DNA), DNA was quantitated by liquid scintillation or DNA-protein complexes were solubilized in 100 mM NaHCO_3 , 1% SDS for further analysis.

Hybridization Analysis of Immunoprecipitated DNA

Formaldehyde cross-links were reversed and DNA was prepared from the immunoprecipitated material and total fractions as described previously (Dedon *et al.*, 1991a). Equivalent specific activity ^{32}P -labeled probes were made from these DNAs using random oligonucleotides and Klenow fragment of DNA polymerase I. Gel-purified restriction digestion fragments from plasmids containing rDNA sequences (see Figure 6), other non-rDNA *Tetrahymena* sequences (see Figure 6 legend) or linearized pBR322 were slot blotted onto Magnagraph charged nylon membrane (Micron Separations, Westborough, MA) according to the manufacturer's instructions. Twenty nanograms of each fragment per slot were used except for fragment T for which 10 ng was used so that it was equimolar with fragment H. Filters were hybridized with 1.25×10^5 cpm/ml of probe as described by Dedon *et al.* (1991a). Autoradiographic signals were quantified with a laser densitometer (LKB Ultrascan XL, LKB, Piscataway, NJ).

The Nopp52 cDNA sequence is available from GenBank (accession number U51555).

RESULTS

Nopp-52, an Abundant Macronuclear Phosphoprotein, Is Phosphorylated by CKII

An abundant polypeptide migrating with the apparent molecular mass of 65 kDa (labeled Nopp52 in Figure 1) is readily visible by Coomassie blue staining following two-dimensional gel electrophoresis of acid-soluble macronuclear proteins (Triton X-100-acid-urea by SDS, Figure 1, bottom panel). After continuous growth in [^{32}P]orthophosphate and autoradiography (Figure 1, top panel), Nopp52 is one of the most highly phosphorylated polypeptides in macronuclei along with the major histones H1 and H2A. The abundant nature of Nopp52 suggests a structural role in macronuclei.

To determine what kinase might be responsible for the phosphorylation of Nopp52 in vivo, HPLC-purified Nopp52 was reacted in vitro with several available kinases. Although Nopp52 was an effective substrate for CKII, it was not phosphorylated with $\text{p}34^{\text{cdc}2}$ or cAMP-dependent protein kinase under conditions that effectively label control substrates. Phosphoamino acid analyses demonstrate that serine is the exclusive phosphoamino acid whether Nopp52 is labeled in vivo or in vitro with CKII (our unpublished results). Tryptic phosphopeptide maps are very similar between Nopp52 phosphorylated in vivo and in vitro by CKII (Figure 1, inset). Thus, these data suggest Nopp52 is phosphorylated by CKII.

cDNA Sequence of Nopp52 Predicts a Modular Protein Composed of Distinctive Domains

Six Nopp52 cDNA clones were isolated from a size-selected poly(dT)-primed library; all of the clones had

Figure 1. Nopp52 is an abundant phosphoprotein in *Tetrahymena* macronuclei. Vegetatively growing cultures were labeled directly in growth medium with [^{32}P]orthophosphate (10 $\mu\text{Ci}/\text{ml}$). Acid-soluble macronuclear polypeptides were separated by two-dimensional gel electrophoresis (Triton X-100-acid-urea by SDS) and analyzed by staining (bottom) and autoradiography (top). Inset, Nopp52 phosphorylated in vivo (lane 1) or in vitro (lane 2) by CKII was recovered and digested with trypsin. Phosphotryptic peptides were separated electrophoretically in a 40% alkaline polyacrylamide gel and analyzed by autoradiography.

similar 5' ends (within 45 bp) and one of two poly(A) addition sites (Figure 2A). The size of the longest cDNA (1646 bp) is consistent with the single poly(A)⁺ message of about 1700 nucleotide seen in RNA blots (our unpublished results). The longest open reading frame begins at the first ATG. The predicted amino acid sequence includes two stretches of amino acids (totaling 35 residues) that match exactly the amino acid sequences generated from two internal peptides of HPLC-purified Nopp52 (Figure 2A, underlined). Consistent with the AT richness of nonprotein coding sequences in *Tetrahymena*, the GC content of the 48 bp upstream of this ATG is only 8.8% whereas the next 50

bp are 35% GC. Similarly, the predicted 171-nucleotide untranslated 3' region is only 15% GC.

The amino-terminal half of the predicted Nopp52 protein sequence consists of seven consecutive repeats of the same amino acid pattern (Figure 2C) with the second, third, and fourth repeat being nearly identical at the nucleic acid level (>93%). Each repeat consists of glutamic and aspartic residues mixed with serines (acidic/serine subdomain) followed by a region rich in lysine, proline, alanine, and valine (basic/proline subdomain) residues. More than 90% of the residues in these repeats are one of these seven amino acids.

After the highly charged amino terminus of Nopp52 are two domains (amino acids 262–340 and 354–452; Figure 2A) matching the consensus sequence of an RNA recognition motif (RRM) found in a wide variety of RNA-binding proteins (Figure 2B; for review, Burd, 1994). The extreme carboxyl-terminal tail of Nopp52 contains a glycine-, arginine-, and phenylalanine-rich (GRF) region (14 of 24 amino acids starting at residue 431), residues that are noticeably less abundant in the amino-terminal half. In Nopp52 as well as other proteins, secondary structure predictions of GRF-rich regions predict a repeated β -turn structure that has been hypothesized to be involved in nucleic acid interactions (Ghisolfi *et al.*, 1992). Collectively, these data suggest that Nopp52 is a modular protein containing discrete domains that may participate in distinct functions.

Nopp52 Is Similar in Structure to Vertebrate Nucleolin and Yeast NSR1

Although each of the domains present in Nopp52 are found in many other proteins, only two other known proteins contain all of the structural features exhibited by Nopp52 (Figure 2D). Interestingly the two, vertebrate nucleolin and yeast NSR1, are localized to nucleoli (Lapeyre *et al.*, 1987; Lee *et al.*, 1991) as is Nopp52 (see below). However, there exist several clear differences between them. First, an alignment of the three protein sequences indicates sequence similarity (50%) but not a high degree of sequence identity (30%).

However, even within vertebrate nucleolins, sequence identity is not high (57% between chicken and hamster), especially in the amino terminus and the first two RRM regions (Maridor *et al.*, 1990). Second, in their amino-terminal domains, Nopp52 and nucleolin are more similar to each other than to NSR1. NSR1 has very short basic regions (without prolines) interspersed with predominantly serine stretches. Third, both NSR1 and Nopp52 have two RRMs, whereas nucleolin has four. Finally, the carboxyl-terminal tails of NSR1 and nucleolin are more GRF-rich and repetitive than Nopp52.

Nopp52 Is Localized to Macronuclei and Enriched in Nucleoli

HPLC-purified Nopp52 was used to generate a highly selective polyclonal antiserum. Affinity-purified antibodies from this antiserum react specifically with a 65-kDa polypeptide in immunoblots of total acid-soluble macronuclear proteins (Figure 3, B and E, arrows in lanes 2). Little, if any, Nopp52 immunoreactivity is observed in immunoblots of total acid-soluble micronuclear proteins (Figure 3, B and E, lanes 1). Macronuclear cross-contamination in our micronuclear preparations was judged to be low when parallel blots are probed with macronuclear-specific histone antibodies directed against H1 (Figure 3C) and hv1 (Figure 3D).

The macronuclear specificity of Nopp52 was confirmed by immunocytochemistry at both the light and

Figure 3. Nopp52 is a macronuclear-specific polypeptide. Total acid soluble micronuclear (lanes 1) and macronuclear (lanes 2) proteins were electrophoresed in a one-dimensional 12% SDS gel and either stained with Coomassie (A) or blotted onto nitrocellulose and reacted with affinity-purified Nopp52 antibodies followed by detection with ECL (B). Loads were normalized by running equivalent amounts of core histones for both samples (A). Purity of the micronuclear preparations is indicated by probing the top half of a parallel immunoblot with antibodies against macronuclear-specific H1 (C) or the bottom half with antibodies against a minor H2A variant, hv1 (D), followed by detection with alkaline phosphatase. The blot shown in C was then reprobbed with affinity-purified Nopp52 antibodies and detected by using ECL (E). Arrows point to Nopp52 in the stain and corresponding immunoblots. Nuclei were purified by sedimentation at unit gravity.

electron microscopic level. In growing or starved cells, staining of Nopp52 was limited to macronuclei with no significant staining of micronuclei or cytoplasm. Still, it remains a formal possibility that a small amount of Nopp52 may be associated with the single copy of rDNA in micronuclei. At the light level (Figure 4A), Nopp52 staining within macronuclei is strikingly punctate and peripheral, highly suggestive of the multiple nucleoli that characteristically line the periphery of macronuclei in *Tetrahymena* (Gorovsky, 1973). The pattern of nucleolar staining observed for Nopp52 matches that of the known nucleolar antigen fibrillarin (our unpublished results).

To better examine Nopp52's apparent enrichment in nucleoli, in situ localizations of ribosomal RNA genes (rDNA) and Nopp52 were conducted simultaneously. The results are shown in Figure 4B, where starved cells were subjected to fluorescent in situ hybridization (FISH) of rDNA sequences as well as immunofluorescence localization of Nopp52. In starved cells, rDNA FISH detects a series of peripherally localized

nucleoli (Figure 4B, left panel) and the corresponding image of Nopp52 distribution (Figure 4B, right panel) demonstrates a one-to-one correspondence between the two signals. These data suggest that Nopp52 may have an association with rDNA in nucleoli in vivo (see below).

Verification of Nopp52 enrichment in nucleoli was provided by immunocytochemistry and electron microscopic analyses. Gold particles decorate peripherally localized nucleoli (NO) observed in the thin section of the macronucleus presented in Figure 5. Little, if any, gold is observed over the nucleoplasm or the adjacent micronucleus, further demonstrating the specificity of Nopp52's localization.

Nopp52 Is Associated with rDNA In Vivo

To directly test the hypothesis that Nopp52 is part of rDNA chromatin architecture, formaldehyde cross-linking followed by immunoprecipitation (Dedon *et al.*, 1991a,b) was used. If Nopp52 is associated with

Figure 4. Nopp52 displays a peripheral punctate staining pattern in macronuclei that closely colocalizes with rDNA. (A) Vegetatively growing (top row) and starved (bottom row) cells were fixed and incubated with affinity-purified Nopp52 antibodies. Immune localizations were detected indirectly with rhodamine-conjugated secondary antibodies; nuclei in the same cells were detected with the DNA-specific dye DAPI. Top and middle, different optical sections. Note the peripheral disposition of Nopp52-positive bodies. Arrows point to micronuclei, which are not stained with Nopp52 antibodies. (B) Starved cells were doubly stained with rDNA (FISH, fluorescein isothiocyanate-conjugated avidin secondary) and anti-Nopp52 (immunofluorescence, rhodamine-conjugated secondary antibody). The rDNA hybridization results (left) are shown (see arrowheads) as well as the corresponding Nopp52 localization (right, arrowheads). B, 400 $\times$.

Figure 5. Nopp52 is enriched in nucleoli. Starved cells were fixed and processed for immunogold analysis using affinity-purified Nopp52 antibodies. A macronucleus (Ma) and adjacent micronucleus (Mi) are shown. Note the presence of gold particles over the peripherally located nucleoli (NO). Bar, 1 μ m. Inset, a higher magnification of a single nucleolus.

rDNA, then rDNA sequences should be enriched in cross-linked material immunoprecipitated with Nopp52 antisera. Equal amounts of similar specific activity probes made from the starting material (total) or immunoprecipitated DNA were hybridized to fragments of macro- or micronuclear rDNA (Figure 6A) bound to filters by using conditions of target excess. Therefore, the enrichment for any particular sequence is equal to the relative hybridization of immunoprecipitated versus total DNA probes. In this procedure, the level of fixation was optimized to allow for solubility and fracturing of DNA into smaller fragments (200–1000 bp) and consequently only captured a fraction of DNA-protein interaction (10–20% for chromatin components, Dedon *et al.*, 1991b). Therefore, the amount of enrichment seen for sequences will underestimate the level of protein binding.

In preliminary experiments, three lines of evidence indicated specificity of immunoprecipitation of DNA by Nopp52 antiserum (our unpublished data). First, HPLC-purified Nopp52, but not histone H2A, quantitatively competed immunoprecipitation of cross-linked DNA to background levels seen with preimmune serum. Second, although crude serum was used in these assays, affinity-purified antibody also precipitated the same amounts of DNA if used at levels where it had equivalent reactivity on immunoblots. Finally, immune serum depleted of anti-Nopp52 immunoglobulins by preincubation with filter-bound

Nopp52 had similarly diminished reactivity to Nopp52 on immunoblots and reduced DNA immunoprecipitation relative (50% of standard conditions). No changes in immunoprecipitation were seen when serum was affinity depleted with histone H2A. Additionally, DNA immunoprecipitation was reduced to less than 10% of that seen with standard conditions if either preimmune sera or no sera was used or in the absence of formaldehyde fixation.

An example of hybridization to rDNA targets with DNA immunoprecipitated by Nopp52 antibodies is shown in Figure 6B, and quantitative data from experiments using growing or starved cells are presented in Figure 6C. All fragments of rDNA are consistently enriched (three- to ninefold) in cross-linked DNA immunoprecipitated from both growing and starved cells with anti-Nopp52 serum. In contrast, no enrichment is observed for six protein-coding genes in various states of transcription (Stargell *et al.*, 1990) assayed in parallel experiments (Figure 6D).

The most 3' fragment of the macronuclear rDNA (Figure 6A, fragment T) also contains telomere sequences. To analyze just the nontelomeric sequences, the micronuclear copy of this region was also used (Figure 6A, fragment H). The increased hybridization to fragment T versus H with all probes indicates that hybridization to fragment T is predominately due to its telomeric sequences. In *Tetrahymena*, half of the telomeres are on the extrachromosomal rDNA mole-

Figure 6. Immunoprecipitation of rDNA sequences with anti-Nopp52 antibodies. (A) Map of rDNA sequences analyzed. The *Hind*III fragments used as hybridization targets are shown by lines below their plasmid source. prD1 (Yu *et al.*, 1988) plasmid contains one copy of the macronuclear rDNA gene signified by a box. The region and direction of transcription are denoted by an arrow. Telomere sequences are indicated by black boxes. pD5H8 (Yao and Yao, 1991) plasmid contains the micronuclear version of the rDNA gene that includes the macronuclear rDNA sequences (without telomeres) flanked by micronuclear specific sequences (horizontal striped boxes). Vector sequences are indicated by thin lines. (B) Autoradiograph of a filter of rDNA fragment targets hybridized with probe made from either immunoprecipitated (IP) or total (T) DNA from growing cells. (C) Quantitation from autoradiographs of relative hybridization of immunoprecipitated vs. total DNA probes for each of the rDNA fragments. The average of two experiments with growing cells (gray boxes) and two with starved cells (diagonal shaded boxes) are shown. The range of the values indicated by the error bars. (D) Relative hybridization of immunoprecipitated vs. total DNA probes from growing cells for a number of non-rDNA sequences. Target sequences and transcription rates for α -tubulin, TATA-binding protein (TBP), and high mobility group B (HMG-B), as well as the starvation-specific gene BC-11 and conjugation-specific gene PC-1, are described in Stargell *et al.* (1990). The full-length cDNA clone was used for Nopp52 (see Figure 2).

cules so some enrichment would be expected even if Nopp52 is only associated with rDNA. Alternatively, Nopp52 could be associated with all telomeres. There is *in vitro* evidence that NSR1 binds telomeres although the *in vivo* significance is not clear (Lin and Zakian, 1994).

The pattern of rDNA enrichment observed with Nopp52 antisera was not found with antisera to three other DNA binding proteins in *Tetrahymena* (Dedon *et al.*, 1991b and our unpublished results). In particular, although *Tetrahymena* histone H1 is present equally on nucleolar DNA and non-rDNA (Colavito-Shepanski and Gorovsky, 1983), H1 antisera did not selectively immunoprecipitate rDNA while enrichment was seen for other sequences (Dedon *et al.*, 1991b).

When RNA was degraded by extensive treatment with NaOH prior to immunoprecipitation, rDNA sequences were still enriched in the immunoprecipitate (two- to fourfold). A similar overall diminution of immunoprecipitation is seen with an anti-histone antibody and may be due to general protein denaturation during alkaline treatment. There was no overall decrease in immunoprecipitation of DNA with Nopp52 antisera when material was pretreated with RNase A (our unpublished results). These experiments do not address whether Nopp52 also binds

RNA but suggest that the association of Nopp52 to DNA is not through an RNA intermediate. Hybridization of fragments across the entire length of the rDNA suggests that Nopp52 is associated with all regions of rDNA, although a slight bias is consistently observed with the transcribed region. The same quantity and pattern of association is seen in either growing or starved cells.

DISCUSSION

We have identified an abundant phosphoprotein that is enriched in nucleoli of transcriptionally active macronuclei and is missing from transcriptionally silent micronuclei in *Tetrahymena*. The polypeptide, termed Nopp52, exhibits a highly modular domain structure with alternating acidic/basic repeats distinguishing its amino-terminal half. A similar organization of alternating acidic/basic stretches has also been described in many nucleolar proteins including nucleolin, NSR1, Np146, Nopp140, and B23/No38 (Dingwall *et al.*, 1987; Lapeyre *et al.*, 1987; Lee *et al.*, 1991; Meier and Blobel, 1992; Shan *et al.*, 1994). Many of this class of polypeptides, including Nopp52, have aberrantly slow mobility in SDS gels and are phosphorylated by CKII. It has been suggested that their shared proper-

ties may enable nucleolar proteins to catalyze the assembly of ribosomal proteins with pre-rRNA and also may regulate histone binding to chromatin (Egyhazi *et al.*, 1988; Erard *et al.*, 1988).

The carboxyl-terminal half of Nopp52 contains two RRM domains that have been identified in a variety of RNA binding proteins including small nuclear ribonucleoproteins and heterogeneous nuclear ribonucleoproteins (hnRNPs), alternative splicing regulators, and poly(A) binding proteins, as well as in many nucleolar proteins such as nucleolin, NSR1, Nop3, and SSB (for review, Burd, 1994). The extreme carboxyl-terminal tail of Nopp52 is rich in glycine, arginine, and phenylalanine. Similar GRF-rich regions have been found in fibrillarin and other nucleolar snoRNP proteins, as well as hnRNP-A, nucleolin and NSR1 (for review, Burd, 1994). Thus, *Tetrahymena* Nopp52 exhibits many of the hallmark features of nucleolar polypeptides in other organisms. Consequently, these properties extend to organisms as distantly related as ciliates and humans.

Vertebrate nucleolin and yeast NSR1 are the only two proteins to have all of the structural domains found in Nopp52 and, interestingly, these motifs are present in the same order in all three proteins. These data suggest that Nopp52, nucleolin, and NSR1 are members of a protein family with potentially related functions. However, nucleolin does not rescue the slow growth phenotype of yeast NSR1 mutants (Lee *et al.*, 1992) and, therefore, complete functional identity cannot be assumed from structural similarities.

The domain structure, localization, and similarity to nucleolin and NSR1 indicate Nopp52 is likely to bind rRNA and have a role in rRNA processing. Both nucleolin and NSR1 appear to be involved in pre-rRNA processing. In yeast NSR1 mutants, there is a disruption of pre-rRNA processing (Kondo and Inouye, 1992; Lee *et al.*, 1992). Nucleolin is associated with pre-rRNA (Herrera and Olson, 1986) and this association appears to involve both the RRM domains and the GRF-rich regions (Sapp *et al.*, 1989; Ghisolfi *et al.*, 1992).

An additional role for this group of proteins in rDNA metabolism is supported by *in vitro* experiments with nucleolin. Nucleolin, through its amino-terminal region, can modulate the overall condensation state of rDNA chromatin (Egyhazi *et al.*, 1988; Erard *et al.*, 1988) and regulate transcription of ribosomal genes positively (Belenguer *et al.*, 1990) or negatively (Bouche *et al.*, 1984; Erard *et al.*, 1990). In apparent contrast to our data, a recent article that analyzed the *in situ* localization of nucleolin in chromosome spreads did not observe signal above background over the rDNA strands (Ghisolfi-Nieto *et al.*, 1996). However, it is difficult to disprove by these techniques that the small fraction of nucleolin proposed to be involved in this second site of regulation

is bound to rDNA; it may not be detectable, available, or stable under their experimental conditions.

Nopp52 immunocytochemistry and rDNA FISH indicate that Nopp52 colocalizes with rDNA. To test the hypothesis that Nopp52 is associated with rDNA chromatin, we used the short-length cross-linking agent formaldehyde to stabilize *in vivo* associations followed by immunoprecipitation. We found that Nopp52 is associated with rDNA along the length of the molecule with a slight bias over the transcribed region. This association was not exclusively through an RNA intermediate. This result suggests that Nopp52 is intimately associated with rDNA *in vivo*, although the exact nature of this association is not known.

No difference in Nopp52 immunoprecipitation of rDNA sequences was found between growing and starved cells where a 2.5-fold difference in rRNA transcription has been reported (Stargell *et al.*, 1990). The simplest explanation for this result is that Nopp52 does not have a role in regulating levels of rRNA transcription, although it may be required to facilitate the high rates of transcription seen even in starved cells. Alternatively, it could regulate transcription rates by being in different forms in growing and starved cells. Such a model of regulation based on phosphorylation states has been proposed for nucleolin (for review, Jordan, 1987).

In conclusion, a polypeptide resembling vertebrate nucleolin and yeast NSR1 has been identified in *Tetrahymena* macronuclei. Nopp52 is closely associated with rDNA *in vivo*, suggesting a potential role in ribosomal gene structure and metabolism. These proteins share the properties of nucleolar localization, similar motif structure, and CKII phosphorylation and may comprise a family of related proteins.

ACKNOWLEDGMENTS

We are grateful to Dr. Fred Warner for his electron microscopy assistance, Dr. Richard Levy and the biology department of Syracuse University for extensive use of their electron microscope facility, and Dr. Meng Chao Yao and Katie Mickey for their FISH technique instruction. We also thank Drs. David Beach and Claire Glover for purified kinases, and Dr. David Spector for the anti-fibrillarin antibodies used in this study. This work was supported by research grants from the National Institutes of Health (GM-53512 and GM-21793 to C.D.A. and M.A.G., respectively).

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