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Human Nop5/Nop58 is a component common to the box C/D small nucleolar ribonucleoproteins

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ABSTRACT

We have identified an apparent human homolog of the yeast Nop5/Nop58 protein. hNop5/Nop58 codes for a protein of predicted molecular weight 59.6 kDa and is 46.8% identical to *Saccharomyces cerevisiae* Nop5/Nop58. Immunofluorescent staining with antibodies against hNop5/Nop58 indicate that it is localized primarily to the nucleolus, and coimmunoprecipitation from nuclear extracts demonstrates that hNop5/Nop58 interacts with the box C/D family of snoRNAs. Thus, hNop5/Nop58 is a common component of the box C/D snoRNPs, and joins fibrillarin as the second such component identified and characterized in metazoans.

Keywords: genomics; nucleolus; ribosome biogenesis; snoRNPs

INTRODUCTION

In eukaryotic cells, three of the four RNA components of ribosomes are transcribed in the nucleolus as a single polycistronic transcript encoding 18S, 5.8S, and 28S/25S rRNAs. This nascent pre-rRNA transcript is cleaved, processed, and modified to generate the mature rRNAs, which are assembled together with 5S rRNA and ribosomal proteins to generate functional ribosomes. At least two different kinds of nucleotide modification take place on the pre-rRNA: pseudouridylation and 2′-*O*-ribose methylation. Small nucleolar ribonucleoproteins (snoRNPs), which consist of a small nucleolar RNA (snoRNA) and a complex of proteins, are required for both the cleavage and modification reactions.

One major class of snoRNPs, the box C/D snoRNPs, is named for the short, conserved box C (UGAUGA) and box D (CUGA) sequences present in their RNA moiety. The majority of these snoRNPs carry out 2′-*O*-ribose methylation of pre-rRNA and other RNAs (Cavaille et al., 1996; Kiss-Laszlo et al., 1996; Nicoloso et al., 1996; Tycowski et al., 1996). The nucleotide to be methylated in the target RNA is directed by the fifth nucleotide upstream of the box D sequence, which resides within a long stretch of complementarity. The box C/D sequences are also required for multiple aspects of snoRNA biogenesis, including: RNA stability, intronic processing, nucleolar targeting, nuclear retention, and 5′ trimethylguanosine cap formation (Baserga et al., 1992; Huang et al., 1992; Peculis & Steitz, 1994; Terns & Dahlberg, 1994; Balakin et al., 1996; Caffarelli et al., 1996; Watkins et al., 1996; Lange et al., 1998a, 1998b; Samarsky et al., 1998), and are likely to be protein-binding sites (Baserga et al., 1991; Caffarelli et al., 1998; Watkins et al., 1998). The box C/D sequences occur at the 5′ and 3′ ends of the snoRNA, respectively, although in a few cases box C occurs internally. Many of the box C/D snoRNPs also bear internal sequences similar to boxes C and D, called box C′ and D′ (Kiss-Laszlo et al., 1998), which are important in some cases for specifying ribose methylation.

Although the snoRNA components of the box C/D snoRNPs are well characterized, most of the protein components have remained undefined. It is likely that there are proteins common to all of the box C/D snoRNAs (common proteins) and proteins specific for snoRNPs with specialized functions in cleavage like U3, U22, U8, and U14 (specific proteins). The first common protein described was fibrillarin (34 kDa; Lischwe et al., 1985). Cross-linking experiments in vertebrate cell extracts have suggested that proteins of molecular weight 40, 65, or 68 kDa associate with the box C/D sequence, and these are also likely candidates for common proteins (Caffarelli et al., 1998; Watkins et al., 1998). In yeast, two other box C/D snoRNP common proteins have been identified: Nop56p and Nop5p/Nop58p

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(Gautier et al., 1997; Wu et al., 1998; Lafontaine & Tollervey, 1999; D.L.J. LaFontaine & D. Tollervey, pers. comm.). These two proteins are highly related to each other, and each bears characteristic KKE/D repeat sequences. Interestingly, homologs for Nop56p have been proposed both in human cells and in Archaea by sequence similarity (Gautier et al., 1997; Lafontaine & Tollervey, 1998; Wu et al., 1998).

We report here the identification and characterization of an apparent human homolog of the yeast Nop5/Nop58 protein. Human Nop5/Nop58, which was found in an assay for proteins that interact with the nuclear import receptor subunit, importin α, is a protein of predicted molecular weight 59.6 kDa. It bears 46.8% identity to the yeast Nop5/Nop58 protein throughout its length. Antibodies generated to two peptide sequences detect a protein of approximately 66 kDa on Western blots, and localize hNop5/Nop58 primarily to the nucleolus. Immunoprecipitations indicate that hNop5/Nop58, like its yeast homolog, is a protein common to the box C/D snoRNPs. Therefore hNop5/Nop58 joins fibrillarin as the second box C/D snoRNP common protein identified and characterized in metazoans.

RESULTS AND DISCUSSION

Identification of the hNop5/Nop58 protein and cloning of its full-length cDNA

An importin α blot overlay assay was the basis for a biochemical screen directed at identifying nuclear proteins bound to importin α in a manner that was not mediated by a nuclear localization sequence. This screen was carried out on rat liver nuclear envelopes (NE) and nucleoplasm fractions, and two proteins of 60 and 66 kDa were identified (data not shown).

Peptide microsequence obtained from a rat liver NE fraction enriched in p60 and p66 revealed that p60 was equivalent to a known nucleolar protein in rat, Nap57 (Meier & Blobel, 1994). Peptide sequence derived from p66 corresponded to two human expressed sequence tag (EST) fragments in the GenBank database, which allowed us to design PCR primers to clone its human homolog by screening a HeLa cDNA library using 3′ and 5′ RACE (rapid amplification of cDNA ends) methodology (see Materials and Methods). This screen resulted in the isolation of a human cDNA coding for an open reading frame (ORF) that contains a stretch of amino acids (216–235) very similar to the peptide microsequence originally obtained from rat p66 (17 of 20 residues identical). The ORF encoded by the human p66 cDNA is 98% identical to an ORF encoded by an uncharacterized rat cDNA (accession number AF069782), and amino acids 216–235 of this rat ORF are identical to the peptide sequence derived from rat p66. Thus, the ORF of the human cDNA that we cloned includes a peptide sequence that closely corresponds to the sequence originally obtained from a tryptic digest of rat p66.

A search of GenBank revealed that p66 was 46.8% identical to the *Saccharomyces cerevisiae* protein Nop5 (Wu et al., 1998), also known as Nop58 (Gautier et al., 1997)—a value that is akin to the 50.7% identity observed between hNop56 and yNop56 (see Fig. 1B). This observed sequence similarity, in conjunction with functional data (see below), strongly suggested that p66 was the human homolog of yNop5/Nop58, so we have designated p66 as hNop5/Nop58.

The human Nop5/Nop58 cDNA encodes a protein with a predicted molecular weight of 59.6 kDa and a predicted pI of 8.9, as compared to yNop5/Nop58, which has a predicted molecular weight of 56.9 kDa and a predicted pI of 9.1 (Wu et al., 1998). hNop5/Nop58, like yNop5/Nop58, migrates more slowly than its predicted weight on SDS-polyacrylamide gels. This discrepancy in hNop5/Nop58 migration, like that of yNop5, is probably due to the highly charged C-terminal domain common to both yNop5 (Gautier et al., 1997; Wu et al., 1998) and to hNop5/Nop58.

hNop5/Nop58 is a member of a highly conserved protein family

A comparison of the protein sequence of hNop5/Nop58 with its homolog in *S. cerevisiae* (yNop5/Nop58; Gautier et al., 1997; Wu et al., 1998) and with the human Nop56 (hNop56; Gautier et al., 1997) and *S. cerevisiae* Nop56 (yNop56; Gautier et al., 1997), also known as Sik1p (Morin et al., 1995), revealed several blocks of sequence that were largely identical in all four proteins (Fig. 1A). A search of GenBank using the BLAST algorithm identified a number of proteins that fell into the Nop5/Nop58–Nop56 family. We used a Lipman–Pearson alignment in the program Megalign (DNASTAR) to generate a similarity index for each of these related proteins in successive pair-wise combinations with hNop5/Nop58, yNop5/Nop58, hNop56, and yNop56 (Fig. 1B). The similarity index represents a combination of two factors: the percentage of residues that are identical between the two sequences, and a penalty factor that takes into consideration the number and length of gaps that must be introduced to produce an alignment.

The similarity indices shown in Figure 1B suggest that Nop5/Nop58 homologs exist in a number of organisms: hNop5/Nop58 is 46.8% identical to yNop5/Nop58, but more closely related apparent homologs exist in rat, mouse, *Arabidopsis thaliana*, pea, rice, and *Caenorhabditis elegans*. These proteins are more similar to hNop5/Nop58 and yNop5/Nop58 than to hNop56 and yNop56, and thus may represent the functional equivalents of Nop5/Nop58 in these organisms. The BLAST search also revealed proteins in chickpea, the fission yeast *Saccharomyces pombe*, and *C. elegans* that have greater similarity to Nop56 than to Nop5/

A

B

Related proteins	hNop5/Nop58	yNop5/Nop58	hNop56	yNop56
hNop5/Nop58	◊ ◊ ◊	46.8	37.3	37.8
Rat *	98.0	48.2	41.0	38.3
Mouse *	93.0	49.0	37.5	39.9
A. thaliana.1 °	61.3	51.3	43.2	43.3
P. sativum.1 (pea) *	55.5	48.1	37.0	40.3
P. sativum.2 (pea) *	55.3	49.9	37.2	40.7
O. sativa (rice) *	54.4	48.0	38.0	38.9
C. elegans.1 °	53.9	45.9	38.1	39.8
A. thaliana.2 °	48.1	45.3	38.5	37.9
yNop5/Nop58	46.8	◊ ◊ ◊	36.7	40.6
C. arietinum (chickpea) *	38.5	36.1	54.9	50.2
S. pombe °	38.1	38.4	51.7	59.0
yNop56	37.8	40.6	50.7	◊ ◊ ◊
hNop56	37.3	36.7	◊ ◊ ◊	50.7
C. elegans.2 °	35.5	37.0	56.2	47.6

* the gene product encoded by this cDNA is uncharacterized

° this is a hypothetical gene product resulting from an ORF predicted by sequence data

FIGURE 1. hNop5/Nop58 is a member of a conserved family of proteins. **A**: Comparison of the hNop5/Nop58 sequence to yNop5/Nop58, hNop56, and yNop56. Alignments were generated with the program Megalign. **B**: Relatedness of the members of the Nop5/Nop58 and Nop56 families. Accession numbers for the sequences cited are as follows: hNop5/Nop58, AF123534; rat, AF069782; mouse, AF053232; *Arabidopsis*.1, O04658; pea.1, AF061963; pea.2, AF061962; rice, AB015431; *C. elegans*.1, AF043704; *Arabidopsis*.2, O04656; yNop5/Nop58, O12499; chickpea, AJ012686; *S. pombe*, AL035216; yNop56, O12460; hNop56, O00567; *C. elegans*.2, O21276.

Nop58 and may be a family of Nop56 homologs. However, because Nop5/Nop58 and Nop56 are themselves related (37.3% identity between hNop5/Nop58 and hNop56; 40.6% identity between yNop5/Nop58 and yNop56), these putative Nop56 homologs retain a significant degree of identity to human and yeast Nop5/Nop58.

Immunolocalization of hNop5/Nop58

Antibodies were raised against peptides corresponding to N- and C-terminal regions of hNop5/Nop58. As hNop5/Nop58 and hNop56 share several highly conserved domains, the regions of sequence selected for the peptides were specifically chosen to be in areas of

hNop5/Nop58 that were not common to hNop56 to minimize the potential for cross-reactivity. In a blot of HeLa cell extract (Fig. 2A), affinity-purified antibodies to the N-terminal portion of hNop5/Nop58 recognize primarily a single protein whose size corresponds to p66, the factor originally identified in the importin α overlay screen. Antibodies to the C-terminal portion also strongly recognize a protein of ~66 kDa, although they react more weakly with a faster migrating band, possibly a degradation product of hNop5/Nop58. Thus, the 66 kDa antigen that these antibodies recognize is likely to be identical to p66.

Affinity-purified antibodies to hNop5/Nop58 were used to examine its subcellular localization in HeLa cells. Confocal immunofluorescence microscopy using either the N- or C-terminal antibodies showed a strong nucleolar stain that largely coincided with the staining pattern delineated by the nucleolar marker protein fibrillarin (Fig. 2B; Lischwe et al., 1985). Interestingly, fibrillarin staining was enhanced at the periphery of the nucleo-

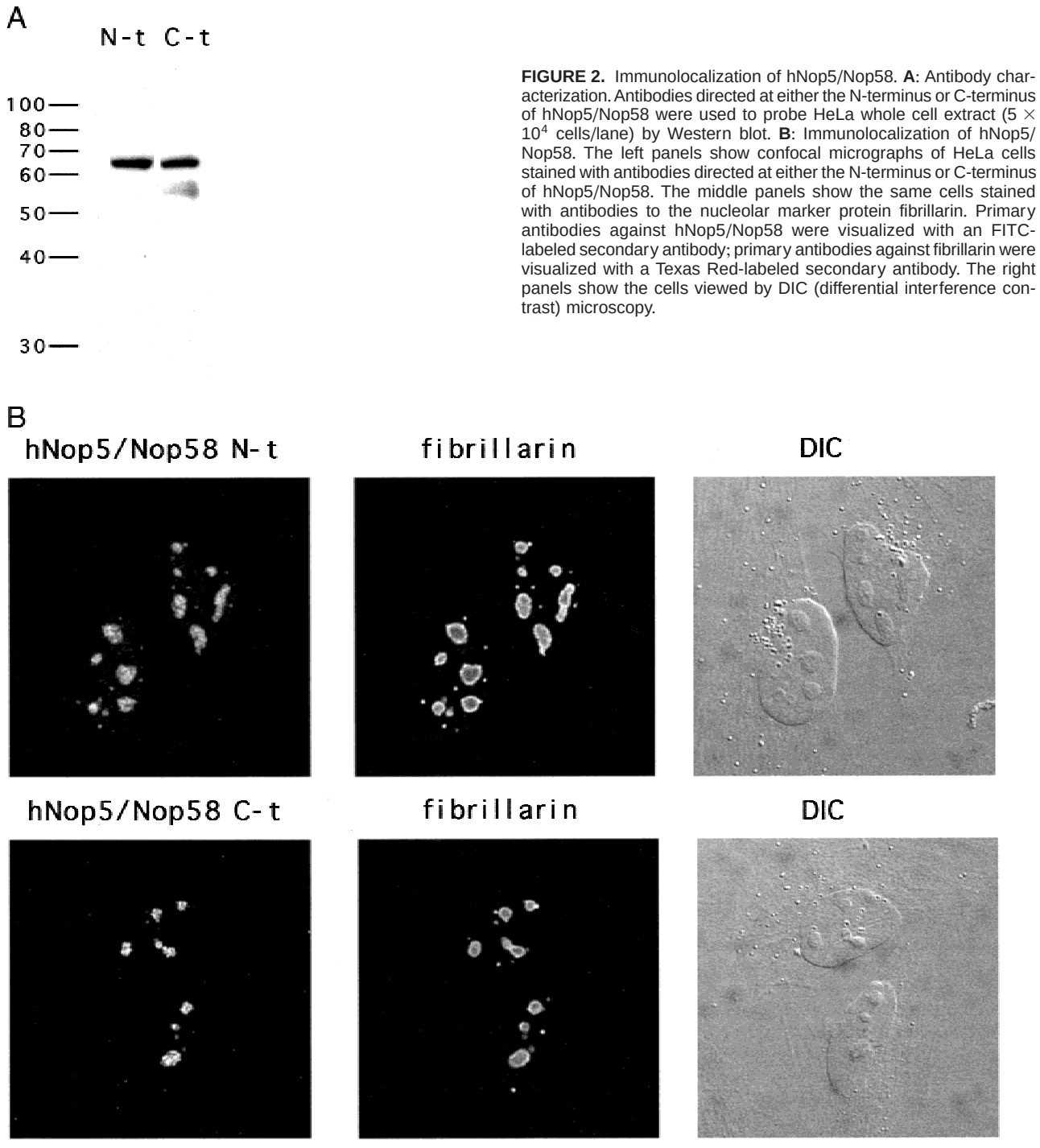

FIGURE 2. Immunolocalization of hNop5/Nop58. **A**: Antibody characterization. Antibodies directed at either the N-terminus or C-terminus of hNop5/Nop58 were used to probe HeLa whole cell extract (5×10^4 cells/lane) by Western blot. **B**: Immunolocalization of hNop5/Nop58. The left panels show confocal micrographs of HeLa cells stained with antibodies directed at either the N-terminus or C-terminus of hNop5/Nop58. The middle panels show the same cells stained with antibodies to the nucleolar marker protein fibrillarin. Primary antibodies against hNop5/Nop58 were visualized with an FITC-labeled secondary antibody; primary antibodies against fibrillarin were visualized with a Texas Red-labeled secondary antibody. The right panels show the cells viewed by DIC (differential interference contrast) microscopy.

lus whereas hNop5/Nop58 staining was not. However, overlapping staining between hNop5/Nop58 and fibrillarin did occur within the main body of the nucleolus. Fibrillarin antibodies, and to a lesser extent, hNop5/Nop58 antibodies, also decorated distinct foci in the nucleus that may represent coiled bodies. In mouse cells (NIH 3T3), *Drosophila* S2 cells, and chick embryo fibroblasts (CEC-32), both the N- and C-terminal antibodies recognized an antigen that showed a localization similar to hNop5/Nop58 (data not shown). Western blot analysis demonstrated that the N- and C-terminal antibodies also recognized primarily a single band of ~64–68 kDa in these cell lines, suggesting that a homolog to hNop5/Nop58 is present in these organisms as well (data not shown).

hNop5/Nop58 is a protein common to the box C/D snoRNPs

The hNop5/Nop58 protein is a nucleolar protein with high similarity to the yeast Nop5/Nop58 protein. If the sequence similarity extends to functional similarity, then it should also be a component of the box C/D snoRNPs. We investigated this question by carrying out coimmunoprecipitation with anti-hNop5/Nop58 antibodies and examining the snoRNAs associated with the precipitated protein. Immunoprecipitations with anti-C-terminal hNop5/Nop58 bound to Protein A Sepharose were performed on extracts from HeLa cells prepared by sonication. RNA was isolated from the pellet, directly labeled with ^{32}pCp, and analyzed by denaturing gel electrophoresis. The pattern of the resulting RNAs was compared to that obtained using antibodies to fibrillarin, a protein common to the box C/D snoRNPs. As an indication of the RNAs bound nonspecifically to the Protein A Sepharose beads, a HeLa cell extract was mixed with Protein A Sepharose beads alone, and the resultant bound RNAs were isolated and analyzed. The immunoprecipitations were compared to 3′ end-labeled total HeLa cell RNA. The results are shown in Figure 3A. Strikingly, the pattern of snoRNAs obtained with anti-hNop5/Nop58 is identical to that obtained with anti-fibrillarin antibodies (Fig. 3A, compare lanes 3 and 4). The anti-N-terminal hNop5/Nop58 antibody was also tested and yielded an identical pattern of coimmunoprecipitating snoRNAs (data not shown). The anti-hNop/Nop58 coimmunoprecipitated snoRNAs can be identified by their position on the gel with reference to the anti-fibrillarin coimmunoprecipitated snoRNAs (Tyc & Steitz, 1989; Westendorf et al., 1998). Anti-hNop5/Nop58 coimmunoprecipitated the U3, U8, U13, U15, and U22 snoRNAs as well as many smaller snoRNAs and tRNAs. The appearance of 5S and 5.8S rRNAs in immunoprecipitates of the box C/D snoRNAs has been observed with other unrelated sera (anti-fibrillarin, anti-Mpp10, and anti-Myc epitope; Dunbar et al., 1997; Westendorf et al., 1998; Lee & Baserga, 1999) and probably results from their abundance relative to the box C/D snoRNAs and from nonspecific association with the bead-bound antibody.

To investigate whether the association of human Nop5/Nop58 with the box C/D snoRNAs was stable in high salt, immunoprecipitations (extracts and washes) were performed in 0.5 M NaCl. The results are shown in Figure 3B. As in lower salt, in high salt anti-human Nop5/Nop58 specifically coimmunoprecipitated the box C/D snoRNAs. The levels of the snoRNAs immunoprecipitated in high salt appears to be higher than in low salt because the levels of the 5S and 5.8S rRNAs and of tRNAs are reduced at the higher stringency. This additional coimmunoprecipitation provides compelling evidence that human Nop5/Nop58 is a stable, core component of the box C/D snoRNPs.

To verify that the end-labeled RNAs immunoprecipitable with anti-hNop5/Nop58 antibodies represent box C/D snoRNAs, immunoprecipitations were performed and analyzed by Northern blotting with riboprobes to the U3 and U8 snoRNAs, two box C/D snoRNAs (Fig. 3C). Both U3 and U8 snoRNAs are coimmunoprecipitated by antibodies to the hNop5/Nop58 protein (Fig. 3C, lanes 3 and 4), whereas no immunoprecipitation of U3 and U8 is seen with preimmune rabbit serum (Fig. 3C, lane 2). Similarly, the spliceosomal snRNA, U1, was not immunoprecipitable over background with anti-hNop5/Nop58 antibodies. Taken together, these three immunoprecipitations suggest that hNop5/Nop58 is associated with the same snoRNAs as fibrillarin and is therefore a component common to the box C/D snoRNAs.

hNop5/Nop58 is only the second box C/D common protein identified in metazoans. The first was fibrillarin, discovered in mammals in 1985 (Lischwe et al., 1985) and identified in 1989 as a protein common to more than one box C/D snoRNP (Tyc & Steitz, 1989). Because fibrillarin is a target of the immune system in systemic sclerosis, antibodies in patient sera were used to demonstrate that fibrillarin associated with the U3, U8, U13, U15, and U22 snoRNAs. Previously, a tentative partial sequence of the hNop5/Nop58 protein (54% of the full-length protein assembled from overlapping EST sequences; Wu et al., 1998) and a putative human homolog of the yeast Nop56p (Gautier et al., 1997) have been published, but neither protein had been demonstrated to be a component of the box C/D snoRNPs in metazoans.

Although it is clear that hNop5/Nop58 and fibrillarin are both components common to box C/D snoRNPs, from our experiments we cannot say with certainty whether they are both contained within the same RNP at the same time. Fibrillarin and hNop5/Nop58 do colocalize in the nucleolus by immunofluorescence, though the staining does not completely overlap, indicating that there may be some fibrillarin that is not associated with hNop5/Nop58 in the nucleolus.

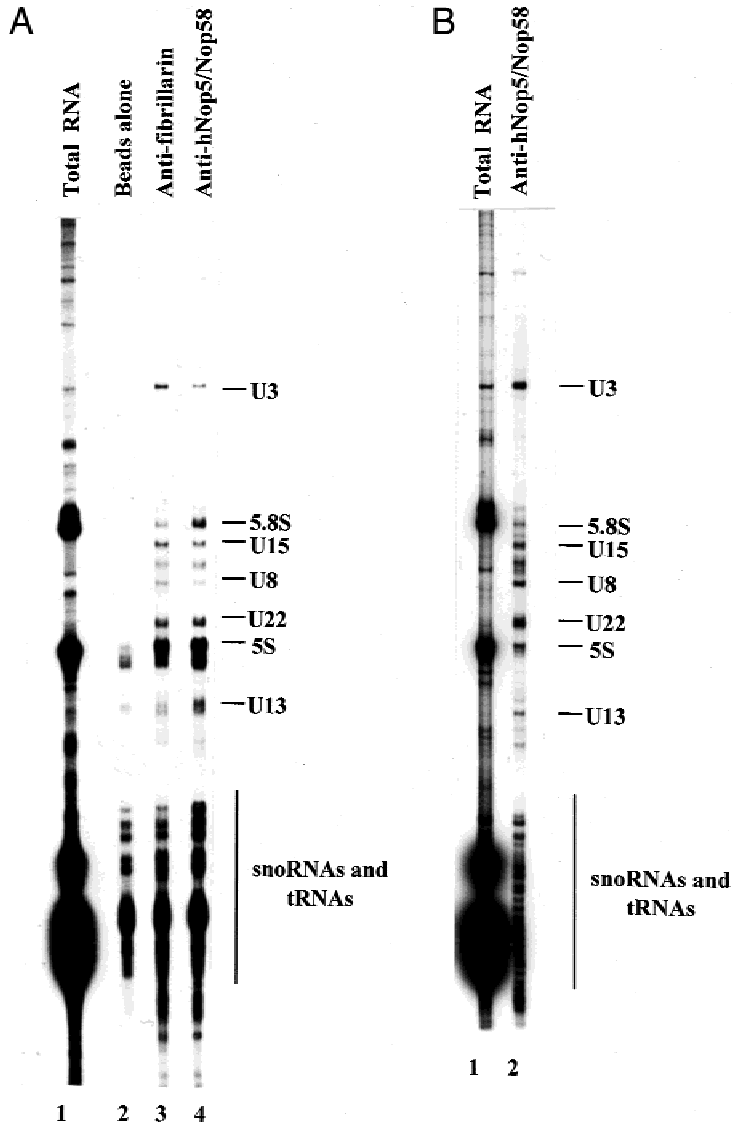

FIGURE 3. hNop5/Nop58 is a component of the box C/D snoRNPs. **A**: Immunoprecipitation of box C/D snoRNAs with anti-hNop5/Nop58. Immunoprecipitations were performed on HeLa cell sonic extract in NET-2 with anti-fibrillarin (lane 3) and anti-C terminal hNop5/Nop58 (lane 4). A control with no added antibody (beads alone) was also performed (lane 2). The resulting bound RNAs were labeled at their 3′ ends and analyzed on an 8% denaturing polyacrylamide gel. For comparison, RNA was isolated from 1% of the amount of extract used for immunoprecipitation and directly 3′ end-labeled (total RNA, lane 1). **B**: The association of hNop5/Nop58 with the box C/D snoRNAs is stable in high salt. An immunoprecipitation was carried out on HeLa cell sonic extract in IPP buffer with anti-C terminal hNop5/Nop58 (lane 2). The resulting bound RNAs were labeled at their 3′ ends and analyzed on an 8% denaturing polyacrylamide gel. For comparison, RNA was isolated from 1% of the amount of extract used for immunoprecipitation and 3′-end labeled (total RNA, lane 1). **C**: Immunoprecipitation of box C/D snoRNAs with anti-Nop5/Nop58 verified by Northern blotting. Immunoprecipitations were performed on HeLa cell sonic extract in NET-2 with anti-fibrillarin (lane 3) and anti-C terminal hNop5/Nop58 (lane 4). A control immunoprecipitation with preimmune serum (lane 2) was also included. The resulting bound RNAs were analyzed on an 8% denaturing polyacrylamide gel and blotted onto Zeta-Probe membrane. For comparison, RNA was isolated from 20% of the amount of extract used for immunoprecipitation (total RNA, lane 1). The blot was probed with riboprobes to the human U3 and U8 box C/D snoRNAs and the spliceosomal snRNA, U1. The exposure time for the U8 signal was three times that for U3 and U2. Experiments from our laboratory suggest that the two bands that hybridize to the U8 probe represent two U8 snoRNAs that differ in sequence in the 3′ terminal 40 nt (C. Chang and S.J. Baserga, pers. comm.).

In yeast, three box C/D common proteins have been described: fibrillarin (Nop1p), Nop5/58p, and Nop56p (Schimmang et al., 1989; Gautier et al., 1997; Wu et al., 1998; Lafontaine & Tollervey, 1999; D.L.J. Lafontaine & D. Tollervey, pers. comm.). yNop5/58p and yNop56p were originally identified in a genetic screen for proteins synthetically lethal with a mutation in fibrillarin that affects rRNA methylation (Gautier et al., 1997). yNop5/58p was also identified independently with a monoclonal antibody to nucleolar proteins (Wu et al., 1998). yNop5/Nop58 and yNop56 are essential proteins that share a high degree of sequence similarity, and both are required for ribosome biogenesis. Interestingly, depletion of yNop5/Nop58p leads to a decrease in the levels of the box C/D snoRNAs, suggesting that it may be required for snoRNA stability or synthesis (Lafontaine & Tollervey, 1999). Because of this, the defect in pre-18S rRNA processing that is observed upon yNop5/Nop58p depletion may be a result of the depletion of key snoRNAs such as U3 and U14.

Candidate box C/D binding proteins, as identified by cross-linking of vertebrate proteins to the box C/D sequence, have estimated sizes of 40, 65, and 68 kDa. hNop5/Nop58 migrates at 66 kDa on SDS-PAGE and may correspond to one of the proteins identified in the cross-linking assay (Caffarelli et al., 1998; Watkins et al., 1998). If so, hNop5/Nop58 may associate with the box C/D sequence and participate in snoRNP biogenesis and localization. hNop5/Nop58 is also likely to interact with fibrillarin, as depletion of yNop5/Nop58 in yeast leads to delocalization of fibrillarin from the nucleolus (Wu et al., 1998). Although further experimentation will be required to precisely define the role of hNop5/Nop58 in snoRNA metabolism, our results demonstrate that it is a highly conserved protein common to the box C/D snoRNPs with the potential for multiple RNA and protein interactions that enable it to carry out its role in ribosome biogenesis.

MATERIALS AND METHODS

cDNA cloning of hNop5/Nop58

For peptide microsequence analysis, a fraction enriched in the importin α-binding proteins p60 and p66 was obtained from rat liver NE: 300 OD_{280} of NE were resuspended in 10 mM HEPES, pH 7.4, 0.1 mM $MgCl_2$, 500 mM NaCl, 1 mM dithiothreitol (DTT), 10% sucrose, and incubated for 5 min on ice. The suspension was underlaid with 0.5× volume of 10 mM HEPES, pH 7.4, 0.1 mM $MgCl_2$, 1 mM DTT, 30% sucrose, and centrifuged at 6,500 × *g* for 20 min at 4 °C. The resultant supernatant was diluted tenfold with Buffer Y (50 mM HEPES, pH 7.4, 5 mM $MgCl_2$, 1 mM DTT, 5 μg/mL each of aprotinin, leupeptin, aprotinin) and incubated with hydroxyapatite resin (Macroprep Type I: 40 μM, BioRad Laboratories, Hercules, California) in batch for 1 h at 4 °C. The resin was washed once in batch with Buffer Y + 300 mM potassium phosphate (pH 7.4) and eluted with Buffer Y + 600 mM potassium phosphate (pH 7.4). p60 and p66 were followed throughout the purification procedure by using blot overlay assay with importin as a detection method.

The eluted fraction, enriched in p60 and p66, was electrophoresed on a 7.5% SDS-polyacrylamide gel and then transferred to Immobilon PVDF membrane (Millipore, Bedford, Massachusetts). The membrane was stained with 0.1% Ponceau S in 1% acetic acid, destained with 1% acetic acid, and the bands corresponding to p60 and p66 were excised (estimated yield ~4–5 μg). Tryptic digestion and subsequent analysis to obtain peptide microsequence were performed by Dr. John Leszyk at the University of Massachusetts Medical School (Shrewsbury, Massachusetts).

The resultant microsequence showed that p60 was equivalent to a known protein, Nap57 (Meier & Blobel, 1994), and that p66 showed similarity to three fragments in the EST database; one from mouse and two from human (accession numbers AA437736, U56656, and AA148805; respectively). The nucleotide sequence of the two human EST fragments overlapped for ~220 bp and the sequence of each of the two fragments within this region of overlap was essentially identical to the other. This 220-bp region was used as a template to design primers to use for the PCR-based cDNA cloning of the human homolog of p66. Upstream and downstream primers derived from the human EST sequences were used in PCR reactions utilizing 3′ and 5′ RACE methodology to screen a HeLa S3 cDNA library (Marathon-Ready cDNA, Clontech Laboratories Inc.). Potential clones were analyzed by sequencing, and we ultimately obtained a full-length ORF that included 224 bp of 3′ UTR followed by a poly A tail.

Antibody production

Polyclonal antibodies were raised against peptides derived from the N-terminal and C-terminal regions of hNop5/Nop58: KKLQEVDSLWKEFETPEK (residues 20–37) and KYEHK SEVKTYDPSG (residues 418–432). Both peptides (synthesized by Research Genetics Inc.) contained an added N-terminal cysteine to facilitate chemical coupling. The N- and C-terminal peptides were cross-linked to bovine serum albumin (BSA) with sulfo-SMCC (succinimidyl 4-(*N*-meleimidomethyl) cyclohexane-1-carboxylate; Pierce) and the resulting conjugates were used to immunize rabbits. Affinity columns were prepared by conjugating the N- and C-terminal peptides to Sulfo-Link resin (Pierce) and antibodies were purified according to standard procedures.

Immunofluorescence microscopy

HeLa cells were grown to ~60% confluency and then fixed with 4% formaldehyde and permeabilized with 0.2% Triton. Fixed cells were blocked with 2% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 15 min at room temperature and incubated with affinity-purified N- or C-terminal hNop5/Nop58 antibodies and with human anti-fibrillarin (346, kindly provided by Dr. Michael Pollard, The Scripps Research Institute) in PBS + 2% BSA for 1 h at room temperature. Cells were washed with PBS and then incubated with fluorescein isothiocyanate- (FITC) conjugated goat anti-rabbit antibody and Texas red-conjugated donkey anti-human antibody (Jackson Immunoresearch Laboratories Inc., West Grove, Pennsylvania) in PBS + 2% BSA for 30 min at room temperature. Cells were examined with a BioRad MRC 1024 confocal microscope and images were digitally recorded.

Immunoprecipitations

Immunoprecipitations were carried out as described (Steitz, 1988). HeLa cell sonic extract from 1×10^6 cells was used for each immunoprecipitation. The antibodies used were: anti-fibrillarin mouse monoclonal (72B9; 100 μL of culture supernatant; Reimer et al., 1987) and anti-C terminal hNop5/Nop58 rabbit polyclonal (300 μg of affinity purified antibody). For Figures 3A and 3C, immunoprecipitations were carried out in NET-2 (150 mM NaCl, 50 mM Tris-Cl, pH 7.4, and 0.05% NP-40). For Figure 3B, immunoprecipitations were carried out in IPP (500 mM NaCl, 10 mM Tris-Cl, pH 8.0, and 0.1% NP-40). The resulting bound RNAs were isolated by extraction with phenol:chloroform:isoamyl alcohol and ethanol precipitation. The coimmunoprecipitated RNAs were then 3′-end labeled with ^{32}pCp and T4 RNA ligase and analyzed by denaturing gel electrophoresis (England et al., 1980). North-

ern blots were performed as described in Dunbar et al. (1997) with riboprobes to human U3, U1, and U8.

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REFERENCES

Balakin AG, Smith L, Fournier MJ. 1996. The RNA world of the nucleolus: Two major families of small RNAs defined by different box elements with related functions. *Cell 86*:823–834.

Baserga SJ, Gilmore-Hebert M, Yang XW. 1992. Distinct molecular signals for nuclear import of the nucleolar snRNA, U3. *Genes & Dev 6*:1120-1130.

Baserga SJ, Yang XW, Steitz JA. 1991. An intact Box C sequence is required for binding of fibrillarin, the protein common to the major family of nucleolar snRNPs. *EMBO J 10*:2645–2651.

Caffarelli E, Fatica A, Prislei S, De Gregorio E, Fragapane P, Bozzoni I. 1996. Processing of the intron-encoded U16 and U18 snoRNAs: The conserved C and D boxes control both the processing reaction and the stability of the mature snoRNA. *EMBO J 15*:1121–1131.

Caffarelli E, Losito M, Giorgi C, Fatica A, Bozzoni I. 1998. In vivo identification of nuclear factors interacting with the conserved elements of box C/D small nucleolar RNAs. *Mol Cell Biol 18*:1023–1028.

Cavaille J, Nicoloso M, Bachellerie JP. 1996. Targeted ribose methylation of RNA in vivo directed by tailored antisense RNA guides [see comments]. *Nature 383*:732–735.

Dunbar DA, Wormsley S, Agentis TM, Baserga SJ. 1997. Mpp10p, a U3 small nucleolar ribonucleoprotein component required for pre-18S rRNA processing in yeast. *Mol Cell Biol 17*:5803–5812.

England TE, Bruce AG, Uhlenbeck OC. 1980. Specific labeling of 3′ termini of RNA with T4 RNA ligase. *Methods Enzymol 65*:65–74.

Gautier T, Berges T, Tollervey D, Hurt E. 1997. Nucleolar KKE/D repeat proteins Nop56p and Nop58p interact with Nop1p and are required for ribosome biogenesis. *Mol Cell Biol 17*:7088–7098.

Huang GM, Jarmolowski A, Struck JC, Fournier MJ. 1992. Accumulation of U14 small nuclear RNA in *Saccharomyces cerevisiae* requires box C, box D, and a 5′, 3′ terminal stem. *Mol Cell Biol 12*:4456–4463.

Kiss-Laszlo Z, Henry Y, Bachellerie JP, Caizergues-Ferrer M, Kiss T. 1996. Site-specific ribose methylation of preribosomal RNA: A novel function for small nucleolar RNAs. *Cell 85*:1077–1088.

Kiss-Laszlo Z, Henry Y, Kiss T. 1998. Sequence and structural elements of methylation guide snoRNAs essential for site-specific ribose methylation of pre-rRNA. *EMBO J 17*:797–807.

Lafontaine DLJ, Tollervey D. 1998. Birth of the snoRNPs: The evolution of modification guide snoRNAs. *TIBS 23*:384–388.

Lafontaine DLJ, Tollervey D. 1999. Nop58p is a common component of the box C+D snoRNPs that is required for snoRNA stability. *RNA 5*:455–467.

Lange TS, Borovjagin A, Maxwell ES, Gerbi SA. 1998a. Conserved boxes C and D are essential nucleolar localization elements of U14 and U8 snoRNAs. *EMBO J 17*:3176–3187.

Lange TS, Ezrokhi M, Borovjagin AV, Rivera-Leon R, North MT, Gerbi SA. 1998b. Nucleolar localization elements of *Xenopus laevis* U3 small nucleolar RNA. *Mol Biol Cell 9*:2973–2985.

Lee SJ, Baserga SJ. 1999. Imp3p and Imp4p: Two specific components of the U3 small nucleolar ribonucleoprotein that are essential for pre-18S rRNA processing. *Mol Cell Biol 19*:5441–5452.

Lischwe MA, Ochs RL, Reddy R, Cook RG, Yeoman LC, Tan EM, Reichlin M, Busch H. 1985. Purification and partial characterization of a nucleolar scleroderma antigen (M_r = 34,000; pI, 8.5) rich in N^G, N^G-dimethylarginine. *J Biol Chem 260*:14304–14310.

Meier UT, Blobel G. 1994. Nap57, a mammalian nucleolar protein with a putative homolog in yeast and bacteria. *J Cell Biol 127*:1505–1514.

Morin JP, Downs JA, Snodgrass AM, Gilmore TD. 1995. Genetic analysis of growth inhibition by GAL4-IκB-α in *Saccharomyces cerevisiae*. *Cell Growth Differ 6*:789–798.

Nicoloso M, Qu LH, Michot B, Bachellerie JP. 1996. Intron-encoded, antisense small nucleolar RNAs: The characterization of nine novel species points to their direct role as guides for the 2′-*O*-ribose methylation of rRNAs. *J Mol Biol 260*:178–195.

Peculis BA, Steitz JA. 1994. Sequence and structural elements critical for U8 snRNP function in *Xenopus* oocytes are evolutionarily conserved. *Genes & Dev 8*:2241–2255.

Reimer G, Pollard KM, Penning CA, Ochs RL, Lischwe MA, Busch H, Tan EM. 1987. Monoclonal antibody from a (New Zealand black × New Zealand white) F1 mouse and some human scleroderma sera target an Mr 34,000 nucleolar protein of the U3 RNP particle. *Arth Rheum 30*:793–800.

Samarsky DA, Fournier MJ, Singer RH, Bertrand E. 1998. The snoRNA box C/D motif directs nucleolar targeting and also couples snoRNA synthesis and localization. *EMBO J 17*:3747–3757.

Schimmang T, Tollervey D, Kern H, Frank R, Hurt EC. 1989. A yeast nucleolar protein related to mammalian fibrillarin is associated with small nucleolar RNA and is essential for viability. *EMBO J 8*:4015–4024.

Steitz JA. 1988. Immunoprecipitations of ribonucleoproteins using autoantibodies. *Methods Enzymol 180*:468–481.

Terns MP, Dahlberg JE. 1994. Retention and 5′ cap trimethylation of U3 snRNA in the nucleus. *Science 264*:959–961.

Tyc K, Steitz JA. 1989. U3, U8, and U13 comprise a new class of mammalian snRNPs localized to the cell nucleolus. *EMBO J 8*:3113–3119.

Tycowski KT, Smith CM, Shu MD, Steitz JA. 1996. A small nucleolar RNA requirement for site-specific ribose methylation of rRNA in *Xenopus*. *Proc Natl Acad Sci USA 93*:14480–14485.

Watkins NJ, Leverette RD, Xia L, Andrews MT, Maxwell ES. 1996. Elements essential for processing intronic U14 snoRNA are located at the termini of the mature snoRNA sequence and include the conserved nucleotide boxes C and D. *RNA 2*:118–133.

Watkins NJ, Newman DR, Kuhn JF, Maxwell ES. 1998. In vitro assembly of the mouse U14 snoRNP core complex and identification of a 65-kDa box C/D-binding protein. *RNA 4*:582–93.

Westendorf JM, Konstantinov KN, Wormsley S, Shu MD, Matsumoto-Taniura N, Pirollet F, Klier FG, Gerace L, Baserga SJ. 1998. M phase phosphoprotein 10 is a human U3 small nucleolar ribonucleoprotein component. *Mol Biol Cell 9*:437–449.

Wu P, Brockenbrough JS, Metcalfe AC, Chen S, Aris JP. 1998. Nop5p is a small nucleolar ribonucleoprotein component required for pre-18 S rRNA processing in yeast. *J Biol Chem 273*:16453–16463.