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# Proteomic Analysis of Human Nop56p-associated Pre-ribosomal Ribonucleoprotein Complexes

## POSSIBLE LINK BETWEEN Nop56p AND THE NUCLEOLAR PROTEIN TREACLE RESPONSIBLE FOR TREACHER COLLINS SYNDROME*[S]

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**Nop56p is a component of the box C/D small nucleolar ribonucleoprotein complexes that direct 2′-*O*-methylation of pre-rRNA during its maturation. Genetic analyses in yeast have shown that Nop56p plays important roles in the early steps of pre-rRNA processing. However, its precise function remains elusive, especially in higher eukaryotes. Here we describe the proteomic characterization of human Nop56p (hNop56p)-associated pre-ribosomal ribonucleoprotein complexes. Mass spectrometric analysis of purified pre-ribosomal ribonucleoprotein complexes identified 61 ribosomal proteins, 16 trans-acting factors probably involved in ribosome biogenesis, and 29 proteins whose function in ribosome biogenesis is unknown. Identification of pre-rRNA species within hNop56p-associated pre-ribosomal ribonucleoprotein complexes, coupled with the known functions of yeast orthologs of the probable trans-acting factors identified in human, demonstrated that hNop56p functions in the early to middle stages of 60 S subunit synthesis in human cells. Interestingly, the nucleolar phosphoprotein treacle, which is responsible for the craniofacial disorder associated with Treacher Collins syndrome, was found to be a constituent of hNop56p-associated pre-rRNP complexes. The association of hNop56p and treacle within the complexes was independent of rRNA integrity, indicating a direct interaction. In addition, the protein compositions of the treacle-associated and hNop56p-associated pre-ribosomal ribonucleoprotein complexes were very similar, suggesting functional similarities between these two complexes with respect to ribosome biogenesis in human cells.**

The ribosome constitutes one of the most fundamental molecular machines in living cells. Given that protein synthesis is essential for cell growth, proliferation, and adaptation, ribosome biogenesis is intimately coupled to the needs of the cell. Ribosome biogenesis is efficiently coordinated in the nucleolus, a subnuclear compartment in eukaryotic cells. Each mature ribosome in the cytoplasm consists of the large subunit (60 S) and the small subunit (40 S) that together comprise over 80 ribosomal proteins organized within and around mature rRNAs. In mammalian cells, the large subunit is composed of ~50 ribosomal proteins and three species of rRNA (28 S, 5.8 S, and 5 S), whereas the small subunit consists of ~30 ribosomal proteins and 18 S rRNA. During ribosome biogenesis, ribosomal DNA for 5.8 S, 18 S, and 28 S rRNAs is transcribed by RNA polymerase I into a large primary precursor (47 S) containing 5′- and 3′-external transcribed spacers (5′-ETS[1] and 3′-ETS) and two internal transcribed spacers (ITS1 and ITS2) (1, 2). Concomitant with the methylation and pseudouridylation of ribose moieties, the 47 S precursor is cleaved at specific sites to produce a series of characteristic intermediates that ultimately result in mature 5.8 S, 18 S, and 28 S rRNAs. Pre-5 S rRNA is transcribed by RNA polymerase III and processed independently of the other three rRNAs (3).

During ribosome biogenesis, a multitude of small nucleolar RNAs (snoRNAs) and trans-acting proteins, neither of which are contained in mature ribosomes, form pre-ribosomal RNP (pre-rRNP) complexes and play crucial roles in the processing and modification of pre-rRNAs as well as the assembly of rRNAs with ribosomal proteins (4–7). Genetic analyses in yeast over the past 3 decades have identified a number of the trans-acting proteins that are involved in ribosome biogenesis, and their ordered association with and dissociation from pre-rRNP complexes have been outlined (5–7). However, the precise functions of these trans-acting factors remain obscure. Furthermore, our knowledge of the function of these factors in mammalian cells has been greatly limited by difficulty in identifying mammalian rRNP components because of limited sample availability and the inability to conduct routine genetic analyses.

Proteomic methodology has been facilitated by advances in tag-based purification methods for protein complexes as well as

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[S] The on-line version of this article (available at http://www.jbc.org) contains Tables I–III and Fig. 1.

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[1] The abbreviations used are: ETS, external transcribed spacer; DDX, DEA(D/H) (Asp-Glu-Ala-Asp/His) box polypeptide; hnRNP, heterogeneous nuclear ribonucleoprotein; ITS, internal transcribed spacer; LC, liquid chromatography; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight; MS, mass spectrometer; PBS, phosphate-buffered saline; pre-rRNP, pre-ribosomal ribonucleoprotein; RNP, ribonucleoprotein; snoRNA, small nucleolar RNA; snoRNP, small nucleolar ribonucleoprotein; SRP, signal recognition particle; TCS, Treacher Collins syndrome; SSU, small subunit.

the development of highly sensitive mass spectrometric techniques and search engines that access huge amounts of genomic data. The advent of proteomic analyses allows the characterization of large complexes consisting of >100 proteins (8, 9). In fact, proteomic analysis of yeast pre-rRNP complexes has proven highly fruitful for the characterization of trans-acting factors involved in ribosome biogenesis (10–13). We recently purified and characterized the nucleolin- and the parvulin-associated pre-rRNP complexes, both of which are involved in human ribosome biogenesis (14, 15). These studies clearly demonstrated the applicability of proteomic analysis to the study of human ribosome biogenesis, and also identified a number of mammalian counterparts of yeast trans-acting factors as well as additional trans-acting factors that had not been previously identified in yeast.

Here we present a proteomic analysis of Nop56p-associated pre-rRNP complexes in human cells. Nop56p is a member of the core proteins of box C/D snoRNP complexes (16, 17) that direct 2′-*O*-methylation of pre-rRNA ribose moieties during the early stages of pre-rRNA processing (16, 18–23). The box C/D snoRNAs are characterized by their conserved box C (RUGAUGA) and box D (CUGA) motifs that are essential for function (18, 24–27). In addition to Nop56p, the core proteins Nop58p, Nop1p (fibrillarin), and Snu13p (a 15-kDa protein in human) form the mature box C/D snoRNP complex. The box C/D snoRNP complex has been reconstituted *in vitro*, and fibrillarin is responsible for its methylation activity (21). Mouse Nop56p is 36% identical and 58% similar to Nop58p at the amino acid level (17). Because only a single ortholog of Nop56p/Nop58p has been identified in Archaea, these two proteins most likely diverged from the archaeal protein (17). Genetic analysis of yeast Nop56p indicates that it is responsible for cleaving 35 S pre-rRNA (the primary precursor in yeast) at sites $A_0$, $A_1$, and $A_2$ to produce 25 S rRNA (the mature rRNA corresponding to human 28 S rRNA) (16). The C-terminal region of yeast Nop56p contains repeated KK(E/D) motifs (16) that are also present in the yeast pseudouridine synthetase Cbf5p (22), and a similar motif (KK*X*) is found in the yeast putative ATP-dependent RNA helicase Dbp3p (27). However, the KK(E/D) motifs are not essential for Nop56p function in yeast, and therefore the relevance of this motif to Nop56p activity is not clear. Human Nop56p (hNop56p) lacks these specific motifs but contains a stretch of highly basic residues within its C-terminal region (16, 17). Although Nop56p is known to play a significant role as a protein trans-acting factor in ribosome biogenesis in yeast, the precise function of this protein remains unknown, especially in mammalian cells (16). Our present data demonstrate that, within hNop56p-associated pre-rRNPs, hNop56p interacts directly with treacle, the Treacher Collins Syndrome (TCS) gene product.

## EXPERIMENTAL PROCEDURES

*Materials*—Human kidney cell line 293EBNA, Opti-MEM, and LipofectAMINE were obtained from Invitrogen. Dulbecco's modified Eagle's medium (DMEM), anti-FLAG M2 affinity gel, FLAG peptide, IGEPAL CA-630, RNase A, and α-cyano-4-hydroxycinnamic acid were from Sigma. Hybond $N^+$ membranes, alkaline phosphatase-conjugated anti-mouse IgG, and [γ-$^{32}$P]ATP (3000 Ci/mmol) were from Amersham Biosciences. Alexa Fluor 488-conjugated rabbit anti-mouse IgG was from Molecular Probes, Inc. (Eugene, OR). Trypsin (sequence grade) was from Promega (Madison, WI) and *Achromobacter lyticus* protease I was from Wako Pure Chemicals (Osaka, Japan). ZipTipC18 was from Millipore (Bedford, MA). LATaq DNA polymerase was from Takara (Shiga, Japan). All other reagents were purchased from Wako Pure Chemicals.

*Epitope-tagged hNop56p and Treacle Vectors and Expression in 293EBNA Cells*—A DNA fragment encoding hNop56p with the FLAG tag (DYKDDDDK) at its N terminus was amplified by PCR using a human placenta cDNA library template (OriGene Technologies, Inc., Rockville, MD) with the primers 5′-ATATAGCTAGCGCCACCATGGACTACAAGGACGACGACGACAAGGCGGGACGTGGCGCCATGGTG-3′ and 5′-TATATGGATCCTTATTTTCTTCCTTCGACCAT-3′. For treacle, a DNA fragment encoding human treacle with the FLAG tag at its C terminus was amplified as above using the primer set 5′-TATAGCTAGCGCCACCATGGCCGAGGCCAGGAAGCGGCGG-3′ and 5′-TATAGGATCCTCACTTGTCGTCGTCGTCCTTGTAGTCTACAGTCTGCTCTGCTGTCTTC-3′. The hNop56p and treacle fragments were cut with the *Nhe*I and *Bam*HI, respectively, and ligated into the corresponding restriction sites of the expression vector pcDNA3.1(+) (Invitrogen).

Human 293EBNA cells were grown in DMEM supplemented with 10% heat-inactivated fetal calf serum, streptomycin (0.1 μg/ml), and penicillin G (100 units/ml) at 37 °C in an incubator with an atmosphere of 5% $CO_2$. Subconfluent cells in 90-mm dishes were transfected individually with 10 μg of the appropriate expression plasmid using LipofectAMINE according to the manufacturer's instructions, and the transfected cells were grown for 48 h at 37 °C.

*Isolation of hNop56p- and Treacle-associated Complexes*—At 48 h post-transfection, 293EBNA cells were harvested and washed with PBS and then lysed in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% IGEPAL CA630, 1 mM phenylmethanesulfonyl fluoride) on ice for 30 min. The soluble fraction was obtained by centrifugation at 15,000 rpm for 30 min at 4 °C and was incubated with 20 μl of anti-FLAG M2-agarose with gentle mixing overnight at 4 °C to immunoprecipitate hNop56p- or treacle-associated complexes. After washing the agarose five times with lysis buffer and then once with 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, the bound complexes were eluted with 20 μl of 50 mM Tris-HCl, pH 7.5, 150 mM NaCl containing 500 μg/ml of the FLAG peptide. The isolated complexes were analyzed by SDS-PAGE on 12.5% gels.

*Ribonuclease Treatment of hNop56p-associated Complexes*—Immunoprecipitated hNop56p-associated complexes (bound to anti-FLAG M2-agarose) were incubated in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl containing 10 μg/ml RNase A for 10 min at 37 °C, washed twice with lysis buffer and once with 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and eluted with the FLAG peptide in the same manner described above.

*Immunocytochemistry*—293EBNA cells were grown on 8-well culture slides (Biocoat, BD Biosciences). Subconfluent cells were transfected with the expression plasmids using LipofectAMINE as above. Prior to fixation, the cells were washed with PBS, followed by incubation with 3.7% formaldehyde in PBS. After several washes with PBS-T (PBS containing 0.05% (w/v) Tween 20), the cells were incubated with PBS containing 0.1% (w/v) Triton X-100 for 5 min at room temperature. The cells were then blocked using 3% (w/v) non-fat dried milk in PBS and were incubated with anti-FLAG for 1 h at room temperature. The cells were washed with PBS-T and then incubated with Alexa Fluor 488-conjugated anti-mouse IgG for 1 h at room temperature, followed by three washes with PBS-T. The cells were then examined with a FluoView confocal laser-scanning microscope (Olympus, Tokyo, Japan).

*Northern Blotting*—RNAs were isolated from hNop56p-associated complexes using the Micro-to-Midi total RNA purification system (Invitrogen). Total RNA was subjected to electrophoresis on a 1.0% agarose gel that was transferred to Hybond $N^+$ nylon membranes as described (28). The membranes were blocked with prehybridization solution consisting of 6× SSC (diluted from 20× SSC: 3 M NaCl, 0.3 M sodium citrate), 5× Denhardt's solution (diluted from 50× Denhardt's solution: 1% Ficoll 400, 1% polyvinylpyrrolidone, 1% bovine serum albumin), 0.5% SDS, 100 μg/ml denatured/fragmented salmon sperm DNA for 3 h at 60 °C. Oligonucleotide probes were designed to hybridize to ribonucleotides 221–250 (5′-ETS), 4251–4280 (18 S), 6741–6770 (5.8 S), 7161–7190 (ITS2), and 9411–9440 (28 S), respectively, of the human 47 S rRNA precursor. Each probe was labeled at the 5′-end using MEGALABEL (Takara) and [γ-$^{32}$P]ATP (3000 Ci/μmol). Each RNA blot was incubated with prehybridization solution containing a single radiolabeled probe (~$1 \times 10^6$ cpm) overnight at 50 °C. The blots were washed twice with 2× SSC at room temperature for 15 min and once with 2× SSC containing 0.1% SDS for 30 min at 50 °C. The hybridized blots were exposed to a PhosphorScreen and analyzed by STORM (Amersham Biosciences).

*Protein Identification by Peptide Mass Fingerprinting*—SDS-PAGE gel fragments containing proteins were cut out and subjected to in-gel protease digestion as described (29). Peptides generated by the digestion were recovered and analyzed for peptide mass fingerprint using a PE Biosystems MALDI-TOF/MS (Voyager DE-STR) as described (29). Peptide masses were searched with 50 ppm mass accuracy using the data base fitting program MS-Fit (prospector.ucsf.edu), and protein identification was carried out using the criteria described previously (15).

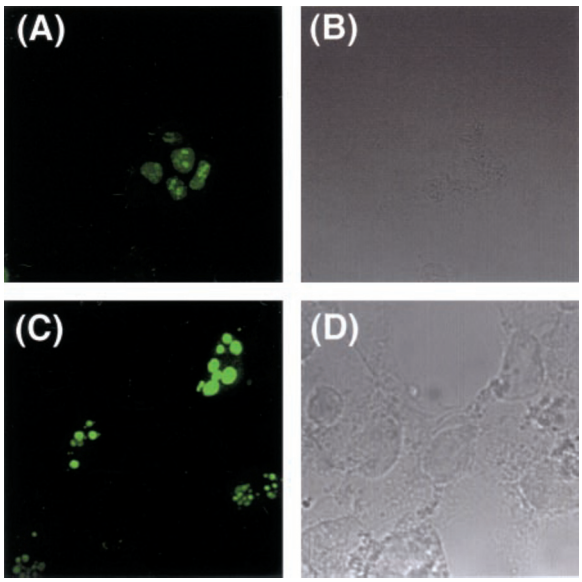


FIG. 1. **Cellular localization of FLAG-tagged-hNop56p and -treacle in 293EBNA cells.** Cells were transfected with FLAG-tagged hNop56p (*A*) or FLAG-tagged treacle (*C*) and stained with an antibody against FLAG and an Alexa Fluor 488-conjugated secondary antibody. *B* and *D* show transmitted images corresponding to *A* and *C*, respectively.

*Protein Identification by LC-MS/MS Analysis*—hNop56p-associated complexes were digested with proteases, and the resulting peptides were analyzed by an LC-MS/MS system at the femtomole level as described (30). The peptide mixture was separated on a frit-less Mightysil-C18 (3-$\mu$m particle, Kanto Chemical, Osaka Japan) column (45 $\times$ 0.150 mm inner diameter) using a gradient of acetonitrile (0–40%) in 0.1% formic acid over 80 min at a flow rate of either 50 or 25 nl/min by direct nano-LC system (Nanosolution, Tokyo, Japan). The eluted peptides were sprayed directly into a quadrupole time-of-flight hybrid mass spectrometer (Q-Tof2, Micromass, Manchester, UK). MS/MS spectra were acquired by data-dependent collision-induced dissociation, and the MS/MS data were analyzed using MASCOT software (Matrix Science, Wyndham Place, UK) for peptide assignment.

## RESULTS

*Purification of hNop56p-associated RNPs*—To purify hNop56p-associated complexes, we constructed an expression plasmid encoding hNop56p fused to the FLAG epitope (FLAG-hNop56p). In 293EBNA cells, transiently overexpressed FLAG-hNop56p localized primarily to the nucleolus (Fig. 1*A*). hNop56p-associated complexes that formed in FLAG-hNop56p-transfected cells were purified via immunoprecipitation using anti-FLAG-conjugated beads. SDS-PAGE indicated that the hNop56p-associated complexes contained more than 50 proteins spanning a broad molecular weight range (Fig. 2, *lane 2*). Of these, four proteins were also immunoprecipitated from non-transfected control cells (Fig. 2, *lane 4*) thus reflecting nonspecific binding. RNase treatment of the complexes while bound to the immunoprecipitating beads resulted in dissociation of almost all protein constituents except for FLAG-hNop56p and its degradation products (Fig. 2, *lane 3*). These results indicated that the purified complexes were ribonucleoproteins (RNPs) and that the association of the protein components was RNA-dependent.

*Protein Components of hNop56p-associated RNPs*—The protein components of the immuno-isolated hNop56p-associated RNP complexes were identified by mass spectrometry. Protein identification was first carried out for individual bands excised from SDS-PAGE gels using in-gel digestion and MALDI-TOF/MS. This analysis yielded 35 ribosomal proteins (29 large subunit proteins and 6 small subunit proteins) and 17 non-ribosomal proteins (Fig. 3,and Supplemental Material Tables I–III).

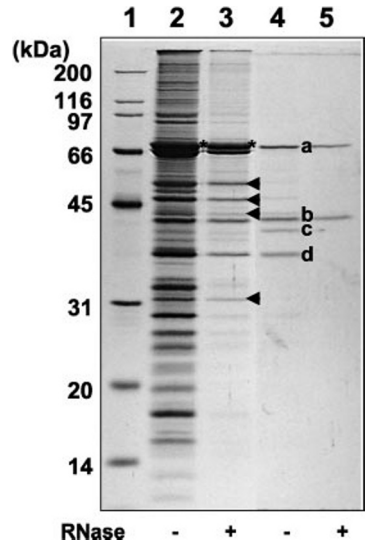


FIG. 2. **Isolation of hNop56p-associated complexes and RNA dependence of protein binding.** hNop56p-associated complexes were isolated by immunoprecipitation from 293EBNA cells transfected with the hNop56p construct. The immuno-isolated complexes were resolved by SDS-PAGE on a 12.5% gel followed by silver staining (14). Untransfected 293EBNA cells were used in control experiments. RNA dependence of protein association was investigated by RNase A treatment. *Lane 1*, molecular mass markers; *lanes 2* and *3*, the hNop56p-associated complexes without and with the RNase treatment; *lanes 4* and *5*, control without and with RNase treatment. *Asterisks* indicate FLAG-tagged hNop56p. The nonspecific binding of four proteins to anti-FLAG M2-agarose beads is shown in the control experiment (*lane 4*). These proteins were identified as SKB1-homolog (NM_006109; gi:20070219) (*a*), MEP50 protein (NM_024102; gi:20127622) (*b*), phosphoribosylpyrophosphate synthetase-associated protein 2 (NM_002767; gi:2253848) (*c*), and phosphoribosylpyrophosphate synthetase 1 (NM_002764; gi:19923737) (*d*), respectively, by MALDI-TOF/MS. hNop56p degradation products are indicated by *arrowheads*.

We described previously a highly sensitive direct nano-flow LC-MS/MS system to identify proteins in limited quantities of multiprotein complexes (30). Therefore, intact immuno-isolated hNop56p-associated RNPs were digested with *A. lyticus* protease I, and the digest was analyzed directly by nano-LC-MS/MS. More than 2,500 MS/MS spectra were obtained, from which ~500 peptides were assigned to 104 proteins using the Mascot search software. Of these, 61 were ribosomal proteins (42 large subunit proteins and 19 small subunit proteins) and 43 were non-ribosomal proteins (Tables I and Table II, and Supplemental Material Tables I–III). This LC-MS/MS analysis identified all the proteins documented by the electrophoresis-based analyses except three. A total of 107 proteins were identified in the hNop56p-associated RNP complex using the electrophoresis- and LC-based analyses. Of these, 62 were ribosomal proteins and 45 were non-ribosomal proteins (Tables I and II, and Supplemental Material Tables I–III).

A functional comparison of the 45 non-ribosomal proteins with yeast orthologs revealed that 16 proteins represent putative trans-acting factors involved in ribosome biogenesis (Table I). These proteins were classified into three functional groups, namely box C/D snoRNP proteins, RNA helicases, and other proteins. Together with Nop56p, fibrillarin, Nop5/Nop58p, and non-histone chromosome protein 2-like 1 (equivalent to a 15.5-kDa protein) form the core of box C/D snoRNPs that direct 2′-*O*-methylation of pre-rRNAs at specific sites. One of the box C/D snoRNPs, U3, processes yeast pre-rRNAs at the $A_0$, $A_1$, and $A_2$ sites (16, 18–20). Two RNA helicases, DEA(D/H) polypeptide box 9 (DDX9) and DEA(D/H) polypeptide box 21 (DDX21), were identified in the hNop56p-associated RNP. The RNA helicases of the DDX and related families belong to the

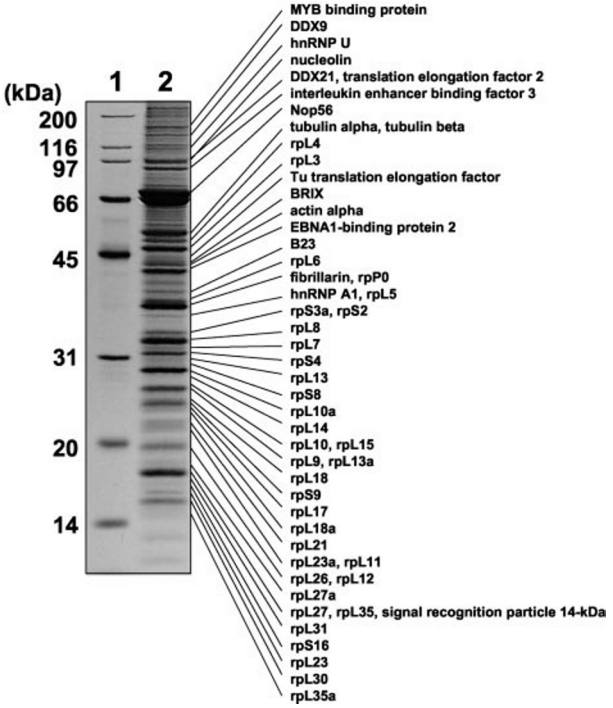


FIG. 3. **MALDI-TOF mass spectrometry of protein components involved in hNop56p-associated complexes.** Protein components of hNop56p-associated complexes were resolved by SDS-PAGE as described in Fig. 2. Each protein band was excised, subjected to in-gel protease digestion, and analyzed by the MALDI-TOF/MS. The resulting peptide mass fingerprints were matched to a data base, and the identity of each protein is shown to the *right* of the gel. *Lane 1*, molecular weight markers; *lane 2*, proteins of the hNop56p-associated complexes.

largest class of trans-acting factors involved in ribosome biogenesis in yeast (7). The hypothetical protein FLJ10377, peter pan homolog, EBNA1-binding protein 2, BRIX, DKFZP564M182 protein, and a putative nucleotide-binding protein (estradiol-induced) have not been reported to function in ribosome biogenesis in mammals although their yeast counterparts are constituents of pre-ribosomal particles (Table I). The roles of nucleolin and B23 in pre-rRNA processing are partly established in mammals. Nucleolin plays multiple roles in early processing of rRNAs and in the packaging of nascent rRNAs (31–34). B23 is involved in the late stage of rRNA processing (35). Nucleolar and coiled-body phosphoprotein 1, designated Nopp130 in human and Nopp140 in mouse, associates with snoRNPs required for rRNA modification and processing and appears to function as a chaperone for ribosome biogenesis and intranuclear transport (36, 37). Heterogeneous nuclear ribonucleoprotein U (hnRNP U) is implicated in RNA metabolism because it interacts with other protein components of hnRNP complexes (38) as well as the C-terminal domain of RNA polymerase II (39). Its yeast ortholog, Nop3p, is an essential nucleolar protein, and its depletion leads to pre-rRNA processing defects at the middle stage of 60 S ribosome subunit biogenesis (40). The identification of these putative trans-acting factors strongly suggested that the hNop56p-associated RNP is involved in ribosome biogenesis.

In addition to the 16 trans-acting factors, 29 non-ribosomal proteins that are not known to function in ribosome biogenesis were identified in hNop56p-associated RNPs. Among these, at least 21 proteins exhibited nucleolar and/or nuclear localization (Table II), suggesting that they may indeed be involved in ribosome biogenesis.

*Pre-rRNA Species in hNop56p-associated RNPs*—Given that our proteomic analysis indicated that hNop56p-associated RNPs might participate in ribosome biogenesis, the RNP complexes were examined for the appropriate rRNA species. For this purpose, RNAs were isolated from the complexes and subjected to Northern blot analysis using oligonucleotide probes corresponding to the 5′-ETS, 18 S and 5.8 S, ITS2, and 28 S regions of 47 S pre-rRNA (Fig. 4*A*). The pre-rRNA species 47 S, 45 S, 41 S, 32 S, 30 S, and 26 S were identified (Fig. 4*B*). Because the hNop56p-associated RNPs also contained 16 possible trans-acting factors involved in ribosome biogenesis, we concluded that this RNP constituted *de facto* pre-ribosomal particles (pre-rRNP complexes). In addition to the pre-rRNAs, the complexes also contained mature rRNAs (5.8 S, 18 S, and 28 S), whereas neither pre-rRNAs nor mature rRNAs were detected in the corresponding preparation isolated from untransfected 293EBNA cells (data not shown). This suggested that the mature rRNAs were endogenous components of the hNop56p-associated complexes. We reported previously that human parvulin-associated pre-rRNP complexes also contain mature rRNAs even though the complexes were purified from the nuclear fraction (15). Thus, the presence of the mature rRNAs in these complexes suggests that pre-rRNA maturation may occur within the nucleolus or nucleus *in vivo* and/or possibly during the purification of the complexes *in vitro*.

*hNop56p Interacts Directly with Treacle, the Treacher Collins Syndrome Gene Product*—No clear protein bands were observed on the SDS gel of the RNase-treated hNop56p-associated pre-rRNPs, except for those of FLAG-hNop56p and proteolytic fragments thereof (Fig. 2, *lane 3*). However, when the same preparation was analyzed directly by LC-MS/MS without gel separation, one particular protein, treacle, was identified specifically (*i.e.* seven peptide hits; see Supplemental Material Fig. 1). The two independent LC-MS/MS analyses also identified this protein in hNop56p-associated pre-rRNPs (14 and 18 peptide hits, respectively, as shown in Fig. 5 and Supplemental Material Fig. 1 as well as Table II and Supplemental Material Table III). Thus, treacle remained associated with hNop56p even after RNase treatment of the hNop56p-associated pre-rRNPs, suggesting that it binds directly to hNop56p in a manner that is independent of rRNA integrity. These data provided the first evidence of a physical and perhaps functional relationship between hNop56p and treacle, the Treacher Collins syndrome gene (*TCOF1*) product that is responsible for a craniofacial disorder (41–43). Treacle contains 1411 amino acids with a calculated molecular mass of 144,228 Da. However, this protein reportedly migrates as a diffuse smear at ~220 kDa during SDS-PAGE, due presumably to hyper-phosphorylation (44). Thus, the fact that treacle was not identified by our gel-based MALDI-TOF/MS analysis of the hNop56p-associated pre-rRNPs (Fig. 3) most likely reflects its anomalous migration during SDS-PAGE.

*Treacle-associated RNP Complexes Contain hNop56p*—Treacle is thought to belong to a class of nucleolar phosphoproteins on the basis of its amino acid sequence (42, 43) and its subcellular localization within the nucleolus (45). To confirm that this protein is indeed a component of hNop56p-associated pre-rRNPs, a reverse pull-down experiment was performed in which treacle-associated complexes were isolated from 293EBNA cells using FLAG-tagged treacle as bait. Exogenously expressed FLAG-tagged treacle localized to the nucleolus (Fig. 1, *C* and *D*) and migrated at ~220 kDa during SDS-PAGE (Fig. 6; identification by MALDI-TOF/MS analysis: 36 peptides matched; 27% peptide coverage; mean error = 1.68 ppm). These data suggested that FLAG-tagged treacle, like the endogenous protein (44), became phosphorylated when expressed in 293EBNA cells. The protein profiles of the treacle-associated and hNop56p-associated complexes were very simi-

TABLE I

*hNop56p-associated trans-acting factors involved in ribosome biogenesis*

The proteins are classified into functional groups, and NCBI accession numbers are given in parentheses. The known functions in mammals were extracted from the NCBI database. Yeast orthologs and their involvement in the pre-ribosomal complexes are shown (6).

| Protein | Known function in mammal | Yeast homologs assigned as protein trans-acting factors | Involvement in the pre-ribosomal complexes in yeast | Refs. |
|---|---|---|---|---|
| Box C/D snoRNP proteins | | | | |
| (NM_006392) nucleolar protein 5A (56 kDa with KKE/D repeat) | Similar to *S. cerevisiae* Sik1p, which is a nucleolar KK(E/D) repeat protein involved in pre-rRNA processing | Sik1/Nop56 | 90 S pre-ribosome | 16, 79 |
| (NM_015934) nucleolar protein NOP5/NOP58 | Putative snoRNA binding domain. This family consists of various pre-RNA processing ribonucleoproteins. The function of the aligned region is unknown; however, it may be a common RNA or snoRNA or Nop1p binding domain | Nop5/Nop58 | 90 S pre-ribosome | 80 |
| (XM_001436) fibrillarin | A component of a nucleolar small nuclear ribonucleoprotein (snRNP) particle thought to participate in the first step in processing preribosomal RNA. It is associated with the U3, U8, and U13 small nuclear RNAs and is located in the dense fibrillar component (DFC) of the nucleolus | Nop1 | 90 S pre-ribosome | 23, 81–83 |
| (NM_005008) non-histone chromosome protein 2-like 1 | Nucleolar protein that associates with the checkpoint protein RAD17; highly similar to *S. cerevisiae* Snu13p | Snu13 | 90 S pre-ribosome | 84, 85 |
| RNA helicases | | | | |
| (NM_001357) DEA(D/H) (Asp-Glu-Ala-Asp/His) box polypeptide 9 (RNA helicase A, nuclear DNA helicase II; leukophysin)(DDX9) | RNA helicase A; has both DNA and RNA helicase activity *in vitro;* member of the DEA(D/H) box ATP-dependent RNA and DNA helicase family | | | 84, 86 |
| (NM_004728) DEA(D/H) (Asp-Glu-Ala-Asp/His) box polypeptide 21 (DDX21) | Gu protein; member of the DEA(D/H) box ATP-dependent RNA helicase family | Dbp3 | | 87–89 |
| Others | | | | |
| (NM_018077) hypothetical protein FLJ10377 | Contains an RNA recognition motif (RRM, RBD, or RNP) | Nop4/Nop77 | Pre-60 S $E_1$ | 90, 91 |
| (NM_020230) peter pan homolog | Peter pan homolog; has weak similarity to *S. cerevisiae* splicing factor Ssf1 | Ssf1 | Pre-60 S $E_1$ | 92–94 |
| (NM_006824) EBNA1-binding protein 2 | Has multiple predicted transmembrane domains | Ebp2 | Pre-60 S $E_1$/pre-60 S $E_2$ | 95–97 |
| (NM_018321) BRIX | BRIX $\sigma$70-like motif-containing protein | Brx1 | Pre-60 S $E_1$/pre-60 S $E_2$ | 84 |
| (XM_085525) DKFZP564M182 protein | Unknown | Nsa3/Cic1 | Pre-60 S $E_2$/pre-60 S M | 92 |
| (NM_014366) putative nucleotide binding protein, estradiol-induced | Putative nucleotide binding protein, estradiol-induced protein | Nug 1 | Pre-60 S M | 84, 98 |
| (NM_005381) nucleolin | Nucleolar phosphoprotein and RNA-binding protein that participates in the assembly of ribosomes | Nsr1/She5 | | 50 |
| (NM_002520) nucleophosmin | Nucleophosmin (nucleolar phosphoprotein B23, numatrin, protein B23); RNA-binding nucleolar phosphoprotein | | | 108 |
| (NM_004741) nucleolar and coiled-body phospho-protein 1 (NOLC-1) | Nucleolar phosphoprotein; functions as a nucleolar phosphoprotein. Human counterpart of Nopp140 | Srp40 | | 99, 100 |
| (NM_031844) heterogeneous nuclear ribonucleoprotein U | Binds RNA and scaffold-attached region DNA; contains an RGG box domain; component of hnRNP complexes: may play a role in hnRNP structure or processing. | Nop3/Npl3/ Mts1/Mtr13 | | 40, 101–103 |

lar (Fig. 6). In addition, MALDI-TOF/MS analysis of treacle-associated complexes identified hNop56p (11 peptides matched; 21% peptide coverage; mean error = −8.61 ppm) as well as a number of components found in common with the hNop56-associated pre-rRNP, including hnRNP U (10 peptides matched; 16% peptide coverage; mean error = −6.73 ppm), nucleolin (21 peptides matched; 32% peptide coverage; mean error = −8.31 ppm), and B23 (nine peptides matched; 30% peptide coverage; mean error = −20.17 ppm) (Fig. 6). We also confirmed that the association of all these protein components with treacle-associated complexes required RNA integrity (using RNase as described for hNop56-associated pre-rRNPs; data not shown). Thus, it is likely that treacle, like hNop56p, is contained within an RNP complex and participates in certain stages of ribosome biogenesis in human cells.

## DISCUSSION

We purified hNop56p-associated complexes from 293EBNA cells via transfection with a FLAG-tagged hNop56p construct. hNop56p-associated complexes were shown to be pre-rRNPs

TABLE II

*Non-ribosomal protein hNop56p-associated trans-acting factors of unknown function in ribosome biogenesis*

NCBI accession numbers are given in parentheses. The known functions in mammals were extracted from the NCBI database. Yeast orthologs are shown in the 4th column.

| Protein | Known function in mammal | Cellular localization | Yeast homologs | Refs. |
|---|---|---|---|---|
| (NM_014520) MYB-binding protein (P160) 1a | Homolog of murine Mybb1a, which is a nucleolar protein that binds the leucine zipper motifs of MYB | Nucleolus[a] | | 109 |
| (NM_000356) Treacher Collins Franceschetti Syndrome 1 (TCOF1) | Treacle; similar to nucleolar trafficking proteins | Nucleolus[a]/cytoplasm | | 41, 42 |
| (NM_004516) interleukin enhancer binding factor 3, 90 kDa | Subunit of nuclear factor of activated T-cells (NF-AT); DNA-binding transcription factor | Nucleus | Rnq1 | 92, 110, 111 |
| (NM_001961) eukaryotic translation elongation factor 2 | Catalyzes the movement of mRNA relative to the ribosomes | Cytoplasm | Eft2 | 92, 112, 113 |
| (NM_003237) SFRS protein kinase 1 (SRPK1) | Protein kinase for Ser-and Arg-rich (SR) RNA splicing factor family; may act to control localization of splicing factors within nucleus | Nucleus | Sky1 | 92, 113, 114 |
| (NM_003286) topoisomerase (DNA) I | Catalyzes the transient breaking and rejoining of a single strand of DNA which allows the strands to pass through one another, thus altering the topology of DNA | Nucleolus[a]/nucleus | Top1 | 92, 113, 115 |
| (NM_017988) hypothetical protein FLJ10074 | May be a protein kinase domain; contains a eukaryotic protein kinase domain | Unknown | Scyt1 | 92, 113, 116 |
| (NM_005968) heterogeneous nuclear ribonucleoprotein M | *N*-Acetylglucosamine thyroid receptor; initiates recycling of immature thyroglobulin through the Golgi back to the apical membrane | Plasma membrane | Gbp2 | 92, 113, 117 |
| (NM_006546) IGF-II mRNA-binding protein 1 | Binds to the 5′-untranslated region of the IGF-II (IGF2) leader 3′ mRNA, may repress translation of IGF-II during late development; contains a KH domain | Cytoplasm | Pbp2 | 92, 113, 118 |
| (NM_007062) nuclear phosphoprotein similar to *S. cerevisiae* PWP1 | Member of the WD-40 family; similar to the *S. cerevisiae* PWP1, which is implicated in regulation of cell growth and transcription | Nucleolus[a]/nucleus | Pwp1 | 92, 113, 119 |
| (NM_007279) U2 small nuclear ribonucleoprotein auxiliary factor (65 Da); splicing factor U2AF 65 kDa | Large subunit of U2AF65 auxiliary factor of U2 snRNP; component of pre-mRNA splicing factor; contains RNA-binding and arginine/serine-rich motifs | Nucleolus[a]/nucleus | Rna15 | 92, 113, 120 |
| (NM_006009) tubulin $\alpha$3 | Polymerizes to form microlubules; member of a family of structural proteins | Microtubule | Tub1 | 92, 113, 121 |
| (NM_001402) eukaryotic translation elongation factor 1 $\alpha$1 | Functions in protein synthesis; has a guanine nucleotide-binding site | Nucleolus[a]/cytoplasm | Tef1 | 92, 113, 122 |
| (NM_030773) tubulin $\beta$1 | Polymerizes to form microtubules; member of a family of structural proteins | Nucleolus[a]/microtubule | Tub2 | 92, 113 |
| (NM_003321) Tu translation elongation factor, mitochondrial | Mitochondrial translation elongation factor Tu | Nucleolus[a]/mitochondrion | Tuf1 | 92, 113, 123 |
| (NM_016019) CGI-74 protein; CGI-59 protein | mRNA splicing | Unknown | Luc7 | 92, 113, 124 |
| (NM_004537) nucleosome assembly protein 1-like 1 | Similar to *S. cerevisiae* Nap1p, which is a nucleosome assembly protein | Nucleus | Nap1 | 92, 113, 125 |
| (NM_004515) interleukin enhancer binding factor 2, 45 kDa | Subunit of NF-AT: DNA-binding transcription factor | Nucleolus[a]/nucleus | | 92, 113, 126 |
| (NM_017816) hypothetical protein FLJ20425 | Ly1 antibody reactive clone; cell-growth regulating nucleolar protein | Nucleolus[a] | | 127 |
| (NM_001613) actin $\alpha$2 | Involved in cell motility, structure, and integrity | Cytoskeletal | Act1 | 92, 128 |
| (NM_002136) heterogeneous nuclear ribonucleoprotein A1 | May function as a temperature dependent RNA carrier during export from the nucleus to the cytoplasm | Nucleolus[a]/nucleus/cytoplasm | Hpr1 | 92, 113, 129 |
| (NM_004559) nuclease-sensitive element-binding protein 1 | Binds CCAAT boxes and regulates the expression of HLA class II genes; contains DNA-binding domain for CCAAT and nuclear localization signal | Nucleus | | 92, 113, 130 |
| (NM_001152) solute carrier family 25 | Fibroblast adenine nucleotide translocator (ADP/ATP carrier) | Integral to plasma membrane | Aac3 | 92, 113, 131 |

TABLE II—*continued*

| Protein | Known function in mammal | Cellular localization | Yeast homologs | Refs. |
|---|---|---|---|---|
| (NM_006758) U2 small nuclear RNA auxiliary factor 1 | Subunit of U2 small nuclear ribonucleoprotein auxiliary factor; functions as a pre-mRNA splicing protein | Nucleus | | 132 |
| (NM_001615) actin γ2 | Cell motility, and maintenance of the cytoskeleton | Nucleolus[a]/cytoskeletal | Act1 | 92, 113, 133 |
| (NM_006026) H1 histone family, member X | Helps compact DNA into nucleosomes and high order chromatin structures | Nucleus | | 92, 113, 134 |
| (NM_005320) H1 histone family, member 3 | Helps compact DNA into nucleosomes and high order chromatin structures | Nucleus | Hho1 | 92, 113, 135 |
| (NM_003134) signal recognition particle 14 kDa | Signal recognition particle (SRP) subunit | Nucleolus[a]/cytoplasm | | 92, 113, 136 |
| (NM_003521) H2B histone family, member E | Involved in compaction of DNA into nucleosomes | Nucleolus[a]/nucleus | Htb1 | 92, 113, 137 |

[a] Protein localization in the nucleolus is adopted from the results as described (63, 64).

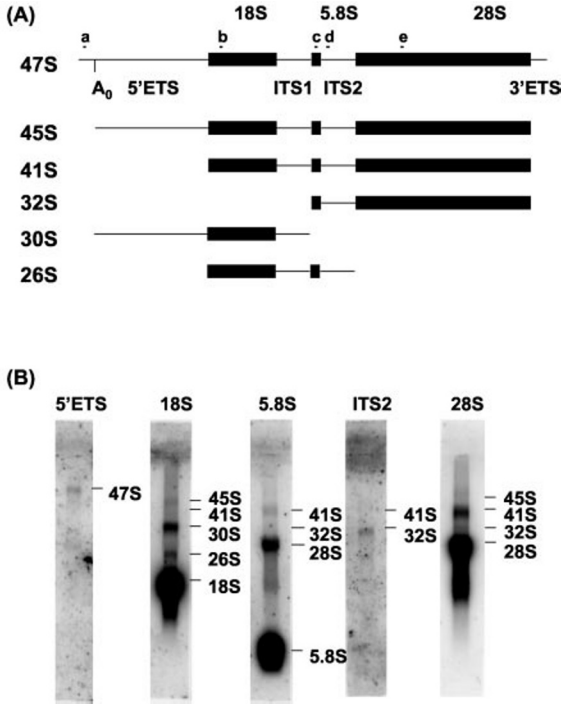


FIG. 4. **Analysis of rRNAs in hNop56p-associated complexes.** *A*, schematic representation of the pre-rRNAs detected in the Northern blots. In the 47 S pre-rRNA, the mature rRNAs (5.8 S, 18 S, and 28 S), the two internal transcribed spacers (ITS1 and ITS2), and the two external transcribed spacers (5′-ETS and 3′ETS), and the first cleavage site ($A_0$) are indicated. The positions of the oligonucleotide probes used for the Northern blot are indicated (*a*, 5′-ETS; *b*, 18 S; *c*, 5.8 S; *d*, ITS2; *e*, 28 S). *B*, RNA was isolated from the hNop56p-associated complexes and subjected to Northern blot analysis. The pre-rRNA oligonucleotide probes are indicated on the *top* of each blot. The rRNA and pre-rRNA species identified are shown to the *right* of the blots. The 5.8 S probe recognized 28 S rRNA non-specifically due to the abundance of 28 S rRNA in the isolated RNA sample.

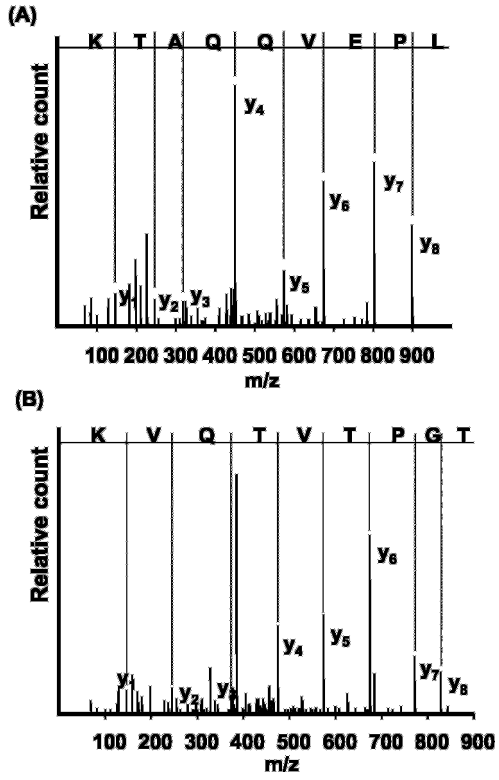


FIG. 5. **Representative MS/MS spectra used to identify treacle as a component of hNop56p-associated complexes.** The doubly charged ions at $m/z$ = 507.34 (*A*) and 465.81 (*B*) separated by LC were fragmented, resulting in the production of ion series containing the C termini (y series). The sets of ion series yielded the amino acid sequences LPEVQQATK and TGPTVTQVK that correspond to residues 1053–1061 and 670–678 of human treacle, respectively.

based on the fact that the complexes contained pre-rRNAs as well as many ribosomal proteins and potential trans-acting factors involved in ribosome biogenesis. Because the pre-rRNP complexes associated with hNop56p contained the 47 S primary precursor rRNA that corresponds to the yeast 35 S rRNA, the hNop56p-associated RNP possibly contains a pre-ribosome particle formed at a very early stage of ribosome biogenesis. In yeast, the primary 35 S pre-rRNA assembles a number of 40 S subunit processing factors and the small subunit (SSU) processome containing the four known core proteins of U3 snoRNP complexes and forms the 90 S ribosomal precursor (6, 46, 47). hNop56p-associated pre-rRNPs contained human orthologs of all four core proteins of yeast U3 snoRNPs, namely hNop56p, fibrillarin, hNop5/Nop58p and a 15.5-kDa protein. These results suggest a role for hNop56p in pre-rRNA modifications during early stages of ribosome biogenesis, as is the case for yeast Nop56p. However, we did not identify any other trans-acting factors of the SSU processome or 40 S processing factors in the hNop56p-associated pre-rRNPs. Thus, hNop56p-associated pre-rRNPs differ from isolated yeast 90 S pre-ribosomes that contain most of the 28 components of the SSU processome and many 40 S processing factors (47).

A variety of 60 S pre-ribosomes have been purified from yeast cells using a series of trans-acting factors as bait proteins. These pre-ribosomes are products of late stage ribosome biogenesis and contain a number of trans-acting factors that probably function as 60 S processing factors (11–13, 48). Thus, we compared the non-ribosomal proteins in the hNop56p-asso-

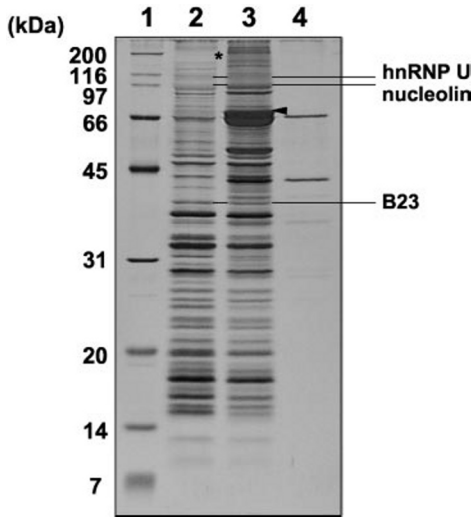


FIG. 6. **Comparison of treacle- and hNop56p-associated complexes.** Protein components of the treacle- and hNop56p-associated complexes were analyzed by SDS-PAGE followed by silver staining. *Lane 1*, molecular mass markers; *lane 2*, treacle-associated complexes; *lane 3*, hNop56p-associated complexes; *lane 4*, control prepared from untransfected 293EBNA cells. FLAG-tagged treacle (*lane 2*) and hNop56p (*lane 3*) are indicated by an *asterisk* and an *arrowhead,* respectively. Three trans-acting factors (hnRNP U, nucleolin, and B23) identified in both complexes are indicated.

ciated pre-rRNPs with the yeast 60 S processing factors with respect to amino acid sequence similarity. Six proteins were assigned as potential human 60 S processing factors, namely hypothetical protein FLJ10377 (corresponding to yeast Nop4/Nop77p), peter pan homolog (Ssf1p), EBNA1-binding protein 2 (Ebp2p), BRIX (Brx1p), DKFZP564M182 protein (Nsa3/Cic1p), and a putative nucleotide-binding protein (Nug1p). Fatica and Tollervey (6) proposed that at least four stable pre-rRNP complexes form during biogenesis of the yeast 60 S ribosomal subunit, and they classified the pre-60 S rRNP complexes into four categories: pre-60 S $E_1$ forms early during stage 1, pre-60 S $E_2$ during stage 2, pre-60 S M during the middle stage, and pre-60 S L forms during the late stage. By these criteria, hNop56p-associated pre-rRNPs most likely represent pre-60 S $E_1$ because these complexes contain the largest number of trans-acting factors (Nop4/Nop77p, Ssf1p, Ebp2p, and Brx1p) found in common with the yeast pre-60 S $E_1$ purified using Ssf1p as bait (11).

hnRNP U is another potential trans-acting factor that we assigned to hNop56p-associated pre-rRNPs. This protein is thought to play a role in hnRNA processing, although its involvement in ribosome biogenesis has not yet been established in mammals. Its yeast ortholog, Nop3p, exhibits 37% sequence identity with human hnRNP U and binds RNA through its glycine- and arginine-rich domain as well as two RNA recognition motifs (49) (Supplemental Material Table II). Nop3p is essential for yeast ribosome biogenesis as its depletion results in a defect in 27 SB pre-rRNA, a component of pre-60 S complexes $E_1$ and $E_2$ that participates in 60 S subunit maturation (49). In addition, we identified the two RNA helicases DDX9 and DDX21 in hNop56p-associated pre-rRNPs as additional trans-acting factors possibly involved in ribosome biogenesis in mammalian cells. The yeast ortholog of DDX21, Dbp3p, is required for efficient conversion of the pre-rRNA 27 $SA_2$ to 27 $SA_3$ in pre-60 S $E_1$ (23). Thus, the human orthologs of Nop3p and Dbp3p support our proposal that hNop56p-associated pre-rRNPs represent the human equivalents of the yeast pre-60 S $E_1$.

Other trans-acting factors found in hNop56p-associated pre-rRNPs include nucleolin and B23, both of which are unique to mammalian pre-rRNPs. Nucleolin participates in multiple stages of early pre-rRNA processing such as rDNA transcription, pre-rRNA processing, and assembly of ribosomal proteins and rRNAs (31–34). The specific interaction of nucleolin with 5′-ETS sequences is required for the first step of pre-rRNA processing (33), which is consistent with the observation that the yeast counterpart Nsr1p is required for efficient pre-rRNA processing at sites $A_0$ to $A_2$ (50, 51). Thus, it is well established that nucleolin and its yeast ortholog exhibit functional similarities in ribosome biogenesis. However, whereas nucleolin is found associated with hNop56p- and parvulin-associated pre-rRNPs isolated from mammalian cells (15), Nsr1p has never been identified in yeast pre-ribosomal complexes, suggesting that the mode of action of nucleolin in ribosome biogenesis differs from that of its yeast ortholog.

On the other hand, no yeast orthologs have been identified for another trans-acting factor, B23. This protein is an endoribonuclease that processes the ITS2 within the pre-rRNA, suggesting its involvement in pre-60 S complexes during the middle to late stages of 60 S biogenesis (35). Additionally, there are no reports of a yeast endoribonuclease that processes the ITS2. Rather, yeast remove the ITS2 using an enzyme complex composed of 11 exoribonucleases termed the "exosome" during the late stage of pre-60 S processing (52, 53). However, we found no evidence of exosome components in hNop56p-associated pre-rRNPs. In total, the data indicate that hNop56p appears to function in pre-ribosomes during the early to middle stages of 60 S ribosome subunit maturation. However, the presence of the mammalian-specific trans-acting factor B23 and the unique association of nucleolin with the mammalian pre-rRNP complexes indicate that some of the fundamental processes of mammalian ribosome biogenesis differ from those of yeast.

A striking finding of this study is that, within the hNop56-associated pre-rRNP, hNop56p interacts directly with treacle, the TCS gene product. The TCS disorder affects craniofacial development and has an incidence of approximately 1 in 50,000 live births (54, 55). Whereas TCS is inherited as an autosomal dominant allele of the *TCOF1* gene, 60% of the cases result from a *de novo* mutation (56). Almost all *TCOF1* mutations reported to date have been either deletions, insertions, splice-site mutations, or nonsense mutations, indicating that TCS results from loss-of-function of the *TCOF1* gene product (41, 43, 57, 58). Furthermore, generation of *Tcof1* heterozygous mice by targeted mutagenesis elicits the TCS phenotype. These observations suggest that treacle haploinsufficiency underlies this disorder. However, the normal function of treacle remains unclear.

Positional cloning identified *TCOF1* (41) as a gene encoding the serine/alanine-rich protein, treacle (42, 43). The treacle amino acid sequence includes several intriguing features. First, the protein localizes to the nucleolus via several nuclear localization signals within its C-terminal region (45, 59). Second, it has a large central repeat domain containing numerous potential casein kinase II phosphorylation sites, indicating a possible regulatory mechanism. Third, treacle exhibits weak but significant similarity to the mouse nucleolar phosphoprotein Nopp140 (60) that is believed to act as a chaperone that mediates pre-rRNP export from the nucleus as well as ribosomal protein import from the cytoplasm during ribosome biogenesis. Because of the similarities between treacle and Nopp140, treacle may also function as an RNP chaperone (45). Thus, our finding that there is a direct interaction between hNop56p and treacle within hNop56p-associated pre-rRNP complexes provides the first experimental evidence supporting this new putative function for treacle.

The fact that hNop56p and treacle interact directly suggests

that hNop56p may act as a scaffold that enables hNop56p-associated pre-rRNPs to bind treacle and thereby export RNPs from the nucleolus. Interestingly, we also identified the nucleolar and coiled-body phosphoprotein 1 (NOLC-1), a human counterpart of Nopp140, as a component of the hNop56p-associated pre-rRNP. However, we were not able to prove that NOLC-1 binds directly to hNop56p using either MALDI-TOF/MS or LC-MS/MS of RNase-treated complexes. Although the possibility still remains that the repeated MS analyses simply failed to identify NOLC-1 in the RNase-treated complexes, our observation suggests that treacle and NOLC-1 (Nopp140) differ in their mode of action during ribosome biogenesis. We also note that, unlike NOLC-1 (Nopp140), treacle neither localizes to small RNP-rich Cajal bodies nor associates with snoRNPs (44) and that its amino acid sequence is unrelated to NOLC-1. Thus, although both of these proteins act as chaperones, their functions may be somewhat distinct. This assertion suggests that hNop56p-associated pre-rRNPs may utilize these two distinct chaperones at different stages during ribosome biogenesis.

Although we demonstrated the possible involvement of treacle in ribosome biogenesis and its direct association with hNop56p, it remains unclear as to whether the functions of hNop56p and treacle in ribosome biogenesis are related to the molecular mechanism underlying TCS. The analysis of *Tcof1* heterozygous mice demonstrated that the correct dosage of treacle is essential for survival of cephalic neural crest cells that contribute significantly to formation of branchial arches. Hence, dysmorphogenesis of the lower face may be a consequence of defective neural crest cell migration from the hindbrain to the branchial arches during early embryogenesis (61, 62). If the functions of hNop56p and treacle in ribosome biogenesis are directly related to TCS, then a possible underlying mechanism is that a sufficient supply of ribosomes is essential for normal development. Proteomic analysis of developmental profiles of hNop56p- and treacle-associated pre-rRNPs during TCS progression in model animals may suggest plausible mechanisms for the TCS disorder.

Apart from treacle, 29 non-ribosomal proteins of unknown function in ribosome biogenesis were identified in this study. Of these, 20 proteins reportedly localize to the nucleus and/or the nucleolus (63, 64) (Table II and Supplemental Material Table III). The subcellular localization of the remaining nine proteins has not been reported to date. Whereas the nucleolus constitutes the site of ribosome biogenesis, recent data indicate that the nucleolus may also play crucial roles in other cellular processes (65, 66), particularly given that it associates with other subnuclear domains (67, 68). Among the 29 non-ribosomal proteins, SFRS protein kinase 1, U2 small nuclear ribonucleoprotein auxiliary factor (65 kDa), CGI-74 protein, U2 small nuclear RNA auxiliary factor 1, and hnRNP A1 are categorized as proteins involved in mRNA processing and export. It has been suggested that spliceosomal small nucleolar RNP biogenesis and mRNA processing/export occur within the nucleolus (69, 70). Four proteins that we identified, namely eukaryotic translation elongation factor 2, IGF-II mRNA-binding protein 1, eukaryotic translation elongation factor 1 $\alpha$1, and Tu translation elongation factor (mitochondrial), are involved in protein translation. Recently, several lines of evidence suggested that translation mechanisms such as nonsense-mediated mRNA decay (71) may exist within the nucleus. Nonsense-mediated mRNA decay is thought to contribute to the mRNA quality control mechanism by reducing the level of mRNAs that contain nonsense mutations. Furthermore, functional ribosomes exist in a number of distinct intranuclear foci (72), suggesting that protein synthesis may occur in the nucleus. Signal recognition particle (SRP) biogenesis may also occur within the nucleolus (73). In fact, the 14-kDa SRP protein was identified in hNop56p-associated pre-rRNPs. Although the significance of SRPs in the nucleolus remains obscure, SRP assembly is believed to be coordinated with ribosome assembly within the nucleolus or nucleus as a mutual quality control mechanism that ultimately ensures the proper translation of each mRNA (74). We also identified nine proteins in hNop56p-associated pre-rRNPs that are classified as transcriptional regulator proteins (Table II and Supplemental Material Table III). These include MYB-binding protein (P160) 1a, interleukin enhancer binding factor 3, DNA topoisomerase I, a nuclear phosphoprotein similar to *Saccharomyces cerevisiae* PWP, nucleosome assembly protein 1-like factor 1, interleukin enhancer binding factor 2, H1 histone family member X, H1 histone family member 3, and H2B histone family member E. The functional relationship between the ribosome and transcriptional regulators is not presently understood. However, given that ribosomal components are found associated with the site of transcription (75), these proteins may be involved in the transcription of pre-ribosomal components. The remaining proteins identified in hNop56p-associated complexes are cytoskeletal proteins such as $\alpha$-tubulin, $\beta$-tubulin, $\alpha$-actin, and $\gamma$-actin. Yeast Nop56p contains C-terminal KK(E/D) repeats, and similar motifs in human microtubule-associating proteins (76, 77) mediate microtubule binding (78). It has been speculated that KK(E/D) repeats aid the efficient and accurate segregation of the pre-rRNA processing machinery during mitosis (16). hNop56p has no KK(E/D) repeats but rather contains highly basic repeats at its C terminus, suggesting a similar function for hNop56 during mitosis. The involvement of hNop56p-associated pre-rRNPs in the above pathways remains unproven, and these RNPs may act independently of pre-ribosomal complexes in some cases. Clearly, additional studies are needed to elucidate the mode of action of hNop56p in pre-rRNP complexes and delineate its role in the newly discovered functions of the nucleolus and the nucleus.

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