# SUPPLEMENTARY MATERIAL

## ARCHITECTURE AND ASSEMBLY OF MAMMALIAN H/ACA SMALL NUCLEOLAR AND TELOMERASE RIBONUCLEOPROTEINS

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MATERIALS AND METHODS

UV-crosslinking

The short synthetic and site-specifically $^{32}$P-labeled rRNA substrates used for crosslinking corresponded to the sequences in human 28S rRNA surrounding the pseudouridylation sites at uridine 4380 and 4363, which are targeted by H/ACA snoRNAs E3 and U65, respectively. The synthesis and labeling of the rRNA substrates was identical to the previously described substrates 2 and 3 except that the target uridines were replaced by 4-thiouridines (Wang et al., 2002). The substrates were incubated for 90 minutes at 30°C with immunopurified H/ACA snoRNPs under standard assay conditions for in vitro pseudouridylation (Wang et al., 2002). Subsequently, the assay mixture was transferred to a 96-well plate on ice and irradiated for 15 minutes with 365nm UV light to specifically crosslink 4-thiouridine. To digest the RNA to single nucleotides, it was incubated for 45 minutes at 37°C with 16pg nuclease P1 and 60U RNase T1 (Sigma, St. Louis, MO). The protein A-Sepharose beads with the residual snoRNPs were then washed with PBS and the proteins analyzed by 15% Tricine SDS-PAGE (Schagger and von Jagow, 1987) followed by autoradiography. For re-immunoprecipitation after crosslinking, the beads were incubated with 100μl 1% SDS for 5 minutes at room temperature to dissociate the snoRNPs and the SDS quenched with four volumes of buffer containing 1.25% Triton, 20mM Tris-HCl (pH7.5), 10mM NaCl. After centrifugation, the supernatant was immunoprecipitated with anti-NAP57 or anti-GAR1 antibodies as described previously (Wang et al., 2002).

DNA templates and plasmids

The DNA template for rat NAP57, pTM575, was described earlier (Meier and Blobel, 1994). The templates for rat NHP2, rat NOP10, mouse GAR1, human fibrillarin and HA-tagged rat NOP10 under the T7 promoter were generated by PCR amplification from plasmids pTM132, pTM133, pTM137, EGFP-fibrillarin (Platani et al., 2000), and pCW10, respectively, using the primers U116 and U118 (NHP2), U122 and T3 (GAR1), U119 and U120 (NOP10), U247 and U248 (fibrillarin), and U119 and U120 (HA-NOP10) (see Table I). Note that mouse and rat GAR1 are 99% identical except for two short insertions in the mouse GAR domains. pTM132, pTM133,

and pTM137 were from American Type Culture Collection (Manassas, VA) and correspond to their numbers 2009673, 2001390, and 1258976, respectively. The HA-NOP10 template (pCW10) was created by amplification of the NOP10 cDNA from pTM133 (using primers T7 and U131) and cloning it into the BamHI and XhoI sites of pACT2 (Clontech, Palo Alto, CA).

The DNA templates, pTM145, pTM147, pTM149, pTM151, pTM154, and pTM156, for transcription/translation of HA-NAP57 and its derivatives were generated by excising their DNA fragments with BglII out of plasmids pTM113, pTM45, pYY42, pYY43, pTM80, and pTM84, respectively, and inserting them into the BamHI site of pBluescript II SK$^+$ (Stratagene, LaJolla, CA). The clones with the constructs under the T7 promoter were selected. The originating plasmids were generated as follows (primers for PCR amplification in parentheses, see Table I); for pTM113 (HA-NAP57), NAP57 was amplified from pTM575 (ME01 and SK) and inserted into the NcoI and EcoRI sites of pACT2 (Clontech); for pTM45 (HA-NAP57ΔC), the NAP57 fragment encoding amino acids 1-466 was excised with NcoI and EcoRI from pET22b/pelB/NAP57 and inserted into those sites of pACT2; for pET22b/pelB/NAP57, NAP57ΔC was amplified from pTM575 (ME01 and ME03) and cloned into the NcoI and EcoRI sites of pET22b (Novagen, Inc., Madison, WI); for pYY39 (NAP57,F37V) pTM575 was amplified with two complementary primers (U72 and U73) carrying the point mutation and digested with DpnI before transformation; for pYY42 (HA-NAP57,F37V), the mutated NAP57 was amplified from pYY39 (ME01 and SK) and cloned into the NcoI and EcoRI sites of pACT2; for pYY43 (HA-NAP57,D126A), the mutated NAP57 was generated by two-step PCR from pTM575 (T7 and U130, and U129 and T3 in the first and T7 and T3 in the second round) and inserted into the NcoI and EcoRI sites of pACT2 (both point mutations were confirmed by sequencing); for pTM80 (HA-NAP57-N terminal half), the NAP57 fragment encoding amino acids 1-259 was amplified (U14 and U16) and cloned into the BamHI and XhoI sites of pACT2; for pTM84 (HA-NAP57-C terminal half), the NAP57 fragment encompassing amino acids 252-466 was amplified (U15 and ME04) and inserted into the BamHI and XhoI sites of pACT2.

Coupled in vitro transcription/translation

Proteins were expressed in vitro using the TNT T7-Quick Coupled Transcription/Translation System (Promega, Madison, WI). According to the manual, a 20μl-reaction contained 16μl of reticulocyte lysate, 0.4μl of T7 enhancer, 0.8μl (~10μCi) of $^{35}$S-methionine easy tag (PerkinElmer, Wellesley, MA) and DNA templates, and was incubated for 90 minutes at 30°C. For the coexpression of several proteins, the DNA amount of individual templates was titrated to ensure a distinct and similar signal from each protein. Routinely, 2μl translation mixture were analyzed for transcription/translation efficiency on 15% Tricine SDS-polyacrylamide gels as described (Meier and Blobel, 1992) and the remainder used for immunoprecipitation. For RNase treatment, 2μg RNaseA (Calbiochem, San Diego, CA) were added and the translation mixture incubated for an additional hour at 37°C. For arginine methylation, 0.2μg of recombinant yeast arginine methyltransferase Hmt1p (Xu et al., 2003; kind gift from Michael Henry), and/or 0.5nmoles S-adenosyl methionine were added post translation and incubation at 30°C continued for 1 hour.

Immunoprecipitation of in vitro translated proteins

Immunoprecipitation was performed as described previously with affinity-purified anti-NAP57 antibodies (0.8μg; Meier and Blobel, 1994) or anti-HA monoclonal antibodies (1μg; Molecular Probes, Eugene, OR). Briefly, antibodies were added to the translation mixture, incubated for ~1 hour at room temperature, the mixture diluted with 10 volumes of wash buffer (10mM HEPES,

pH 7.9, 150mM NaCl, 2mM $MgCl_2$, 0.1% Triton X-100, 0.02% SDS), centrifuged for 10 minutes at 14,000xg, the supernatant transferred to protein A-Sepharose beads, and incubated at room temperature for ~1 hour. To precipitate HA-NOP10, the diluted translation products were transferred to monoclonal Anti-HA Agarose Conjugate beads (Sigma, St. Louis, MO). The beads were washed three times with 1ml wash buffer and once with 1ml PBS before elution of the proteins with sample buffer and analysis on 15% Tricine SDS-PAGE and fluorography. The protein bands were quantitated with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) and the molar ratios of NOP10/NHP2 calculated considering the number of methionines in each protein, 3/4, respectively. When protein-RNA interactions were tested in this system, $^{32}$P-labeled RNA was either added simultaneously with the DNA templates or at the end of translations followed by an additional 1 hour incubation at 30°C. Since the results were independent of the order of addition, the experiments shown in the figures were carried out under the former conditions. For quantitation of the $^{32}$P signal of the RNA in the presence of comigrating $^{35}$S-methionine labeled proteins, the $^{35}$S signal was blocked with a transparency.

<u>RNA mobility shift assays with recombinant proteins</u>

Plasmids pCW3 (GST-NHP2) and pCW5 (GST-NOP10) were constructed by amplification from pTM132 (primers U100 and T3) and from pTM133 (U99 and T7), respectively, and insertion of the products into the BamHI and SacI sites of pGEX-KG (Guan and Dixon, 1991). GST-NHP2 and GST-NOP10 were expressed in BL21(DE3) cells and affinity purified on glutathione-Sepharose 4B (Pharmacia, Piscataway, NJ) as described previously (Meier, 1996). In RNA binding assays, the recombinant proteins (0.04, 0.1, 0.2, 0.4, 1μg of GST-NHP2 and 1μg of GST-NOP10) were incubated with ~20fmol labeled RNA in 20μl 50mM Tris-HCl pH7.5, 100mM KCl, 1mM $MgCl_2$, 1mM DTT, 5% glycerol at room temperature for 20 minutes in the presence of 1μg of E.coli tRNA, 1μg of BSA, 10U of RNasin (Panvera, Madison, WI). Subsequently, the incubation mixture was analyzed by 4% native polyacrylamide gel electrophoresis and autoradiography (Konarska and Sharp, 1987). In competition experiments, increasing amounts of unlabeled RNA (200- and 1000-fold molar excess) was added prior to the probe.

<u>Reconstitution of H/ACA snoRNPs in cellular extracts</u>

Reconstitution was essentially performed as described (Dragon et al., 2000). Briefly, a 25-μl reaction contained 5-10μl of cytosolic S-100 or nuclear extract from Hela Cells (prepared according to Dignam et al., 1983 and generously provided by Charles Query), 20mM HEPES-KOH pH7.5, 120mM KCl, 2mM $MgCl_2$, 1pg tRNA, 20U RNasin, 1mM ATP, and ~20fmol of $^{32}$P-labeled (for mobility shift assays) or 500fmol of unlabeled RNA (for activity determination) and was incubated at 30°C for 1 hour. Reconstituted pseudouridylase activity was assayed as described (Wang et al., 2002). For competition experiments, increasing amounts of unlabeled RNA (10-, 100- or 1000-fold molar excess) were added prior to E3 snoRNA except where stated that it was added after reconstitution and incubated for an additional hour at 30°C. Before analysis by 4% native polyacrylamide electrophoresis, 0.5mg/ml heparin and 5% glycerol were added for 10 minutes. For supershift analysis, ~0.2ng of NHP2, NOP10, and fibrillarin antibodies (Aris and Blobel, 1988; Pogacic et al., 2000) were added prior to the RNA probe.

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TABLE I

Primer sequences (5' to 3', T7 promoter is underlined)

| Primer | Sequence |
|---|---|
| ME01 | TACCATGGCGGACGCGGAAG |
| ME03 | GAGAATTCACCCGGGGCGTGGTA |
| ME04 | GACTCGAGCCCGGGGCGTGGTAGT |
| SK | CGCTCTAGAACTAGTGGATC |
| T7 | AATACGACTCACTATAG |
| T3 | ATTAACCCTCACTAAAGGGA |
| U14 | GAAGATCTACATGGCGGACGCGGA |
| U15 | GAAGATCTCGGGGGTCGTGGG |
| U16 | CTGACTCGAGCACGCGCCGCAGCT |
| U72 | CAGCACGCGGAAGATGTCCTGATCAAACCG |
| U73 | CGGTTTGATCAGGACATCTTCCGCGTGCTG |
| U83 | GCATCGATTTTCAGTATCTACGATTCATAG |
| U99 | GCGGATCCATGTTTCTCCAGTATT |
| U100 | GCGGATCCATGACCAAAATAAAGGT |
| U116 | TAATACGACTCACTATAGGGCCACCATGACCAAAATAAAGGTG |
| U118 | TGGGTTGCAGCCCAA |
| U119 | TAATACGACTCACTATAGGGCCACCATGTTTCTCCAGTATTAC |
| U120 | TAACACAAGAGATTAACAA |
| U122 | TAATACGACTCACTATAGGGCCACCATGTCTTTCCGAGGC |
| U129 | GACACTTGCCCCCAAGGT |
| U130 | ACCTTGGGGGCAAGTGTC |
| U131 | GCGGATCCTCATGTTTCTCCAGTATTA |
| U247 | TAATACGACTCACTATAGGGCCACCATGAAGCCAGGATTCA |
| U248 | TTCAGTTCTTCACCTTGGG |
| U249 | AGTGAGCGCGCGTAATACGACTCACTATAGGGCCACCATGGCTTACCCATACGA |
| U250 | TAATACGACTCACTATAGGGTTGCGGAGGGTGGGC |
| U251 | GCATGTGTGAGCCGAGTCCT |