Components of U3 snoRNA-containing Complexes Shuttle between Nuclei and the Cytoplasm and Differentially Localize in Nucleoli: Implications for Assembly and Function

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Submitted June 5, 2003; Revised July 31, 2003; Accepted August 26, 2003
Monitoring Editor: Joseph Gall

U3 small nucleolar RNA (snoRNA) and associated proteins are required for the processing of preribosomal RNA (pre-rRNA) and assembly of preribosomes. There are two major U3 snoRNA-containing complexes. The monoparticle contains U3 snoRNA and the core Box C/D snoRNA-associated proteins and an early preribosome-associated complex contains the monoparticle and additional factors that we refer to as preribosome-associated proteins. To address how and where the U3 snoRNA-containing preribosome assembles and how these processes are temporally and spatially regulated, we have examined the dynamics and distribution of human U3 complex-associated components in cells with active or inactive transcription of rDNA. We found that U3 complex-associated proteins shuttle between the nucleus and the cytoplasm independent of the synthesis and export of preribosomal particles, suggesting that the shuttling of these proteins may either provide opportunities for their regulation, or contribute to or modulate ribosome export. In addition, monoparticle and preribosome associated components predominantly localize to different nucleolar substructures, fibrillar components, and granular components, respectively, in active nucleoli, and partition separately into the two components during nucleolar segregation induced by inhibition of pol I transcription. Although the predominant localizations of these two sets of factors differ, there are significant areas of overlap that may represent the sites where they reside as a single complex. These results are consistent with a model in which U3 monparticles associate with the fibrillar components of nucleoli and bind pre-rRNA during transcription, triggering recruitment of preribosome-associated proteins to assemble the complex necessary for pre-rRNA processing.

INTRODUCTION

RNA polymerase I (pol I) and cofactors transcribe the 28S, 18S, and 5.8S ribosomal RNAs (rRNAs) as a 47S precursor (pre-rRNA) (for reviews, see Hannan *et al.*, 1998; Grummt, 1999) that undergoes multiple cleavages, modifications, and is assembled with ribosomal proteins to form preribosomal subunits. These processes initially take place cotranscriptionally in the fibrillar substructures and subsequently in the granular components of nucleoli. The assembled preribosomal particles then move to the nucleoplasm before export into the cytoplasm. These steps of ribosome synthesis require a large and diverse population of *trans*-acting factors that act in a sequential yet interdependent manner to create mature rRNA (for reviews, see Kressler *et al.*, 1999; Venema

and Tollervey, 1999; Warner, 2001). Many of these functions require one or more of the ~200 small nucleolar ribonucleoproteins (snoRNPs) of mammalian cells. Regions of the RNA components of snoRNPs are complementary to and interact with short sequences in pre-rRNA, whereas snoRNP proteins generally carry out enzymatic or structural functions. SnoRNPs can be divided into two families, box H/ACA or box C/D, based on conserved elements (boxes) within their snoRNAs. The box C/D snoRNPs act as guides that direct 2'-O-methylation or cleavage and some are required for preribosomal particle assembly (for reviews, see Filipowicz and Pogacic, 2002; Kiss, 2002; Terns and Terns, 2002).

The U3 snoRNA is an abundant box C/D snoRNA that is required for some of the pre-rRNA processing and assembly steps that produce the small (40S) ribosomal subunit. U3 contains regions that are complementary to and bind sequences of the pre-rRNA 5' external transcribed spacer and 18S rRNA (Parker and Steitz, 1987; Beltrame *et al.*, 1994; Beltrame and Tollervey, 1995; Hughes, 1996; Mereau *et al.*, 1997; Sharma and Tollervey, 1999). This binding occurs cotranscriptionally because U3 snoRNA and associated proteins are components of the "terminal knobs" seen attached to pre-rRNA in Miller spread analyses (Miller and Beatty, 1969; Mougey *et al.*, 1993; Dragon *et al.*, 2002). Vertebrate U3 snoRNA and its associated proteins use these associations to guide the cleavages at sites A0, 1, and 2 in pre-rRNA that

Article published online ahead of print. Mol. Biol. Cell 10.1091/mbc.E03-06-0363. Article and publication date are available at www.molbiolcell.org/cgi/doi/10.1091/mbc.E03-06-0363.

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Abbreviations used: ActD, actinomycin D; DFC, dense fibrillar component; FC, fibrillar center; GC, granular component; hnRNP, heterogeneous ribonucleoprotein; pol I, RNA polymerase I; pre-mRNA, pre-messenger RNA; pre-rRNA, pre-ribosomal RNA; snoRNP, small nucleolar ribonucleoprotein; snoRNA, small nucleolar RNA; rRNA, ribosomal RNA; rDNA, ribosomal DNA; UBF, upstream binding factor.

separate the 18S precursor from the 5.8S and 28S precursors (Kass and Sollner-Webb, 1990; Hughes and Ares, 1991; Savino and Gerbi, 1991; Mougey *et al.*, 1993; Beltrame and Tollervey, 1995; Enright *et al.*, 1996; Borovjagin and Gerbi, 1999). In addition, a U3 snoRNA-containing complex may also act as a chaperone that prevents the premature formation of the central pseudoknot at the 5' end of 18S rRNA (Hughes, 1996; Sharma and Tollervey, 1999) and may play related roles in ribosome assembly because the depletion of U3 snoRNA and many of its associated proteins inhibits both processing and 40S subunit assembly (for reviews, see Kressler *et al.*, 1999; Venema and Tollervey, 1999; Warner, 2001).

Biochemical analyses identify U3 snoRNA in at least two different complexes in mammalian cells, one of which migrates in gradients at 12S and the other at 60–80S (Granneman *et al.*, 2003). These complexes correspond in size and cosediment with some of the same components that have been identified in the purified yeast U3 snoRNA-containing complexes. The yeast 12S complex, referred as the U3 monoparticle or U3 snoRNP, contains the core snoRNP proteins (Billy *et al.*, 2000; Granneman *et al.*, 2003). Four of these proteins, fibrillarin (Schimmang *et al.*, 1989; Tollervey *et al.*, 1991), Nop56 (Gautier *et al.*, 1997), Nop5/Nop58 (Gautier *et al.*, 1997; Wu *et al.*, 1998; Lafontaine and Tollervey, 1999; Lyman *et al.*, 1999), and 15.5K (Watkins *et al.*, 2000), are also associated with the other Box C/D snoRNAs. The fifth core protein, U3-55K, although not a common box C/D snoRNA binding protein, directly and specifically associates with U3 snoRNA (Lubben *et al.*, 1993; Pluk *et al.*, 1998; Watkins *et al.*, 2000). The larger U3 complex in yeast, called the small subunit (SSU) processome or a preribosomal particle, has been found to contain at least 28 proteins, including all the core proteins, and five small subunit ribosomal proteins (Rps4, Rps6, Rps7, Rps14, and Rps28) (Dragon *et al.*, 2002). The majority of the noncore proteins, which we will refer to as preribosome-associated proteins, have been found to associate with U3 but not other snoRNAs. These include Sof1 (Jansen *et al.*, 1993), Mpp10 (Baserga *et al.*, 1997; Dunbar *et al.*, 1997; Lee and Baserga, 1997; Westendorf *et al.*, 1998), Imp3, Imp4 (Lee and Baserga, 1999), Dhr1 (Colley *et al.*, 2000), Enp1 (Chen *et al.*, 2003), and 17 previously uncharacterized preribosome-associated proteins (Utp1–17) (Dragon *et al.*, 2002). In addition to these proteins, several other proteins have been shown to associate specifically with U3 snoRNA, including Bms1 (Wegierski *et al.*, 2001), Lcp5 (Wiederkehr *et al.*, 1998), P110 (Adamson *et al.*, 2001), and Rcl1 (Billy *et al.*, 2000). These proteins, however, were not found in the purified preribosome but may associate with it in a weak or transient manner in vivo. The two major U3-containing complexes have thus far only been purified from and characterized in yeast, and though the components and U3 snoRNA-containing complex sizes are conserved, clarification of the characteristics of the mammalian complexes will require their purification.

Outside the nucleolus, newly synthesized U3 snoRNA (Narayanan *et al.*, 1999; Verheggen *et al.*, 2002) and the box C/D core proteins fibrillarin (Raska *et al.*, 1990; Gerbi and Borovjagin, 1997), Nop56, Nop58, and 15.5K (Verheggen *et al.*, 2002) are localized to Cajal bodies, subnuclear structures that also contain RNA polymerase II transcription factors, premessenger RNA (pre-mRNA) splicing factors, and other snoRNAs (for reviews, see Gall, 2000; Carmo-Fonseca, 2002; Ogg and Lamond, 2002). It is thought that the common box C/D proteins assemble with U3 snoRNA to create an early U3 snoRNP within Cajal bodies (Narayanan *et al.*, 1999; Kufel *et al.*, 2000; Verheggen *et al.*, 2001, 2002) and that

U3-55K association within the nucleolus completes the monoparticle complex. Because proteomic studies in yeast indicate that the U3-containing complex associated with pre-rRNA is in the form of the preribosome (Dragon *et al.*, 2002), there is apparently a transition between the two identified U3-containing complexes. It remains to be determined whether the U3 monoparticle associates with pre-rRNA and recruits other proteins to form preribosome or whether the preribosomal complex is preassembled elsewhere in the nucleus and recruited to its pre-rRNA target.

To address how and where the U3 snoRNA-containing preribosome assembles, and how all these processes are temporally and spatially regulated, we have examined the dynamics and distribution of human U3 snoRNA and its associated proteins in cells with active or inactive transcription of rDNA. We found that U3 snoRNA complex-associated proteins shuttle between the nucleus and the cytoplasm independent of the synthesis and export of ribosomal particles. In addition, the U3 core and preribosome-associated components localize differentially within transcriptionally active nucleoli and partition separately during nucleolar segregation induced by inhibition of pol I transcription.

MATERIALS AND METHODS

Green Fluorescent Protein (GFP) and FLAG-tagged Fusion Proteins

The pEGFP-C1-fibrillarin, pEGFP-C1-UBF (Chen and Huang, 2001), and pEGFP-C1-PTB clones (Kamath *et al.*, 2001) have been characterized previously. The clones for pEGFP-C3-Imp3 and Imp4 (Granneman *et al.*, 2003) were kindly provided by G. Pruijn (University of Nijmegen, Nijmegen, The Netherlands). The full-length cDNAs for human Sof1p (GenBank accession NM_015420) and Rcl1 (accession AJ276894) were amplified from HeLa RNA by using primers specific for each protein and the Titan One-Tube RT-PCR kit (Roche Diagnostics, Indianapolis, IN). These cDNAs were separately cloned into the *EcoRI* and *BamHI* restriction sites of pEGFP-C1 vectors (BD Biosciences Clontech, Palo Alto, CA) to add amino-terminal GFP fusion tags. Full-length Sof1 was also cloned into the *EcoRI* and *BamHI* restriction sites of the pFLAG-CMV2 vector (Sigma-Aldrich, St. Louis, MO) to add an amino-terminal FLAG tag. Full-length human Mpp10p (hMpp10p) was subcloned from the full-length cDNA (kindly provided by S. Baserga, Yale University, New Haven, CT) into the *EcoRI* site of pEGFP-C1. Human U3-55K was subcloned from the pGEM3Zf+ vector (Pluk *et al.*, 1998) into the *BglII* to *BamHI* restriction sites of the pEGFP-C1 vector.

Antibodies and In Situ Probes

Human anti-fibrillarin antibody was obtained from Sigma-Aldrich (product no. ANA-N) and rabbit anti-*Xenopus* U3-55K antiserum has been characterized previously (Lukowiak *et al.*, 2000). Affinity-purified rabbit antibodies against human Nop56 (Watkins *et al.*, 2002) and Nop58 (Watkins *et al.*, 2000) were generously provided by N. Watkins and R. Luhrmann (Max-Planck-Institut für Biophysikalische Chemie, Göttingen, Germany). The Cy3-labeled in situ oligonucleotide probe used against human U3 snoRNA has been characterized previously (Narayanan *et al.*, 2003).

Cell Culture and Transfection

Human HeLa (cervical carcinoma) cells were grown in DMEM supplemented with 10% fetal bovine serum. For transient transfection, expression constructs were transfected into HeLa cells by electroporation (Sambrook *et al.*, 1989). Briefly, subconfluent cells in 100-mm culture dishes were collected by trypsinization and mixed with 4 μ g of target DNA and 16 μ g of sheared salmon sperm DNA. A 280- μ l mixture of cells in DMEM with 10% fetal calf serum (FCS) and DNA was electroporated in a Bio-Rad (Richmond, CA) electroporator at 250 V and 950 μ F. Subsequently, cells were seeded onto glass coverslips in 35-mm petri dishes and incubated for 24 h before experimentation.

Heterokaryon Assays

HeLa cells were transfected with the appropriate GFP-fusion construct and were seeded on glass coverslips. After 4 h, an equal number of untransfected HeLa cells was added to the coverslips, and these were grown with the transfected cells for 12 h. The cells were then further incubated for an additional 2 h in presence of 100 μ g/ml cycloheximide (Sigma-Aldrich). Cell fusions were carried out by washing the cells with phosphate-buffered saline (PBS), incubating them in 50% (wt/vol) polyethylene glycol 1500 (Roche

Diagnostics) for 2 min, and rinsing two times with PBS (Pinol-Roma and Dreyfuss, 1992). Heterokaryons were incubated further for the given postfusion times in media containing 100 $\mu\text{g}/\text{ml}$ cycloheximide before fixation. For transcription inhibition studies, heterokaryons were incubated with both cycloheximide and either 4 $\mu\text{g}/\text{ml}$ actinomycin D (ActD), 0.04 $\mu\text{g}/\text{ml}$ ActD, 25 $\mu\text{g}/\text{ml}$ 5,6 dichloro-1- β -D-ribofuranosylbenzimidazole (DRB), or 50 $\mu\text{g}/\text{ml}$ α -amanitin in DMEM containing 10% FCS for 2 h before fusion and 2 h after fusion. For the leptomycin B studies, the cells were pretreated with 1 h with 100 $\mu\text{g}/\text{ml}$ cycloheximide followed by 1 h with 0.2 ng/ml leptomycin B and cycloheximide before fusion. The heterokaryons were incubated for 2 h postfusion in the same drugs before fixation. Immunofluorescence was carried out with paraformaldehyde fixation as described above except that cells were stained with 4,6-diamidino-2-phenylindole (200 ng/ml in PBS; Sigma-Aldrich) for 1 min and with rhodamine-phalloidin (1 $\mu\text{g}/\text{ml}$ in PBS; Molecular Probes, Eugene, OR) for 15 min.

Quantitation of Relative Fluorescence Intensity

Fluorescence intensity was measured using MetaMorph (Universal Imaging, West Chester, PA) imaging software. For all constructs besides GFP-polypyrimidine tract binding protein (PTB), the average intensities of the same-sized area of the most brightly stained region of the nucleoli of the transfected and untransfected fused cells were measured in images taken under the same conditions for each data set. Because GFP-PTB is predominantly nucleoplasmic, the intensities of nucleoplasmic regions were used for GFP-PTB heterokaryon quantitation. Heterokaryons were selected with the lowest levels of transfected protein expression to minimize the effect of overexpression and only fusions containing two nuclei were quantitated. The ratio of fluorescence intensity (F_R) in the heterokaryon analyses was calculated as $F_R = F_U - F_B / F_T - F_B$ (Kamath *et al.*, 2001). F_U is the average intensity of the region within a nucleolus from the untransfected cell, F_B is the background fluorescence intensity (measured outside the cells) for each heterokaryon, and F_T is the average intensity of the region within a nucleolus from the transfected cell. When F_U equals F_T , F_R is 1 and the two nucleoli are considered to be at equilibrium for the given protein.

Transcriptional Inhibition, In Situ Hybridization, and Indirect Immunofluorescence

For untreated and transcription inhibition studies on human cells, untransfected HeLa cells or cells at 24 h posttransfection were grown on coverslips in the absence of drug or incubated for 3 h in 0.04 $\mu\text{g}/\text{ml}$ ActD (Sigma-Aldrich) in DMEM containing 10% FCS. The cells used for both in situ hybridization and immunofluorescence were fixed in 4% paraformaldehyde in PBS for 15 min and permeabilized with 0.5% Triton X-100 in PBS for 5 min.

In situ hybridization coverslips were washed once with 2 $\times$ SSC (American Bioanalytical, Natick, MA) and 50% formamide (Sigma-Aldrich). The coverslips were then incubated with 30 ng of Cy3-conjugated U3-antisense probe for 3 h at 37°C in hybridization buffer (10% dextran sulfate, 1 μl of RNase Out [Invitrogen, Carlsbad, CA]), 0.02% RNase-free bovine serum albumin, 40 μg of *Escherichia coli* tRNA, 2 $\times$ SSC, and 50% formamide). Coverslips were washed two times in 2 $\times$ SSC and 50% formamide and two times in 2 $\times$ SSC. Coverslips were then fixed for 5 min in 1% paraformaldehyde in PBS, washed one time in PBS, and subjected to immunofluorescence to stain fibrillarin.

For immunofluorescence, fixed, permeabilized cells were incubated for 1 h with primary antibodies in PBS at room temperature. All coverslips were stained with anti-fibrillarin antibody (1:10; Sigma-Aldrich) and some untransfected HeLa were simultaneously incubated with either rabbit anti-Nop56 (1:200), rabbit anti-Nop58 (1:200), or rabbit anti-U3-55K antiserum 397 (1:200). Subsequently, cells were incubated with Texas Red-conjugated anti-human antibody (1:200), and the untransfected cells were also stained with fluorescein isothiocyanate-conjugated goat anti-rabbit antibody (1:200; Vector Laboratories, Burlingame, CA) for 1 h at room temperature followed by 3 $\times$ 10-min washes in PBS. The coverslips were mounted onto glass slides with mounting medium containing 90% glycerol in PBS with 1 mg/ml parapenylenediamine (Sigma-Aldrich) as an antifade agent. The mounting medium was adjusted to pH 8.0 with 0.2 M bicarbonate buffer. Cells were observed on Nikon Eclipse E800 epifluorescent microscope, and images were acquired with a SenSys cooled charge-coupled device camera (Photometrics, Tucson, AZ) by using MetaView version 4.5 software (Universal Imaging).

The immunofluorescence figures in this report show representative data. Each experiment was reproduced multiple times, and the cells shown are representative of the overall effects observed under each set of conditions.

RESULTS

U3 Complex-associated Proteins Shuttle between the Nucleus and Cytoplasm

To characterize the intracellular dynamics of U3-associated proteins, we asked whether the core and preribosome-associated U3 snoRNA binding proteins shuttle between the nucleus and cytoplasm based on the occasional weak cyto-

plasmic labeling of some of these proteins. The intercompartmental shuttling of GFP or FLAG-tagged fusion proteins were assayed using HeLa-HeLa cell heterokaryons. Although interspecies heterokaryons are often used to assay the shuttling process, we believe that cell fusion of the same species are more accurate for evaluating shuttling kinetics. Donor cells were transfected with expression constructs of the fusion proteins, and these cells were fused with recipient (untransfected) cells and grown in the presence of a protein synthesis inhibitor for 1 to 3 hours postfusion. If a protein shuttles between the nucleus and cytoplasm, we expect that the fusion protein will occur in the nuclei of the recipient cells. The heterokaryons expressing FLAG proteins were immunostained with anti-FLAG antibodies, and all heterokaryons were stained with the actin dye rhodamine phalloidin (to identify fused actin networks) and the DNA dye DAPI (to delineate nuclei and nucleoli).

The nucleocytoplasmic shuttling of two core proteins (GFP-fibrillarin and -U3-55K) and five preribosome-associated proteins (GFP-Imp3, -Imp4, -Mpp10, -Rcl1, -Sof1, and FLAG-Sof1) were assayed. To evaluate whether the fusion proteins behave similarly to their endogenous counterparts, their cellular localization and their interactions with normal partners were evaluated. All of these tagged proteins localized similarly to their endogenous counterparts (Figure 1). The localization and intermolecular interactions for GFP-Imp3, -Imp4, -Mpp10 (Granneman *et al.*, 2003), and -fibrillarin (Dundr *et al.*, 2000; Snaar *et al.*, 2000; Chen and Huang, 2001) have been characterized previously. GFP- or FLAG-Sof1 was immunoprecipitated in complex with ribosomal protein S6 (our unpublished data), indicating that these proteins interact with components of the U3 snoRNA-associated preribosome. To reflect the endogenous protein shuttling as closely as possible, cells with the lowest expression levels were evaluated in the heterokaryon assays. To control for the integrity of the assay, we also evaluated the shuttling of GFP-polypyrimidine tract binding protein (PTB), which has been shown to be a nucleocytoplasmic shuttling protein (Kamath *et al.*, 2001), and GFP-upstream binding factor (UBF), a protein that does not shuttle between the nucleus and the cytoplasm (Kamath and Huang, unpublished data). PTB (heterogenous nuclear ribonucleoprotein [hnRNP] I) is a heterogenous nuclear ribonucleoprotein that binds some pre-mRNAs and participates in splice site selection (Valcarcel and Gebauer, 1997), polyadenylation (Lou *et al.*, 1996, 1999), and translation (Hellen *et al.*, 1994; Kaminski *et al.*, 1995; Witherell *et al.*, 1995; Pickering *et al.*, 2003). UBF is an RNA polymerase I-specific transcription factor (for reviews, see Hannan *et al.*, 1998; Grummt, 1999).

Representative cells from 2 h postfusion are shown in Figure 1. Thick arrows indicate the nucleoli from the donor (transfected) cells, whereas thin arrows indicate the nucleoli from the recipient cells. In these assays GFP-fibrillarin, -U3-55K, -Imp3, -Imp4, -Mpp10, -Rcl1, -Sof1 (Figure 1A), and FLAG-Sof1 (our unpublished data) were all detected in the nucleoli from the recipient cells. GFP-UBF, a nonshuttling protein, was not detected in the recipient cells, whereas GFP-PTB, a positive control (Kamath *et al.*, 2001), was detected in the nucleoplasm of the recipient cells. These results demonstrate that U3 complex-associated proteins shuttle between the nucleus and the cytoplasm.

To quantitatively analyze the shuttling dynamics of all assayed proteins, F_R , the ratio of average fluorescence intensities of the recipient nucleoli versus the donor nucleoli, was determined at different time points postfusion (Kamath *et al.*, 2001) (see MATERIALS AND METHODS). Because GFP-PTB does not localize to nucleoli, the F_R values for this

Figure 1. U3 snoRNP proteins shuttle between nuclei and the cytoplasm at different rates. (A) Untransfected HeLa were fused in heterokaryon assays with HeLa transfected with constructs encoding either negative (GFP-UBF) or positive (GFP-PTB) controls, U3 snoRNP core proteins GFP-fibrillarin or -U3-55K (left) or the preribosome-associated proteins GFP-Imp3, -Imp4, -Mpp10, -Rcl1, or -Sof1 (right). Two cell heterokaryons were analyzed 2 h after fusion. DNA was labeled with DAPI to delineate nuclei and nucleoli. Thick arrows indicate nucleoli of transfected cells, thin arrows the nucleoli of untransfected cells. Bar, 10 μ m. (B) Quantitative analyses of the heterokaryon assays represented in A were done to compare the relative shuttling rates of the proteins analyzed in A. For all constructs besides GFP-PTB, the F_R (y-axis) was measured as fluorescence intensity in the untransfected cell nucleolus (F_U)/fluorescence intensity in the transfected cell nucleolus (F_T) within a same-sized area. For GFP-PTB, nucleoplasmic rather than nucleolar fluorescence intensity was used.

protein were calculated using nucleoplasmic fluorescent intensities. The F_R value rises as more tagged proteins move from the donor nucleus to the common cytoplasm and are imported into the recipient nucleus. An F_R value of 1 indicates that the shuttling of tagged proteins has reached equilibrium between the donor and recipient nucleoli. The F_R values for the GFP-fusions are plotted versus hours postfusion in Figure 1B. These results demonstrate a large range of shuttling kinetics among the different U3 complex-associated proteins. As a basis of comparison for this and following studies, the F_R values at 2 h postfusion will be compared because these values seem to be representative of the overall shuttling behavior of the proteins. At 2 h postfusion, GFP-fibrillarin and -U3-55K have the lowest F_R values (0.075 ± 0.038 and 0.068 ± 0.017 , respectively). Although these values are low, they, like GFP-PTB, did increase over time, whereas the F_R value of GFP-UBF, the negative control, did not. GFP-Imp3 and -Imp4 have slightly higher F_R values (0.117 ± 0.067 and 0.138 ± 0.043 , respectively) than GFP-fibrillarin and GFP-U3-55K, whereas GFP-Mpp10 and -Rcl1 yielded F_R values (0.162 ± 0.057 and 0.227 ± 0.047 , respectively) that were 2 to 3 times higher. In contrast to the other proteins tested, GFP-Sof1 yielded a much higher F_R value of 0.391 ± 0.084 , almost twice that of GFP-Rcl1. To address whether these large variations could be due to differential effects that the GFP tags may have on the tagged proteins, the F_R values for GFP-Sof1 and FLAG-Sof1 at 2 h postfusion were com-

pared and found to be similar (our unpublished data). In addition, all GFP-tagged proteins localized similarly to the endogenous proteins. Therefore, the large differences in shuttling kinetics could not be completely attributed to presence of the large GFP tag, at least in the case of Sof1. Furthermore, cells with the lowest expression levels were generally evaluated in the heterokaryon assay. Therefore, the large differences in shuttling kinetics among U3 binding proteins, both core and preribosome associated, most likely reflect that the U3 binding proteins do not shuttle between the nucleus and cytoplasm as parts of the same complex.

The Nucleocytoplasmic Shuttling of U3 Complex-associated Proteins Is Not Dependent on Continuous rRNA Synthesis or Crm1-mediated Export

To gain insight into the mechanism by which U3 complex-associated proteins shuttle between the nucleus and cytoplasm, we asked whether the shuttling of these proteins is dependent on ribosome synthesis and/or export. The transcription of pre-rRNA was selectively inhibited by treating heterokaryons with a low concentration of ActD (Perry, 1963; Dousset *et al.*, 2000) before cell fusion. If the nucleocytoplasmic shuttling of U3-associated proteins is coupled with maturing 40S ribosomes export, we expected that the loss of ribosome synthesis should change the nucleocytoplasmic shuttling dynamics of these proteins. In these experiments, a mix of untransfected HeLa and HeLa trans-

Figure 2. Nucleocytoplasmic shuttling of U3 snoRNP proteins does not require rDNA transcription or Crm1-mediated export. (A) Untransfected HeLa were fused in heterokaryon assays with HeLa transfected with constructs encoding either core proteins GFP-fibrillarin or -U3-55K or the preribosome-associated proteins GFP-Imp3, -Imp4, -Mpp10, -Rcl1, or -Sof1. The cells were pretreated for 2 h and grown postfusion for 2 h in the presence or absence (untreated) of 0.04 μ g/ml actinomycin D. Quantitative analysis was done to compare the relative shuttling rates of the proteins. The F_R (y-axis) was measured as fluorescence intensity in the untransfected cell nucleolus (F_U)/fluorescence intensity in the transfected cell nucleolus (F_T) within a same sized area. Error bars indicate standard deviations. (B) Heterokaryon assays were done as described above except that the cells were pretreated for 1 h pre-fusion and grown for 2 h postfusion in the presence of 0.2 ng/ml leptomycin B. Quantitation was done as described above.

ected with the GFP-fusions of U3 complex-associated proteins were treated with cyclohexamide and 0.04 μ g/ml ActD for 2 h before fusion. After fusion, the heterokaryons were incubated in the same drugs for 2 h before fixation and prepared for observation by using epifluorescence microscopy. The nucleoli from both donor and recipient cells exhibited the segregation phenotype typical of inhibition of pol I transcription (Rivera-Leon and Gerbi, 1997; Douset *et al.*, 2000). In mammalian cells, the granular component remains in the center of the nucleolar space, whereas the fibrillar components move to the periphery of the nucleoli and form one or more nucleolar "caps" (Thiry and Thiry-Blaise, 1989; Derenzini *et al.*, 1990; Puvion-Dutilleul *et al.*, 1992; Wachtler *et al.*, 1992; Jimenez-Garcia *et al.*, 1993). The results of these assays showed that the inhibition of pol I transcription did not prevent any of the tested proteins from shuttling because all of the fusion proteins were detected in the segregated nucleoli of the recipient cells (our unpublished data). Quantitative analyses of their nucleocytoplasmic shuttling kinetics demonstrated that the loss of pre-rRNA synthesis altered slightly but did not substantially affect the shuttling dynamics of most of the proteins (Figure 2A). The differences of F_R values among the different fusions were generally maintained whether or not pol I was active. These results indicate that the nucleocytoplasmic shuttling of GFP-fusions of the core proteins fibrillarin and U3-55K and the preribosome-associated proteins Imp3, Imp4, Mpp10, Sof1, and Rcl1 are not dependent on pre-rRNA synthesis.

Late-stage preribosomal particles move from the granular component of nucleoli to the nucleoplasm and are exported to the cytoplasm via a Crm1-mediated pathway (Moy and Silver, 2002), which can be specifically and effectively disrupted by leptomycin B (Fukuda *et al.*, 1997). As an alternative approach to determining whether the nucleocytoplasmic shuttling of U3-associated proteins is coupled to ribosome export, we examined whether the nucleocytoplasmic shuttling of these proteins is affected by treating heterokaryons with leptomycin B. Specifically, a mix of transfected and untransfected HeLa cells were treated with cyclohexamide for 1 h, 0.2 ng/ml leptomycin B was added for another hour before fusion, and the heterokaryons were incubated in the same drugs for 2 h before fixation.

The two fusion proteins that were examined in this assay were GFP-fibrillarin, a representative of the core proteins, and GFP-Sof1, a preribosome-associated protein. In the pres-

ence of leptomycin B, both GFP-fibrillarin and -Sof1 were detected in the heterokaryon nuclei from untransfected cells (our unpublished data), demonstrating continued shuttling between the nucleus and cytoplasm. Calculations of their F_R values showed that the shuttling kinetics of these proteins was not significantly altered when Crm1-mediated export pathway was blocked (Figure 2B). These results further indicate that the synthesis and export of 40S ribosomal subunits are not necessary for the nucleocytoplasmic shuttling of either core or preribosome-associated proteins.

U3 snoRNA, Core, and Preribosome-associated Proteins Exhibit Different Subnucleolar Localization in Transcriptionally Active Nucleoli

To analyze the spatial relationship between the components of U3 monparticles and U3 snoRNA-associated preribosomes, we examined the subcellular distribution of these components in HeLa cells. U3 snoRNA localization was examined using in situ hybridization. Antibodies were used to immunofluorescently label endogenous proteins (fibrillarin, Nop56, Nop58, and U3-55K) in HeLa cells. GFP-tagged fusion constructs were transfected into HeLa to explore the localization of the human homologues of Imp3, Imp4, Mpp10, and Rcl1. As described above, these fusion proteins exhibit localization patterns similar to those described for endogenous proteins and are present in complexes with endogenous U3 snoRNA-associated proteins. Although we have antibodies against some preribosome-associated proteins, these antibodies only label the proteins in cells fixed by highly extractive reagents such as methanol that significantly distort the known localization of some proteins, including fibrillarin. Thus, GFP fusion proteins were used for the localization studies of these and other preribosome-associated proteins to most accurately reflect the localization of these proteins. In addition, only cells with lowest expression levels of the GFP-fusion proteins were examined.

Although all the proteins tested localized to nucleoli (Figure 3, large arrowheads), their subnucleolar localization was not identical. The distribution patterns can be divided into two types. The first, exemplified by U3 snoRNA, fibrillarin, Nop56, Nop58, and U3-55K, is the labeling of concentrated zones within the nucleoli, visible as colocalization with fibrillarin in the strong, yellow-colored foci in the overlay images (Figure 3A). All of these proteins also exhibited a weaker localization outside of these foci. The second distribution pattern, exemplified by GFP-Imp3, -Imp4, -Mpp10,

Figure 3. In active nucleoli, U3 snoRNA and the core proteins localize to different subnucleolar structures than preribosome-associated proteins. (A) The subcellular localization of U3 snoRNA HHeHe (top) in HeLa cells was determined by in situ hybridization against U3 snoRNA and immunolabeling with antibodies against fibrillarin (middle). The subcellular localization of core U3 snRNP proteins (bottom four panels) was determined by double immunolabeling HeLa cells with antibodies against fibrillarin (middle) and antibodies against either Nop56, Nop58, or U3-55K (left). Overlay images are shown in the right panels. Arrows indicate Cajal bodies. Bar, 10 μ m. (B) Subcellular localization of preribosome-associated U3 snoRNA-associated proteins was determined by transfecting HeLa cells constructs encoding either GFP-Imp3, -Imp4, -Mpp10, or -Rcl1 (left column) and immunolabeling the cells with antibodies against fibrillarin (middle column). Overlay images are shown in the right column. Arrows indicate Cajal bodies. Bar, 10 μ m.

and -Rcl1, is defined by a more diffuse labeling predominantly outside of the nucleolar foci with some weaker labeling within the foci, as observed in their weak colocalization with punctate fibrillarin (Figure 3B). Although the two patterns are clearly distinguishable, there are substantial overlaps between the two groups of factors at the rims and subnucleolar areas outside of the fibrillarin-labeled foci (Figure 3B).

As characterized previously, U3 snoRNA (Narayanan *et al.*, 1999; Verheggen *et al.*, 2002), fibrillarin (Raska *et al.*, 1990; Gerbi and Borovjagin, 1997), Nop56, Nop58, and 15.5K (Verheggen *et al.*, 2002) were also enriched in Cajal bodies (small arrowheads), as verified by their immunofluorescent colocalization with coilin (our unpublished data). However, the core protein U3-55K was not detected in Cajal bodies, consistent with previous observations (Lukowiak *et al.*, 2000). In contrast to the U3 core proteins, we found that the preribosome-associated proteins were not concentrated in the Cajal bodies (Figure 3, A and B, small arrowheads).

These results show differential subnuclear distributions of U3 core versus preribosome-associated proteins. The common box C/D snoRNP proteins (fibrillarin, Nop56, and Nop58) are enriched in Cajal bodies, and all of the core proteins (fibrillarin, Nop56, Nop58, and U3-55K) localize predominantly to the foci within nucleolus. In contrast, the preribosome-associated proteins, including GFP-Imp3, -Imp4, -Mpp10, and -Rcl1, are predominantly associated

with regions outside of these nucleolar foci. Thus, there is apparently a spatial separation of the core and preribosome-associated proteins in transcriptionally active nucleoli.

Core and Preribosome-associated Components Differentially Localize in the Absence of rDNA Transcription

When rDNA transcription is selectively inhibited by low doses of ActD (Perry, 1963) and nascent pre-rRNA synthesis is interrupted (Rivera-Leon and Gerbi, 1997; Dousset *et al.*, 2000), nucleoli exhibit a characteristic segregated phenotype. In mammalian cells, the granular component remains in the center of the nucleolar space, whereas the fibrillar components move to the periphery of the nucleoli and form one or more nucleolar caps (Thiry and Thiry-Blaise, 1989; Derenzini *et al.*, 1990; Puvion-Dutilleul *et al.*, 1992; Wachtler *et al.*, 1992; Jimenez-Garcia *et al.*, 1993).

Because a complex containing U3 snoRNA is involved in some early steps of pre-rRNA processing, interruption of pre-rRNA synthesis is expected to have a significant effect on the intranucleolar distribution of its components. Although the localization of U3 snoRNA and some of its associated proteins have been examined, the redistribution of the majority of U3 complex-associated components during nucleolar segregation had not yet been assayed. To compare the effects of the inhibition of pol I transcription and nucleolar segregation on the localization of U3 snoRNA

Figure 4. In transcriptionally inactive nucleoli, U3 snoRNA and the core proteins localize to different subnucleolar structures than preribosome-associated proteins. (A) To inhibit transcription by RNA polymerase I, HeLa cells were grown for 3 h in the presence of 0.04 $\mu\text{g}/\text{ml}$ actinomycin D before fixation. The subcellular localization of U3 snoRNA (top) was determined by in situ hybridization against U3 snoRNA and immunolabeling with antibodies against fibrillarin (top). The subcellular localization of core U3 snoRNA-associated proteins (bottom four panels) under these conditions was determined by double immunolabeling HeLa cells with antibodies against fibrillarin (middle) and antibodies against Nop56, Nop58, or U3-55K (left). Overlay images are shown in the right panels. Arrows indicate fibrillar caps of segregated nucleoli. Bar, 10 μm . (B) The subcellular localization of preribosome-associated U3 snoRNA-associated proteins was determined by transfecting HeLa cells with GFP-Imp3, -Imp4, -Mpp10, or -Rcl1, treating them as described above, and then immunolabeling the cells with antibodies against fibrillarin. Overlay images are shown in the right panels. Arrows indicate fibrillar caps of segregated nucleoli. Bar, 10 μm .

and the proteins that associate with it, we treated cells for 3 h with a low dose (0.04 $\mu\text{g}/\text{ml}$) of ActD that selectively inhibits pol I transcription (Perry, 1963; Dousset *et al.*, 2000). After fixation, some cells were subjected to in situ hybridization to U3 snoRNA, immunofluorescent labeling of fibrillarin (Figure 3A, top), and DAPI staining of DNA to delineate nucleoli. In addition, the immunolocalization of Nop56, Nop58, or U3-55K (Figure 3A) was examined and compared with that of fibrillarin. Treated HeLa cells that had been transfected with GFP-Imp3, -Imp4, -Mpp10, or -Rcl1 were also immunolabeled with anti-fibrillarin (Figure 3B).

As described previously, U3 snoRNA (Puvion-Dutilleul *et al.*, 1992, 1997) and fibrillarin (Ochs *et al.*, 1985; Reimer *et al.*, 1987; Puvion-Dutilleul *et al.*, 1992, 1997; Dousset *et al.*, 2000; Uzbekov *et al.*, 2003) localized to the caps of segregated nucleoli (Figure 4A). We found that other core proteins, including endogenous U3-55K, Nop56, and Nop58, also localized to the caps, as observed by colocalization with fibrillarin (Figure 4A). In addition, all of the core components had a slightly stronger nucleoplasmic label than when pol I was active. This was especially evident for U3 snoRNA, Nop58, and fibrillarin and is consistent with previous studies that found both U3 snoRNA and fibrillarin partially relocating to the nucleoplasm after ActD treatment (Rivera-Leon and Gerbi, 1997; Dousset *et al.*, 2000). In contrast, all the preribosome-associated proteins (GFP-tagged Imp3, Imp5, Mpp10,

and Rcl1) were localized predominantly to the central region of the segregated nucleoli and only a small fraction of each colocalized with fibrillarin in the caps (Figure 4B).

These results indicate that during nucleolar segregation induced by the inhibition of rDNA transcription, U3 snoRNA, and its core proteins partition to a different subnucleolar compartment than do preribosome-associated proteins. This difference in the spatial redistribution of the two groups mirrors that observed in active nucleoli in which U3 snoRNA and the core proteins are concentrated in nucleolar foci, and the preribosome-associated proteins are predominantly associated with nucleolar regions outside these foci.

DISCUSSION

U3 snoRNA-associated Proteins Shuttle between the Nucleus and the Cytoplasm

We demonstrate for the first time that U3 complex-associated proteins shuttle between the nucleus and cytoplasm as assayed by heterokaryons by using GFP-tagged proteins. Although all of the proteins tested shuttle, there is a broad range of dynamics among different U3-associated proteins as evaluated by their F_R values over time. Although some of the F_R values of the U3 complex-associated proteins were

low, all of them show increases over time, unlike GFP-UBF that stays near zero. We believe that the differences in the kinetics of U3 complex-associated proteins are unlikely all due to alterations in protein behavior that may have been generated by tagging and/or overexpression from transient transfections. The GFP fusion proteins localize to similar subnucleolar compartments as endogenous counterparts and interact with appropriate partners (Dundr *et al.*, 2000; Snaar *et al.*, 2000; Chen and Huang, 2001; Granneman *et al.*, 2003). In addition, only those cells expressing the lowest levels of the constructs were evaluated. Thus, the large differences in shuttling kinetics are likely to reflect the endogenous behavior of these proteins.

Although all of the preribosome-associated proteins have faster shuttling kinetics than the U3 core proteins, it is difficult to categorize the relative rates based on the limited number of constructs tested. Similar shuttling kinetics could reflect that the proteins are shuttling in the same or similar complexes. The broad range of U3 complex-associated shuttling rates suggests that the majority of these proteins are shuttling either in complexes separate from the other proteins tested or as individual proteins. Interestingly, the shuttling rates of GFP-fibrillarin and -U3-55K are nearly identical, suggesting that these two proteins could shuttle as part of the same complex.

A large number of nuclear proteins shuttle between the nucleus and cytoplasm, some of which are involved in ribosome biogenesis and/or are RNA binding proteins. These include nucleolin (Borer *et al.*, 1989) and Nopp140 (Meier and Blobel, 1992), both of which are localized to nucleoli and Cajal bodies (Bauer *et al.*, 1994; Isaac *et al.*, 1998; Yang *et al.*, 2000). These proteins interact with U3 snoRNA complexes (Ginisty *et al.*, 1998; Yang *et al.*, 2000) and affect U3 snoRNA localization (Verheggen *et al.*, 2001). The functional relevance of the shuttling of these proteins is unknown, although it has been hypothesized that these are involved in nuclear import and/or export (Meier and Blobel, 1992; Ginisty *et al.*, 1999). It is also unknown whether nucleolin and Nopp140 shuttle as individual proteins or as part of complexes. It is possible that these proteins could shuttle in complexes with U3 snoRNA-associated proteins, although there is no evidence to support this as yet and the shuttling of the different proteins was assayed using very different techniques, making it difficult to compare relative shuttling kinetics.

Other RNA binding proteins that shuttle include some heterogeneous hnRNPs that bind to transcribing pre-mRNAs and are involved in pre-mRNA processing. Some of these hnRNPs are nucleocytoplasmic shuttling proteins and have been implicated in the export of mRNAs and various cytoplasmic functions (for review, see Dreyfuss *et al.*, 2002). In addition, pre-mRNA splicing factors, SR proteins, also are nucleocytoplasmic shuttling proteins (Caceres *et al.*, 1998), and a member of the family has recently been shown to facilitate mRNA export (Huang *et al.*, 2003). The studies of these proteins provide insight into the functional relevance of the nucleocytoplasmic shuttling of some factors involved in RNA metabolism. However, not all nuclear proteins shuttle. For example, some of the hnRNP proteins, including hnRNP C (Pinol-Roma and Dreyfuss, 1992), and the pol I-specific transcription factor UBF do not shuttle, indicating that nucleocytoplasmic shuttling is not a universal phenomenon.

Although the functional relevance of the shuttling of U3 complex-associated proteins is not known, several possibilities exist. The nucleocytoplasmic shuttling could represent one mechanism by which the functional activity of these

proteins is regulated. In addition to their nucleocytoplasmic shuttling, U3 complex-associated components and other proteins involved in ribosome synthesis are highly dynamic within the nucleus (Phair and Misteli, 2000; Chen and Huang, 2001). GFP-fibrillarin moves rapidly between the nucleolus and nucleoplasm (Phair and Misteli, 2000; Snaar *et al.*, 2000; Chen and Huang, 2001) and through Cajal bodies (Snaar *et al.*, 2000). These exchanges are affected by the inhibition of pol I transcription (Phair and Misteli, 2000; Chen and Huang, 2001), suggesting that intercompartmental shuttling may be important for regulating the functional availability of U3 complexes, either by sequestration or modification (Leary and Huang, 2001). Modifications of U3 complex components could include phosphorylation, methylation, or acetylation, and there is evidence that at least some U3-associated proteins are subject to posttranslational modifications. Mpp10 was originally identified as a mitotic phosphoprotein (Matsumoto-Taniura *et al.*, 1996) and both nucleolin (Ginisty *et al.*, 1999) and Nopp140 (Meier and Blobel, 1992) are phosphoproteins, although the relationship of these modifications to U3 complex function is unknown. Moreover, fibrillarin (Christensen and Fuxa, 1988) and nucleolin (Lischwe *et al.*, 1985) undergo arginine dimethylation. The cycling of U3 complex-associated proteins through the cytoplasm may also allow a spatial opportunity to sequester these factors from functioning in the nucleus. This possibility is consistent with a previous observation in which both U3 and fibrillarin became detectable in the cytoplasm when mouse fibroblasts were grown in serum-free medium (Sienna *et al.*, 1996). The relocalization was suggested to be the result of a growth arrest-induced down-regulation of ribosome synthesis that decreased the need for pre-rRNA processing factors in the nucleolus.

A second possibility is that the nucleocytoplasmic shuttling of U3 complex-associated proteins could contribute to or modulate ribosome export. Although the shuttling is not dependent upon ribosome synthesis or export, it remains conceivable that the export of these proteins to the cytoplasm could affect ribosome export. It has previously been hypothesized that U3 snoRNA and/or some U3 complex-associated proteins could transiently or stably associate with 40S preribosomal particles during its later assembly steps (Puvion-Dutilleul *et al.*, 1992; Gerbi and Borovjagin, 1997), and these components could also be involved in export. Several recent proteomic studies in yeast demonstrated that U3 snoRNA and the majority of U3 complex-associated proteins (including all those analyzed in this article) have been detected in complexes with 35S pre-rRNA (Grandi *et al.*, 2002) but not in later preribosomal precursors (Gavin *et al.*, 2002; Schafer *et al.*, 2003), indicating that most U3 complex-associated components dissociate from pre-rRNA soon after their cleavage and chaperone functions are complete. The results of these studies indicate that it is unlikely that the majority of U3 complex-associated proteins stably associate with 40S during its export. However, U3-associated proteins individually or in complexes other than U3 monparticles or early preribosomes might modulate ribosome export through transient interactions with cargo or transport adaptors in a manner similar to that used by the SR proteins (Huang *et al.*, 2003).

Core U3 Complex-associated Components Localize to Different Subnucleolar Structures than Preribosome-associated Proteins in Transcriptionally Active Nucleoli

Electron microscopy studies of the nucleolus have identified three distinct nucleolar substructures: fibrillar centers (FCs), dense fibrillar components (DFCs), and granular compo-

nents (GCs). In situ and immunogold electron microscopic studies have demonstrated that U3 snoRNA is at the highest concentration at the FC/DFC border and within the DFCs of active nucleoli (Puvion-Dutilleul *et al.*, 1991, 1997) and that fibrillarin is concentrated in the same regions (Puvion-Dutilleul *et al.*, 1991). Our light microscopy studies of transcriptionally active nucleoli show that U3 snoRNA and its core binding proteins, including fibrillarin, Nop56, Nop58, and U3-55K, are primarily concentrated in nucleolar foci and exhibit a weaker label throughout the remainder of the nucleolus (Figure 3A). We believe that these foci to which U3 snoRNA and the core proteins localize correspond to the fibrillar structures of nucleoli and that the region outside these foci is the granular components. By fibrillar structures, we are referring to both the FC and DFC because the resolution of light microscopy cannot distinguish the two structures. Our findings are consistent with previous light microscopy studies showing that U3 snoRNA is concentrated in nucleolar foci that probably correspond to fibrillar components and is also localized throughout the nucleolus (Carmo-Fonseca *et al.*, 1991; Jimenez-Garcia *et al.*, 1994). In addition, fibrillarin was originally named based on its association with nucleolar foci that are believed to be DFCs and FCs (Ochs *et al.*, 1985). Together, studies from this report and others demonstrate that U3 and its core binding proteins are predominantly concentrated in the fibrillar components of the nucleolus and that they are less concentrated within GCs.

In contrast to U3 snoRNA and core proteins, preribosome-associated proteins are not concentrated in fibrillar components of active nucleoli, as visible by their weak colocalization with fibrillarin foci. GFP-Imp3, -Imp4, -Mpp10, and -Rcl1 were predominantly concentrated in GCs, and generally the label was lower within the fibrillar components (Figure 3B). Because the GC actually occupies significantly more volume than DFCs (Puvion-Dutilleul *et al.*, 1991), even when staining is equal between the two compartments the majority of the protein is found in the GC. These results are consistent with localization studies of P110 (Adamson *et al.*, 2001), a protein that is associated with U3 snoRNA but was not found in either U3 monparticles or the purified SSU processome. In active nucleoli, P110 was observed evenly distributed through nucleoli. Overall, our observations of multiple preribosome-associated proteins demonstrated a predominant association between these proteins and the GCs of transcriptionally active nucleoli.

Core Components Localize to Different Subnucleolar Structures than Preribosome-associated Proteins in Transcriptionally Inactive Nucleoli

On selective inhibition of pol I, nucleoli undergo characteristic segregation into fibrillar caps and granular components. Light and electron microscopic studies have shown that under these conditions U3 snoRNA (Puvion-Dutilleul *et al.*, 1992, 1997) and fibrillarin (Ochs *et al.*, 1985; Reimer *et al.*, 1987; Puvion-Dutilleul *et al.*, 1992, 1997; Dousset *et al.*, 2000; Uzbekov *et al.*, 2003) localize primarily to the fibrillar caps. In addition to U3 RNA and fibrillarin, we show in this report that other U3 core proteins, including Nop56, Nop58, and U3-55K, also predominantly localize to the fibrillar caps during pol I transcription inhibition (Figure 4A). There is very little labeling of these proteins in the granular components of the segregated nucleoli. These findings demonstrate that U3 snoRNA and the majority of its core proteins are partitioned with pol I transcription machinery and possibly rDNA within the fibrillar components of active nucleoli and the fibrillar caps during inhibition of pol I transcription.

Because the components within fibrillar caps include all of the U3 monparticle proteins tested, the results are consistent with the U3 monparticles being retained in these components independent of nascent pre-rRNA, thus suggesting associations with pol I transcription machinery, rDNA, or other factors within fibrillar components. Such associations have been proposed previously (Gerbi and Borovjagin, 1997; Rivera-Leon and Gerbi, 1997) as a means of storage of U3 complex components.

In contrast to U3 snoRNA and core proteins, the preribosome-associated proteins are not concentrated in fibrillar caps of transcriptionally inactive nucleoli. GFP-Imp3, -Imp4, -Mpp10, and -Rcl1 remain predominantly associated with the granular component in segregated nucleoli (Figure 4B). These results are consistent with localization studies of P110 (Adamson *et al.*, 2001) that localized predominantly to the granular component of segregated nucleoli. However, our results differ from those from a study of endogenous Mpp10 immunolabeling during transcriptional inhibition (Westendorf *et al.*, 1998). On treatments with a higher concentration of ActD (1 μ g/ml) for 4 h, Westendorf *et al.* (1994) showed that Mpp10 is predominantly concentrated in fibrillar caps in HEP-2 cells. Two aspects of this study are not clear. First, treatment with a higher concentration of ActD may substantially inhibit pol II transcription and thus induce a nucleolar segregation phenotype that is different from that observed under selective pol I transcription inhibition. Second, from the only image in the article, a nucleolar label of Mpp10 outside of the caps can be observed, but it is unclear whether this staining is localized in GCs. Unfortunately, the Mpp10 antibody used in these studies is no longer available to examine the localization in greater detail. Overall, our observations of multiple preribosome-associated U3 binding proteins, all of which are components of the SSU processome but not the U3 monparticle, demonstrated a predominant association between these proteins and the GCs of both transcriptionally active and inactive nucleoli.

U3 snoRNA and the proteins associated with it are highly conserved from yeast to mammals as are the processing and assembly steps in which they participate. A recent study of mammalian U3 complexes (Granneman *et al.*, 2003) indicates that human cells contain 12S and 60-80S U3 snoRNA-containing complexes, mirroring the findings in yeast (Billy *et al.*, 2000; Dragon *et al.*, 2002). This study indicates that the core protein U3-55K sediments at 12S and 60-80S in glycerol gradients and that the preribosome-associated proteins Imp3, Imp4, and Mpp10 only cosediment with U3 snoRNA in 60-80S complexes. These data provide indirect evidence that the general structure of U3 complexes has been conserved and that the mammalian core and preribosome-associated proteins are associated with U3 snoRNA in complexes that are probably structurally and functionally equivalent to the yeast U3 monparticle and the U3 snoRNA-containing preribosomal particle. However, definitive identification of human U3 snoRNA complexes will require the purification and proteomic analysis of these complexes.

The two different complexes purified from yeast and likely conserved in mammalian cells may represent two different functional stages of U3 complex activity. Our observations show that although the core and preribosome-associated components primarily localize to different subnucleolar regions, there are significant overlaps between their localization patterns at both the rims of fibrillar components and within the granular components. These localization overlaps may represent the sites where these two classes of proteins come together to form a large complex. Given that pre-rRNA is transcribed within the fibrillar com-

Figure 5. Model for the assembly of U3 monoparticles and the formation and function of the U3 snoRNA-containing preribosome in the context of subnucleolar and subnuclear structures.

ponents of nucleoli, most likely within the DFC (for review, see Huang, 2002), and that the U3 snoRNA containing preribosome forms around pre-rRNA as it is being transcribed (Dragon *et al.*, 2002), we consider the outer rims of DFCs and the surrounding GC as the most likely sites for the formation and function of the large U3 snoRNA-containing preribosome. Such a complex is likely to be transient and may dissociate rapidly when U3 complex function is completed and the later stage preribosomes enter further into the granular components. Such a transient association is supported by evidence that neither U3 snoRNA nor most of the proteins that associate with it have been found associated with later stage preribosomal particles (Gavin *et al.*, 2002; Schafer *et al.*, 2003). The predominant localization of the preribosome-associated proteins in the GCs may represent the functionally active forms that are ready to be recruited to form preribosomes or those that remain associated with preribosomes beyond the initial cleavage and assembly reactions.

Based on our observations and studies from other groups, we propose a model (Figure 5) in which the assembly of U3 monoparticles and the formation of the U3 snoRNA-containing preribosome are explained in the context of subnucleolar and subnuclear structures. In this model, early U3 monoparticles (containing U3 snoRNA and the core U3 binding proteins aside from U3-55K) assemble either in the nucleoplasm or in the Cajal bodies when they are present. These early monoparticles subsequently translocate to the nucleolus and form mature U3 monoparticles upon the association of U3-55K. Mature U3 monoparticles are recruited to the fibrillar structures (FC and/or DFC) where they become associated with pol I transcription machinery, rDNA, or other unknown factors. These associations are supported by data from this and other studies (Gerbi and Borovjagin, 1997; Rivera-Leon and Gerbi, 1997) showing that core components predominantly localize to the fibrillar subnucleolar structures and remain associated with these structures even when pre-rRNA synthesis is inhibited. In contrast, peri-

bosome-associated proteins predominantly localize to GCs upon inhibition of pol I transcription, illustrated in the model by the absence of these proteins from the fibrillar components when pre-rRNA is not being transcribed. When pre-rRNA transcription is activated, the positioning of U3 monoparticles near the sites of transcription ensures efficient recognition of pre-rRNA while it is being transcribed. The binding of U3 monoparticles to nascent pre-rRNA in the fibrillar components may induce structural changes in the monoparticle or in nascent pre-rRNA that trigger the recruitment of preribosome-associated proteins from the granular components, the regions in which they are concentrated, to form functional preribosomes. Based on our localization data, this step most likely occurs at the outer edges of fibrillar components or in the GC surrounding these regions. On inhibition of pol I transcription, the more distinct segregation of core proteins to the fibrillar caps and preribosome-associated proteins to the granular components is most probably due to the lack of U3 monoparticle-pre-rRNA complexes that recruit the other factors. Once U3 snoRNA-containing preribosomes are assembled, pre-rRNA processing and preribosomal particle assembly would commence. Once these functions are completed, the large complex would disassemble, leaving ribosomal proteins and some preribosome components attached to the pre-rRNA for later ribosomal synthesis steps in the GC. On complex disassembly, the majority of the components of the SSU processome, including components of the monoparticle, exit the fibrillar components. The U3 complex-associated proteins are then either available for the next round of function or could be subject to regulatory mechanisms that may involve their shuttling through different cellular compartments, as has been observed in fluorescence recovery after photobleaching studies (Phair and Misteli, 2000; Chen and Huang, 2001) and in our nucleocytoplasmic shuttling assays. Although this model is consistent with the current data, further studies are necessary to verify the nature of these processes *in vivo*.

ACKNOWLEDGMENTS

We thank Drs. Sander Granneman and Ger Pruijn for providing the Imp3 and Imp4 clones, Drs. Nicholas Watkins and Reinhard Luhrmann for antibodies against Nop56 and Nop58, and Dr. Susan Baserga for the Mpp10 clone. We also thank Drs. Danyang Chen, Chen Wang, and Rajesh Kamath for technical assistance. This work was supported by training grant DAMD17-00-1-0387 from the U.S. Army Congressionally Directed Medical Research Breast Cancer Research Program (to D.J.L.), National Institutes of Health grant GM-054682 (to M.P.T.) and National Cancer Institutes grant 5R01 CA077560 (to S.H.).

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