Organization of Small Nucleolar Ribonucleoproteins (snoRNPs) by Fluorescence In Situ Hybridization and Immunocytochemistry

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The organization of the U3, U8, and U13 small nucleolar ribonucleoproteins (snoRNPs) has been investigated in HeLa cells using antisense DNA and 2'-OMe RNA oligonucleotides. Oligomers corresponding to deoxynucleotides that target RNase H degradation of intact RNP particles were synthesized and used for fluorescence in situ hybridization. U3 and U13 are distributed throughout the nucleolus and colocalize with anti-fibrillarin antibodies. U8, however, is organized in discrete ring-like structures near the center of the nucleolus and surround bright punctate regions visualized with anti-RNA polymerase I and anti-UBF/NOR-90 antibodies. In decondensed nucleoli, a necklace of smaller ring-like structures of U8 RNA appear. A model for the recruitment of U8 (and presumably other processing factors) to the sites of rRNA transcription is discussed. Hybridization to mitotic cells showed that unlike pol I and NOR-90, U8 is dispersed into the cytoplasm during mitosis. The subnucleolar organization of U8 is consistent with its demonstrated participation in early intermediate steps in pre-rRNA processing. In contrast, the more dispersed intranucleolar distribution of U3 agrees with its putative involvement in both early and late steps of rRNA maturation. These studies illustrate the feasibility of mapping functional domains within the nucleolus by correlating the in vitro activities of small nuclear RNPs with their in situ locations.

INTRODUCTION

The human nucleolus is a nuclear organelle in which rRNA genes from five different chromosomes come together to perform the critical task of ribosome synthesis. This complex process involves rRNA transcription, rRNA processing, and ribonucleoprotein (RNP) assembly (see Gerbi *et al.*, 1990; Reeder, 1990; Warner, 1990; Sollner-Webb *et al.*, 1994 for reviews). As defined by electron microscopy, the mammalian nucleolus consists of three subregions: the fibrillar center (FC), the dense fibrillar component (DFC), and the granular component. During mitosis the nucleoli disperse as cells enter prophase; some nucleolar com-

ponents remain associated with the condensing chromosomes, whereas others are released. Nucleolar reformation normally begins during telophase as the daughter nuclei are formed. Although in most species nucleolar regeneration is directed by tandemly repeated rRNA gene clusters called nucleolus organizers (NORs), Karpen *et al.* (1988) showed that in *Drosophila* P-element-mediated transposition of a single rRNA repeat unit resulted in nucleolar formation on polytene chromosomes. For complete restoration of nucleolar morphology, the ribosomal chromatin is not sufficient; rRNA transcription is required (Stevens and Prescott 1971; Benavente *et al.* 1987).

A growing number of small nuclear RNPs (snRNPs) has been shown to play important roles in the processing of pre-rRNA (Fournier and Maxwell 1993; Sollner-

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Webb *et al.* 1994). For example, U3 is known to be required for the earliest cleavage event within the 5' external transcribed spacer (ETS) region (Kass *et al.* 1990; Mougey *et al.* 1993a,b). Evidence that U3 also contributes to subsequent pre-18S processing steps comes from studies in yeast (Hughes and Ares 1991; Beltrame and Tollervey 1992). U3 snRNP may also be involved in other steps of pre-rRNA processing (Savino and Gerbi 1990). More recently, Peculis and Steitz have shown that depletion of U8 in *Xenopus* oocytes disrupts several cleavage events necessary for maturation of 5.8S and 28S rRNAs (Peculis and Steitz 1993, 1994). Many small nucleolar RNPs (snoRNPs) share a common 34-kDa protein called fibrillarin (Fb). These so-called Fb snRNPs include U3, U8, U13, U14, U15, U16, U18, U20, U21, and U22 (reviewed in Fournier and Maxwell 1993; Sollner-Webb 1993).

Although aqueous fractionation methods reveal that the snoRNPs involved in rRNA processing are localized

in the nucleolus (reviewed in Baserga and Steitz 1993), these studies give no information regarding the organization of these particles within the nucleolus. Thus we used highly specific oligonucleotide probes for U3, U8, and U13 RNAs and fluorescence in situ hybridization (FISH) to correlate the in vitro RNA processing activities of these snoRNPs with their in situ locations. We find that U3 and U13 are located throughout the nucleolus in a nonhomogeneous pattern, whereas U8 is restricted to regions resembling the DFC. The distribution patterns of U3 and U8 were also compared to those of other proteins known to be involved in rRNA transcription, such as RNA polymerase I (RPI), human upstream binding factor hUBF (an abundant pol I transcription factor, also known as NOR-90), and Fb. Our results reveal that the U3 oligomer-staining pattern most closely resembles that of Fb, and that the U8 hybridization signal forms ring-like structures around RPI and hUBF.

Figure 1. Sequences and secondary structures of human U3, U8, and U13 RNAs. The regions complementary to the antisense oligonucleotides used for in situ hybridization are underlined. All of the oligoprobes were 2'-OMe RNA except U13(2-22), which was DNA. The conserved Box A-D sequences are also shown.

A

B

Figure 2. Oligonucleotide-directed RNase H degradation of U8 and U13 RNAs in their native snoRNP particles. Total HeLa cell extracts were digested with RNase H in the presence of either U8 (A) or U13 (B) specific antisense oligonucleotides. The numbers at the top of each panel indicate the complementary bases. The U3(64–79) oligonucleotide, which has been shown to direct specific RNase H digestion of U3 (Kass *et al.*, 1990), was used as a control. RNA was isolated from each reaction, fractionated by polyacrylamide gel electrophoresis, and probed for (A) U8 and (B) U13 by Northern blot hybridization. The same blots were then reprobed for U3 and U13 (A) or U3 and U8 (B) to control for both U3 RNA degradation and for loading. MW stands for DNA markers, whose sizes (nts) are shown.

MATERIALS AND METHODS

HeLa Cell Extracts

HeLa cell extracts were prepared essentially as described (Tyc and Steitz 1989), except that 5×10^8 cells were sonicated in 2.5 ml of buffer containing 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) pH 7.9, 300 mM KCl, 1 mM MgCl₂, 1 mM dithiothreitol (DTT), and 0.1 mM EDTA. The sonicate was subsequently spun at $100,000 \times g$, and the supernatant was collected, supplemented with glycerol to 10%, and stored at -80°C .

RNase H Digestions and Northern Blot Analysis

Figure 1 depicts the primary and secondary structures of U3, U8, and U13 RNAs. RNase H digestions were performed using antisense DNA oligonucleotides spanning various segments of U8 and U13. In addition, a control oligonucleotide complementary to U3 was also used. The 20- μl reactions included 10 μl of HeLa cell extract, 1 μg of the appropriate antisense oligonucleotide, and 1 U of RNase H (Pharmacia, Piscataway, NJ) in a solution containing 25 mM HEPES pH 7.9, 135 mM KCl, 4 mM MgCl₂, 0.5 mM DTT, 0.05 mM EDTA, and 5% glycerol. After incubation for 10 min at 37°C and 20 min at 30°C , 180 μl of a stop solution containing 0.1% sodium dodecyl sulfate and 5 mM EDTA was added. After phenol extraction RNAs were fractionated on 10% denaturing polyacrylamide gels and electrophoretically transferred to membranes.

Northern blot analyses were performed as described (Kass *et al.* 1990) except that the hybridization temperature was 60°C . For analysis of U3 and U8 RNAs, radiolabeled antisense RNA probes were used. The pSP65 plasmids containing either a rat U3 insert (Stroke and Weiner 1989) or the human U8 sequence (inserted into the *Sma* I site) were linearized with *Hind*III and *Eco*RI, respectively, and transcribed in vitro with SP6 polymerase in the presence of [α - ^{32}P]UTP. The U13 probe was generated as follows. One hundred nanograms each of two oligonucleotides (U13-A and U13-B), whose 3'-terminal 15 nucleotides are complementary, were annealed by heating for 5 min at 85°C and allowed to cool to room temperature over 30 min. The single-stranded regions were then filled in using 3 U of the Klenow enzyme (gift of N. Grindley, Yale University, New Haven, CT) and 100 μCi of [α - ^{32}P]ATP (3000 Ci/mmol) as described (Maniatis *et al.* 1982).

Cell Culture and Slide Preparation

Two strains of HeLa cells were grown either in monolayers on chambered slides (strain ATCC) or in spinner flasks (strain JS1000) as described previously (Matera and Ward 1993). The monolayer (HeLa-ATCC) cells were prepared by removing the medium from the slide chambers and rinsing in phosphate-buffered saline (PBS)-A (PBS without Mg⁺⁺ or Ca⁺⁺). After fixation in 4% paraformaldehyde, 100 mM KCl, 20 mM Na piperazine-*N,N'*-bis(2-ethanesulfonic acid) pH 7.4, 15 mM β -mercapto-ethanol, 2.5 mM MgCl₂, 0.5 mM ethylene glycol-bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid for 10 min at room temperature, the slides were quenched in 0.1 M glycine for 5 min and then permeabilized in -20°C acetone for 10 min. Alternatively, preextraction of the slides for 1 min in 0.5% Triton X-100, 1 $\times$ PBS-A before the fixation step did not significantly alter the nucleolar fluorescence pattern, although cytoplasmic background was somewhat reduced. Strain JS1000 cells were prepared for the mitotic studies by adding methotrexate to 10^{-7} M and incubating overnight. The cells were spun down to remove the medium; block was released by resuspending in fresh medium containing 10^{-5} M thymidine. The cells shown in Figure 6 were fixed after 5 h of culture in the presence of thymidine.

Oligonucleotides, Antisera, and In Situ Hybridization

The oligonucleotides used in this study were as follows: U13-A, 5'-d(TAATACGACTCACTATAGGATCCTTTTGTAGTTCATGAGC-

GTGATGATTGGGTGTTTCATACGCTTGTGTGAGATGTGCC) 3'; U13-B, 5'-d(GATCAGACGAATAATGTGCCCACGTCGT-AACAAGGTTCAAGGGTGGCACATCTCACACA)-3'; U3(64-74), 5'-BBBBUUUCGGUGUC-3'; U8(43-55), 5'-(dC*)₄UGUUCUAA-UCUGC3'; U13(10-22), 5'-(dC*)₄GCUCAUGAACUAC-3'; U13(2-22), 5'-d(GCT*CAT*GAAC*ACAAAAGGAT*T)-3'. Residues preceded by a lower case d are deoxyribose (DNA) linkages; A, G, C, U, and I are 2'-OMe RNA. B is a biotin phosphoramidite residue, whereas residues marked with an asterisk were amine modified and then labeled with digoxigenin as described (Matera and Ward 1992). Previously characterized human autoantibodies against RNA polymerase I (Reimer *et al.* 1987) and hUBF/NOR-90 (Chan *et al.* 1991; Rendón *et al.* 1992) were kindly provided by Dr. J. Craft (Yale University, New Haven, CT). Monoclonal antibody 72B9 (kind gift of Dr. E. Tan, Scripps Institute, La Jolla, CA) was used for detection of Fb.

After equilibrating the slides in 4× SSC, 0.1% Tween-20 for 5–10 min at room temperature, the cells were hybridized in 20 μl 4× SSC, 10% dextran sulfate, ~1 μg/μl competitor oligonucleotide (a random 22-mer), and 1–2 ng/μl-labeled probe oligomer. The probe was allowed to anneal under a 22 × 22-mm coverslip for 30 min at 37°C in a humidified chamber. After the hybridization the slides were washed three times for 5 min in 4× SSC, 0.1% Tween at 37°C, blocked in 250 μl of 4× SSC, 3% bovine serum albumin, 0.1% Tween for 20 min, and then incubated with fluorochrome-conjugated Avidin-DCS (Vector Labs, Burlingame, CA) (1:400 dilution) for 20 min. The unbound avidin was washed off in 4× SSC, 0.1% Tween (3 times for 5 min at 37°C) before counterstaining in 4,6-diamidino-2-phenylindole (DAPI) (Boehringer Mannheim, Indianapolis, IN) (200 ng/ml in 4× SSC, 0.1% Tween) for 1 min, destaining for 1–2 min, and then mounting in a 2.3% wt/vol 1,4-diazobicyclo-2,2,2-octane (DABCO) (Sigma, St. Louis, MO), 0.1 M tris(hydroxymethyl)aminomethane pH 8.0, 90% glycerol antifade solution.

Images were acquired using a Zeiss Axioskop (63×, 1.2 n.a. Plan Neofluar objective, Thornwood, NY) epifluorescence microscope equipped with a cooled charge-coupled device (CCD) camera (Photometrics, Tucson, AZ). The CCD microscope was fitted with highly plane-parallel emission filters that limit image displacement to ±1 pixel when switching filter channels. Image acquisition was performed using CCD image capture (Yale University). Pseudocoloring and merging were accomplished using Gene Join (Yale University) or Registration (Biological Design Systems, Pittsburgh, PA) software running on an Apple Macintosh computer. Figures 3–6 were composed from the individual source or merged images using Adobe Photoshop (Adobe Systems, Mountain View, CA) and printed directly using a digital dye-sublimation printer (Kodak, Rochester, NY).

RESULTS

Oligonucleotide-directed RNase H Digestion

To identify available single-stranded regions within the human U8 and U13 RNAs in their native snoRNP particles, we used an oligonucleotide-directed RNase H digestion assay. HeLa whole cell extracts were incubated with RNase H and either U8 or U13 specific oligonucleotides. For reference the sequences and secondary structures of U3, U8, and U13 RNAs are presented in

Figure 1. Oligonucleotides used in the RNase H experiments are named according to their relative antisense positions. The degradation of U8 and U13 RNAs was monitored by Northern blot analyses (Figure 2, A and B). For both RNAs the sequences upstream of box C are the most accessible for RNase H degradation. Oligonucleotides U8(1–15), U8(39–51), and U8(43–55) elicited efficient degradation of U8 RNA, whereas U8(81–96) targeted U8 only partially. Similarly, oligonucleotides U13(1–13) and U13(10–22) complementary to the 5' region of U13 targeted U13 RNA efficiently, whereas oligonucleotide U13(62–74) caused only partial digestion. Note that boxes C and D, suggested to be sites of protein binding (Baserga *et al.* 1991; Huang *et al.* 1992), are protected from digestion in both RNAs. Biotinylated 2'-OMe RNA oligonucleotides U8(1–15), U8(43–55), and U13(10–22) were further checked for specificity by selection of U8 and U13 RNAs from HeLa cell total RNA using streptavidin agarose (Matera, Tykowski, Steitz, and Ward, unpublished data). Oligonucleotide U8(43–55) specifically selected U8 RNA, whereas U8(1–15) also bound 5.8S rRNA. Presumably because of its low abundance, U13 RNA was selected only marginally above the nonspecific background using the U13(10–22) oligomer. We chose to use U8(43–55) and U13(2–22) as hybridization probes for FISH.

In Situ Localization of snoRNPs

U3, U8, and U13 snRNAs were localized in interphase HeLa cells using antisense DNA and 2'-OMe RNA oligonucleotides and FISH. As shown in Figure 3A, U3 was distributed throughout the entire nucleolus with a slight background in the cytoplasm and nucleoplasm. Although the pattern within the nucleoli was always somewhat heterogeneous, brighter areas were sometimes resolved as discrete spots (Figure 3B). These punctate spots are suggestive of the necklace-like structures often seen with anti-RNA polymerase I or hUBF/NOR-90 antibodies (Haaf *et al.* 1991; Zatzepina *et al.* 1993) (see also Figure 4), and the number of spots is in reasonable agreement with the interpretation that the individual beads of the necklaces represent approximately six active ribosomal RNA cistrons (Hozák *et al.* 1994). This localization pattern is consistent with previous results indicating that the U3 snoRNP remains attached to the nascent rRNA even after 5' ETS cleavage (Kass *et al.* 1990; Mougey *et al.* 1993a). Although the punctate signals seen in Figure 3B have not been pre-

Figure 3. Fluorescence in situ hybridization with U3, U8, and U13 antisense oligomers in HeLa monolayer cells. (A) A typical U3(64–74) signal (bottom right) and the corresponding DAPI-stained nuclei (top left). Fluorescence is detected throughout the nucleoli, although some regions are more intense than others. (B) The bright areas seen in A were sometimes resolved into a punctate pattern, perhaps corresponding to the distribution of rDNA transcription units (bottom). The DAPI-stained nucleus (top) is shown for comparison. (C) Double-label experiment with DAPI (top), U3(64–74) (middle), and anti-Fb (bottom). (D) A cell stained with DAPI (left) and hybridized with U13(2–22) (right). (E) U8(43–55) signal (right) is strikingly different from that of U3 and U13. Ring-like structures are detectable only in the nucleoli (see DAPI stain at left). F demonstrates that the ring-like U8 structures (right) are not readily visible in phase contrast (left) or DAPI (middle).

viously reported (at the level of the light microscope), other laboratories agree that U3 is distributed throughout the breadth of the nucleolus (Carmo-Fonseca *et al.* 1991; Puvion-Dutilleul *et al.* 1991; Jiménez-García *et al.* 1994). The distribution of U13 mirrors that of U3 (Figure 3D), although the signal is far less intense. Of all the snoRNPs that bind the nucleolar protein Fb, U3 is by far the most abundant (Baserga and Steitz 1993). As expected, the Fb-staining pattern closely resembles that of U3 (Figure 3C).

The U8 pattern is strikingly different from that of U3, U13, or Fb. Figure 3E shows that the distribution of U8 is restricted to several ring-like subdomains within the nucleolus. These structures are not readily visible in either the DAPI or phase-contrast images (see comparison with U8 probe in Figure 3F) but are highly reminiscent of the DFC, as seen by low-magnification electron microscopy (Jordan 1978; Puvion-Dutilleul *et al.* 1991). Thus it is reasonable to assume that if U8 is, indeed, restricted to the DFC, then the centers of the rings must correspond to FCs.

Localization of U8 Relative to rRNA Transcription Factors

RNA polymerase I is known to be localized exclusively within FCs (Scheer and Rose 1984; Reimer *et al.* 1987). To further investigate the relationship of the DFC-like rings in Figure 3, E and F to known components of the rRNA transcription machinery, the U8 oligomer was used in double-labeling experiments with antibodies against either RNA polymerase I or hUBF/NOR-90 (Figures 4 and 5). Individual nucleoli stained with pol I or NOR-90 displayed essentially three types of subnucleolar patterns. Some nucleoli exhibit a necklace-like arrangement, some are characterized by large spheroid structures, and others display a combination of both patterns (see Figure 4). Two elegant studies by Zatsepina *et al.* (1993) and Haaf *et al.* (1991) provide an explanation for these findings. As nucleoli become active in transcription, they begin to decondense; during the activation process, the intranucleolar distributions of pol I and NOR-90 change from the dot-like structures to the necklace-like ones. Interestingly, comparison of the raw images from Figure 4 and the merged images (Figure 5) clearly shows that U8 forms ring-like structures around *both* the larger dots and smaller beads. By extrapolating from what we know concerning the larger structures, we hypothesize that the smaller ringlets are mini-DFCs that are organized around each transcriptionally active rDNA repeat unit.

Localization of U3 and U8 in Mitosis

As mentioned above, some nucleolar components remain bound to the NORs during cell division, whereas others become dispersed throughout the cell. Two important cogs in the machinery of rRNA transcription, pol I and UBF/NOR-90, are stably associated with the NORs of metaphase chromosomes (Scheer and Rose 1984; Chan *et al.* 1991; Haaf *et al.* 1991). Jiménez-García *et al.* (1994) have recently shown that U3 snRNA is released into the cytoplasm during mitosis but begins to accumulate in prenucleolar bodies at telophase. In addition to U3, we find that U8 is also dispersed into the cytoplasm during mitosis (Figure 6). Thus it appears that proteins important in pol I transcription remain poised on the DNA in some form of preinitiation or arrested complex (Zatsepina *et al.* 1993). In contrast, the pre-rRNA processing components are released from their sites of action in early prophase and must be recruited back to the newly forming nucleoli as cytokinesis begins.

DISCUSSION

Immunoprecipitation of small RNAs from HeLa cells with anti-Fb antibodies reveals at least 10 different nucleolar species (reviewed in Filipowicz and Kiss 1993; Sollner-Webb 1993). Of these 10 RNAs only U3, U8, and U13 are known to be transcribed from their own promoters and to possess trimethyl-guanosine caps (Tyc and Steitz 1989). The RNAs of the other Fb snRNPs are seemingly derived from secondary RNA processing events within the introns of other gene transcripts (Fournier and Maxwell 1993; Sollner-Webb 1993).

U3 and U13 Colocalize with Fb in the Nucleolus

Using highly specific 2'-OMe oligonucleotide probes, we have shown that U3 and U13 are both inhomogeneously distributed throughout the nucleolus and that the distribution of U3 dominates the anti-Fb-staining pattern (Figure 3C). Moreover, although most of the nucleoli were diffusely (yet still nonuniformly) labeled with the U3 antisense oligomer, some nucleoli revealed a punctate pattern in addition to the general nucleolar fluorescence (Figure 3, A and B).

The U3 snRNP is thought to be involved in both early and late steps in pre-rRNA processing (see Baserga and Steitz 1993, for review). The punctate spots might, therefore, represent the individual rDNA transcription units and thus the site of the 5'-ETS cleavage reaction described by Kass *et al.* (1990) *in vitro*. Mougey *et al.*

Figure 4. Comparison of anti-U8 hybridization with other nucleolar proteins. In all panels the DAPI stain is shown on the left, the U8(43–55) oligo is in the middle, and the antibody stain is on the right. (A and B) U8(43–55) and RNA polymerase I antibodies. (C and D) U8(43–55) and anti-hUBF/NOR-90 serum. Some nucleoli are characterized by large dot-like antibody staining (thick arrows), others display multiple smaller dots (thin arrows), whereas a third type of nucleolus shows both large and small dots (arrowheads).

(1993) hypothesized that assembly of the terminal knobs (now realized to comprise the 5' ETS processing complex) may also aid in later pre-rRNA processing steps. Involvement of U3 in later steps (Savino and Gerbi 1990; Hughes and Ares 1991; Beltrame and Tollervey 1992) could account for the remainder of the hybridization signal. In agreement with this view, U3 has been detected in all three of the nucleolar subcompartments via electron microscopy (Fischer *et al.* 1991; Puvion-Dutilleul *et al.* 1991).

Studies with human autoimmune sera have shown that the nucleolar protein Fb is also detected in the nuclear organelles called coiled bodies (CBs) (Raska *et al.* 1990, 1991). Carmo-Fonseca *et al.* (1993) showed that an antisense 2'-O-allyl oligonucleotide probe did not detect U3 RNA in CBs. However, using a 4-kilobase genomic clone containing a U3 gene, Jiménez-García *et al.* (1994) have recently shown that a low level of U3 is detectable in nucleoplasmic CBs. Using antisense oligos to both U3 and U8, we were also unable to detect these RNAs in CBs, although the snoRNP protein fibrillarin was readily detectable within them.

As the above studies in human cells were taking place, a similar story was unfolding in experiments on *Xenopus* egg extracts. Bell *et al.* (1992) found that they were unable to detect the presence of U3 in CBs using FISH on nuclei formed in vitro using *Xenopus* egg extracts. However, Bauer *et al.* (1994) had no difficulty using tritiated riboprobes to demonstrate the presence of both U3 and U8 in the in vitro-assembled nuclei. As with any experiment, negative evidence must be viewed with caution.

U8 Is Recruited to Distinct Subnucleolar Regions that Resemble the DFC

Studies with anti-RNA polymerase I and anti-hUBF/NOR-90 antibodies have shown that during the course of nucleolar activation, increasing numbers of rDNA transcription units are harnessed to meet the metabolic demands of the cell (Haaf *et al.* 1991; Zatsepina *et al.* 1993). As more and more transcription units are turned on, the ribosomal chromatin decondenses until the antibody staining pattern resembles a nucleolar necklace. The adenosine analogue 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole (DRB) can also be used to decondense nucleoli. Voit *et al.* (1992) have shown that DRB inhibits casein kinase II-mediated phosphorylation of UBF, which may play a role in the decondensation process. Conversely, inhibition of transcription with actinomycin D or through serum deprivation reverses this trend, causing accretion of the protein into intense dot-

like structures (Rendón *et al.* 1992; Zatsepina *et al.* 1993). These larger dots are located within areas of the nucleolus that do not stain with anti-rRNA probes and are called "nucleolar vacuoles" (Matera, unpublished data). Interestingly, U8 RNA is organized in ring-like structures around the periphery of the pol I or UBF signals (Figures 4 and 5).

The precise localization of active rDNA transcription units within the nucleolus is still a matter of debate, but most agree that anti-polymerase I antibodies stain within (or near the periphery of) the FC. The distribution of U8 encircling that of pol I argues that U8 is concentrated in the DFC. Most striking are the images in which both the beads of the nucleolar necklace and the large dot-like vacuoles are present within the same nucleoli (Figure 4C). It is in these partially decondensed nucleoli that U8 can be seen to form mini-ringlets around each of the beads of the necklace and larger rings around the nucleolar vacuoles. Therefore, the recruitment of U8 and presumably other components of the pre-rRNA processing machinery around the pol I transcription complexes plausibly leads to formation of the DFC. Thus, the in situ evidence presented here is compatible with the in vivo experiments of Peculis and Steitz (1993) that suggest that U8 functions in rRNA processing steps subsequent to the initial 5' ETS cleavage event catalyzed by U3.

Dispersal of Nucleolar snRNPs During Mitosis

As cells exit G₂ and prepare for mitosis, the chromosomes begin to condense and ribosome synthesis is shut down. Several key factors involved in rRNA transcription remain tightly associated with the rRNA genes, including RNA polymerase I (Scheer and Rose 1984; Haaf *et al.* 1991), topoisomerase I (Rose *et al.* 1988), and UBF/NOR-90 (Chan *et al.* 1991; Rendón *et al.* 1992; Zatsepina *et al.* 1993). These experiments suggest that the pol I transcription machinery remains ready for action after telophase. Curiously, both U3 and U8 are released from the NORs during cell division. To become relocalized in the nucleoli, it appears that these snRNPs must first find their way into the prenucleolar bodies (PNBs). In support of this model are the recent findings of Bauer *et al.* (1994), who showed that the PNBs formed in *Xenopus* egg extract contain both U3 and U8 (in addition to the spliceosomal RNAs [U1, U2, U4/5/6] and the histone pre-mRNA processing snRNP U7). On the basis of their composition, Bauer *et al.* have termed these PNBs coiled bodies. Thus as the reorganization of the nucleus proceeds, the RNAs from three different processing pathways must be sent to their proper destin-

Figure 5. (A–D) The same cells shown in Figure 4 were pseudocolored and merged to reveal that both the large spheroid structures and the smaller necklace-like ones seen with the anti-RPI and NOR-90 sera (red) are surrounded by larger and smaller rings, respectively, of anti-U8 fluorescence (green). The DAPI counterstain is shown in blue.

Figure 6. U8 is released from the NORs during mitosis. (A) DAPI and (B) U8(43–55). Like U3 and unlike RPI and NOR-90 (see text), the U8 signal is dispersed throughout the metaphase cell (left) and is clustered within the nucleoli of the two interphase cells (right).

ations. Perhaps common proteins (e.g., fibrillarin in the case of the nucleolar RNAs) play a role in this cellular trafficking conundrum.

Thus it seems likely that nucleolar formation is a cascade of events that temporally succeed the initiation of RNA polymerase I transcription. As nascent pre-rRNA chains emerge from the polymerase, they nucleate assembly of the processing machinery. The maturing rRNAs then acquire their various proteins as the large and small ribosomal subunits are assembled and begin their trek to the cytoplasm. It is the convergence of all of these metabolic activities within one confined region of the nucleus that gives rise to the structure of the nucleolus.

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