Pol I Transcription and Pre-rRNA Processing Are Coordinated in a Transcription-dependent Manner in Mammalian Cells[□]

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Pre-rRNA synthesis and processing are key steps in ribosome biogenesis. Although recent evidence in yeast suggests that these two processes are coupled, the nature of their association is unclear. In this report, we analyze the coordination between rDNA transcription and pre-rRNA processing in mammalian cells. We found that pol I transcription factor UBF interacts with pre-rRNA processing factors as analyzed by immunoprecipitations, and the association depends on active rRNA synthesis. In addition, injections of plasmids containing the human rDNA promoter and varying lengths of 18S rDNA into HeLa nuclei show that pol I transcription machinery can be recruited to rDNA promoters regardless of the product that is transcribed, whereas subgroups of pre-rRNA processing factors are recruited to plasmids only when specific pre-rRNA fragments are produced. Our observations suggest a model for sequential recruitment of pol I transcription factors and pre-rRNA processing factors to elongating pre-rRNA on an as-needed basis rather than corecruitment to sites of active transcription.

INTRODUCTION

Nucleoli contain hundreds of tandem rRNA genes and a large number of factors necessary for transcription of ribosomal DNA (rDNA), processing of pre-rRNA, and ribosome assembly. Electron microscopic imaging of nucleoli reveal three distinct subcompartments: the fibrillar centers (FC), dense fibrillar components (DFC), and granular components (GC; Huang, 2002). It is at the FC/DFC border that RNA polymerase I (pol I) and other pol I-specific transcription factors associate to form the pol I preinitiation complex (PIC) at rRNA promoters (Grummt, 2003; Grummt and Pikaard, 2003) for transcription of rDNA (Raska *et al.*, 2004). In vivo activation of the human rRNA promoter requires protein-protein interactions between the DNA-binding protein, upstream binding factor (UBF1), and SL1 for the targeting of pol I and stable PIC formation (Bell *et al.*, 1988; Friedrich *et al.*, 2005). In combination with several other evolutionarily conserved proteins, these and other pol I transcription factors confer pol I promoter specificity to produce a single polycistronic pre-rRNA transcript (45S pre-rRNA in humans; Grummt, 2003; Grummt and Pikaard, 2003).

A large number of small nucleolar ribonucleoproteins (snoRNPs), composed of small nucleolar RNAs (snoRNAs) and their associated proteins, as well as more than 100 protein cofactors, are responsible for processing the 45S transcript (Fromont-Racine *et al.*, 2003; Granneman and Baserga, 2005). The box C/D snoRNA U3 and its associated proteins are required for the initial cleavages of the 5' end of pre-rRNA at sites A₀, A₁, and A₂ and are thereby involved in the release of the 18S precursor (Fromont-Racine *et al.*, 2003; Granneman and Baserga, 2005). Yeast U3 snoRNA exists in at least two types of complexes in vitro: a 12S monoparticle (Billy *et al.*, 2000; Granneman *et al.*, 2003) and a larger ~90S complex termed the small subunit (SSU) processome (Fabrizio *et al.*, 1994; Dragon *et al.*, 2002). The 12S monoparticle presumably includes U3 snoRNA in addition to the core box C/D snoRNPs Nop1 (fibrillarin), Nop56, Nop58, and Snu13 (15.5K; Fabrizio *et al.*, 1994; Watkins *et al.*, 2000) and the U3-specific protein Rrp9 (U3-55K; Lubben *et al.*, 1993). The 90S U3 complex contains these five proteins in addition to several other proteins that associate specifically with U3 snoRNA, including Sof1, Imp3, Imp4, Mmp10p, Rrp9p, and the t-UTPs, among others (Dragon *et al.*, 2002; Terns and Terns, 2002; Granneman and Baserga, 2005). It has been hypothesized that the cleavage and release of pre-40S rRNA at A₂ by the SSU may trigger the association of the large subunit (LSU) processing factors with the remaining rRNA (Tschochner and Hurt, 2003). This pre-60S rRNA then undergoes further processing facilitated by the LSU processome, which contains numerous other factors including B23 (Savkur and Olson, 1998; Itahana *et al.*, 2003). Ultimately, 18S, 5.8S, and 28S ribosomal RNAs and their associated ribosomal proteins are assembled into nearly mature preribosomal particles and exported from the nucleus.

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Ribosome biogenesis is a complex process that involves all three classes of transcription to produce up to 80% of total RNA in a proliferating cell (Warner, 1999; Rudra and Warner, 2004). Consequently, the process must be tightly regulated and coordinated to ensure that there are a sufficient number of ribosomes for protein synthesis under various growth conditions. The transcription of rDNA and pre-rRNA processing are believed to be key steps in the regulation of ribosome biogenesis, and recent studies indicate that the two processes are coordinated in plants and yeast (Granneman and Baserga, 2005). Electron micrographs of yeast have shown that the U3 snoRNA-containing 80S SSU processome is a component of the terminal knobs attached to the 5' ends of nascent pre-rRNA (Dragon *et al.*, 2002) and that the processing of pre-rRNA takes place co-transcriptionally (Osheim *et al.*, 2004). These results indicate that processing factors associate with and process the multiple nascent pre-rRNAs that are simultaneously transcribed from a single rRNA gene (Miller and Beatty, 1969). Although the coordination between pol I transcription and pre-rRNA processing is evident in yeast and plants, it remains unclear how the key steps in this process are associated, especially in mammalian cells. The coordination between transcription and pre-rRNA processing could be facilitated through at least two different mechanisms. The transcription and processing factors could be corecruited to the site of transcription in a large complex, as the pol II system is coordinated, which is an idea that is supported by a number of published studies (Fath *et al.*, 2000; Saez-Vasquez *et al.*, 2004). Alternatively, processing factors could be recruited to the sites of transcription only when the nascent pre-rRNA substrate becomes available.

In this report, we have used both biochemical and cell biological approaches to distinguish between these possibilities and further dissect the association between pol I transcription machinery and pre-rRNA processing in mammalian cells. We found that pol I transcription factors and pre-rRNA processing factors can be in the same complex in mammalian cells as assayed by immunoprecipitations. Our results also suggest that complex formation depends on active pol I transcription. Additionally, microinjection of plasmids containing the rDNA promoter and varying lengths of rDNA fragment demonstrate that pol I transcription factors can be recruited to rDNA promoters regardless of the product that is transcribed. In comparison, subgroups of pre-rRNA processing factors are recruited to the plasmids only when pre-rRNAs specifically are being produced. Altogether, our data demonstrate a coordinated relationship between pol I transcription machinery and subgroups of pre-rRNA processing factors that is sequential and mediated by active RNA synthesis.

MATERIALS AND METHODS

Cell Culture and Transfection

HeLa (human cervical carcinoma) cells were grown in DMEM in 10% FBS and 5% P/S. For transient transfections, expression constructs were electroporated into HeLa cells using standard procedures and incubated for 18–24 h before experimentation (Ausubel *et al.*, 2002).

Immunoprecipitation and Western Blots

Whole cell lysates were prepared from transfected or untransfected HeLa cells by sonication in lysis buffer (10 mM Tris, pH 8.0, 150 mM NaCl, 20% glycerol, 1 mM EDTA, 1 mM DTT, 0.5% NP40, supplemented with protease inhibitor cocktail; cat. no. P-8340, Sigma, St. Louis, MO) before centrifugation. For endogenous proteins, cell lysate was added directly to a 50% slurry of protein A-Sepharose beads (Amersham Biosciences AB, Uppsala, Sweden) in RSB-100 buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, P-8340) and precleared at 4°C for 1 h. The fluorescence of cell lysates con-

taining GFP fusion proteins was measured using a TD-700 Fluorometer (Turner Designs, Sunnyvale, CA), normalized, and then precleared. Lysates were incubated with beads conjugated with the appropriate antibody (see below for dilutions) for 2–4 h at 4°C, washed at least six times with RSB-100/P-8340, resuspended in Laemmli buffer, and boiled before separation using 12% SDS-PAGE. Experiments requiring RNase A treatment were incubated with 200 µg/ml nuclease at 37°C for 1 h, either after preclearing immediately before immunoprecipitation or after RSB-100 washes before adding Laemmli buffer. ActD (4 mg/ml) was added to cells 2 h before harvest and lysate preparation. Analysis of Western blot band intensity was done on a Kodak Image Station 440CF and with the Kodak 1D image analysis software (Kodak Molecular Imaging, New Haven, CT).

Human anti-UBF antibody (Chan *et al.*, 1991; Imai *et al.*, 1994) was used at a 1:500 dilution for immunoprecipitations. Mouse monoclonal anti-UBF (F9) antibody (cat. no. sc-13125; Santa Cruz Biochemicals, Santa Cruz, CA) was used at 1:200 for Western blots. Fibrillarin mAb P2G3 (Lopez-Iglesias *et al.*, 1988) was used at 1:5000 for Western blots. Monoclonal and polyclonal anti-GFP antibodies (Clontech, Palo Alto, CA) were used at 1:500 and 1:5000 dilutions for immunoprecipitations and Western blots, respectively. Affinity-purified rabbit antibodies against human Nop58 (Watkins *et al.*, 2000) were used at a 1:5000 dilution for Western blots. Chicken anti-hImp4 (Granneman *et al.*, 2003) was used at a dilution of 1:5000 for Western blot analysis.

Plasmids

The plasmid pHrB was a gift from J. Sylvester (Wilson, 1982; Maden *et al.*, 1987). The plasmid pHR-BES was a gift from L. Rothblum (Grummt *et al.*, 1982; Miesfeld and Arnheim, 1982). pHR-BESΔrRNA was created by PCR amplification of the pol I promoter from pHR-BES using primers 5'-GGAATTC-CGAGGCCCTTTCGCTCTTCAA-3' and 5'-CCCAAGCTTGGGCCAGAGGA-CAGCGTGTC-3'. The resulting 620-base pair fragment, which lacks all pre-rRNA coding sequences, was cloned into EcoRI and HindIII restriction sites of pBR322. The pEGFP-C1-fibrillarin deletion mutants were subcloned from the full-length construct (Chen and Huang, 2001) into the KpnI and BamHI MCS of pEGFP-C1 (Clontech).

Reverse Transcription PCR

HeLa cells were transfected with plasmids, and total RNA was isolated 24 h later using the RNeasy kit (Qiagen, Chatsworth, CA). cDNA was produced from the RNA using random hexamers and Multiscribe Reverse Transcriptase with the Taqman reverse transcription (RT)-PCR kit (Applied Biosystems-Roche, Branchburg, NJ). RT-PCR for the plasmid-encoded RNAs was performed using primers that annealed within the plasmid backbone (pHrBESΔrRNA) or between 100 and 450 nucleotides upstream of the 3' end of inserted rRNA sequences and 60–250 nucleotides into the plasmid backbone (pHrBES and pHrB). The specific primer pairs are as follows: pHrBESΔrRNA 5'-ATA TCG TCC ATT CCG ACA GC-3' and 5'-AGT GGC TCC AAG TAG CGA AG-3'; pHrBES 5'-GTG CGG CGA CGA TAG TCA TGC CCC-3' and 5'-ACC GTC CTT CTC GCT CCG CCC CGC C-3'; pHrB 5'-GTG CCA CCT GAC GTC TAA GAA ACC-3' and 5'-GGA ATT GAC GGA AGG GCA CCA CCA GG-3'. Controls include reactions run without addition of reverse transcriptase (–RT), reactions using total RNA isolated from untransfected HeLa cells (HeLa Lysate), and reactions containing no RNA (water only).

Microinjection

HeLa cells were seeded onto etched coverslips and grown for 48 h until they reached 50% confluency. Plasmids were passed through a 0.22-µm filter, suspended in 0.5× PBS (68.5 mM NaCl, 1.3 mM potassium chloride, 5 mM phosphate buffer), and quantified spectrophotometrically. An Eppendorf Femtojet microinjection system was used to deliver plasmids (0.35 mg/ml) into HeLa nuclei with an injection pressure of 145 hPa for 0.3 s, as previously described (Gasiorowski and Dean, 2005). Approximately 100 cells were microinjected for each experiment, and all experiments were performed at least three times.

DNA In Situ Hybridization and Immunofluorescence

Two hours after plasmid microinjection, cells were rinsed with 1× PBS, permeabilized in 0.5% Triton X-100 for 45 s, and fixed at –20°C in a 1:1 methanol:acetone solution for 5 min. In situ hybridizations were carried out using nick-translated pBR322 DNA probe as previously described (Dean, 1997). After the final wash of the in situ hybridization protocol, the coverslips were blocked with 1 mg/ml BSA in PBS for 1 h at room temperature, rinsed in PBS, and incubated with primary UBF (1:50; Chan *et al.*, 1991; Imai *et al.*, 1994), fibrillarin (1:500; cat. no. mca38f3; Encor Biotechnology, Alachua, FL), Nop58 (1:500; Watkins *et al.*, 2000), RPA39/40 (1:50; A. Lamond, University of Dundee, Scotland), B23 (1:75; cat. no. B-0556, Sigma), or Sof1 (1:200; DL253, a purified polyclonal rabbit antibody raised against recombinant human Sof1, D.J.L., unpublished data) antibodies in PBS containing 0.5 mg/ml BSA for 2 h at 37°C. Coverslips were washed in PBS and secondary antibodies (1:200) conjugated to Alexa 555 or Alexa 647 fluorophores (Molecular Probes, Eugene, OR) were applied in PBS containing 0.5 mg/ml BSA for 1 h at room

Figure 1. UBF interacts with fibrillarin. (A) Immunoprecipitation reaction using an anti-UBF antibody in HeLa cell lysates and a Western blot against fibrillarin. (B) Immunoprecipitation reaction performed in HeLa cells transiently transfected with GFP-fibrillarin (full length). An antibody against endogenous UBF was used for the Western blot.

temperature. Coverslips were washed in PBS and mounted with DAPI and anti-fade DABCO (Molecular Probes).

For sequential RNA and DNA fluorescence in situ hybridization, HeLa cells seeded on etched CELLocate coverslips (Eppendorf, Hamburg, Germany) were injected and fixed after 2 h in 4% formaldehyde, 10% acetic acid, and PBS for 10 min. The Cy3-labeled in situ oligonucleotide probe against human U3 snoRNA was used as previously described (Narayanan *et al.*, 2003). The etched area of the CELLocate coverslips were imaged for U3 snoRNA, DAPI-stained nuclei, and phase contrast, and then the DNA in situ hybridization was performed as stated above. Individual cells with injected plasmid DNA were mapped back to the U3 snoRNA images and identical subnuclear occupancy was determined.

Microscopy

HeLa cells were observed under a Leica DMRA2 epifluorescence microscope with a 100× objective (NA 1.35; Deerfield, IL). Images were acquired with OpenLab software (Improvision, Lexington, MA). Colocalization was verified by confocal microscopy using a Zeiss LSM510 (Thornwood, NY) or by deconvolution on the Leica DMRA2 microscope using Velocity Restoration software (Improvision).

RESULTS

rRNA Transcription Factor, UBF, Forms a Complex with the pre-rRNA Processing Factor, Fibrillarin

To evaluate the coordination of rDNA transcription and pre-rRNA processing in mammalian cells, we first examined whether pol I transcription and processing factors can be found in the same complex in mammalian cells. Coimmunoprecipitation assays followed by Western blots were performed on HeLa cell lysates to evaluate the association between UBF, a transcription factor, and fibrillarin, a pre-rRNA processing factor and a component of the box C/D snoRNPs (including U3). After immunoprecipitation of HeLa cell lysates with an anti-UBF antibody, we were able to detect fibrillarin by Western blot using a fibrillarin-specific antibody (Figure 1A). The control lane containing beads alone detected no protein, demonstrating the specificity of the immunoprecipitation. This finding suggests that UBF and fibrillarin could be in the same complex in mammalian cells. To confirm this finding, reciprocal immunoprecipitations were carried out using several commercially available antibodies against fibrillarin. Unfortunately, these antibodies were unable to immunoprecipitate fibrillarin, although they can specifically detect fibrillarin in immunofluorescence and Western blot assays. Using an alternative approach, we transfected cells with GFP-tagged fibrillarin, which has been shown to have localization and biochemical extraction properties similar to endogenous fibrillarin (Dundr *et al.*, 2000; Chen and Huang, 2001) and immunoprecipitated the tagged fibrillarin with an anti-GFP antibody. Western blot of the precipitates with an anti-UBF antibody shows the coprecipitation of UBF with tagged

Figure 2. Fibrillarin interacts with UBF in the form of a snoRNP. (A) Schematic representation of N-terminal GFP-fibrillarin fusion proteins with various truncation mutations at indicated amino acid residues. (B) Immunoprecipitations using anti-GFP antibodies in HeLa cells that were transiently transfected with a variety of constructs: beads alone (first lane, –), pEGFP-C1-Fib (second lane, FL), pEGFP-C1-Fib1 (third lane, 1), pEGFP-C1-Fib2 (fourth lane, 2), pEGFP-C1-Fib3 (fifth lane, 3), pEGFP-C1-Fib3.5 (sixth lane, 3.5), pEGFP-C1-Fib4 (seventh lane, 4), pEGFP-C1-Fib5 (eighth lane, 5), pEGFP-C1-Fib6 (ninth lane, 6), pEGFP-C1 (tenth lane, G). No plasmids were transfected into cells for lysate that was loaded into lane 11, nor was this lysate used for an immunoprecipitation. Anti-UBF (top panel), anti-Nop58 (second panel), anti-GFP (third panel), and anti-Imp4 (bottom panel) antibodies were used for detection on Western blot.

fibrillarin (Figure 1B), confirming that they can exist in the same complex in HeLa cells.

UBF Interacts with snoRNPs Rather Than Free Fibrillarin

To determine whether UBF interacts with fibrillarin in its free form or within snoRNPs, we designed a series of GFP-fibrillarin truncation mutants (Figure 2A). The rationale of the experiment is that if UBF only interacts with fibrillarin mutants that are capable of forming snoRNPs, but not those that are unable to be in a complex with their RNP partners, it would suggest that fibrillarin interacts with UBF through snoRNPs rather than in a free form.

Each GFP-tagged deletion mutant was transiently transfected into HeLa cells and visualized by indirect microscopy to determine their nucleolar localization patterns (unpublished data and see Snaar *et al.*, 2000). Of the mutants tested, GFP-Fib5 is the only construct with a localization pattern similar to that of the wild-type protein, indicated by punctate speckles concentrated within the nucleolus. Although GFP-Fib1 and 2, showed a uniform, diffuse localization throughout the nucleoli, GFP-Fib3, 3.5, and 4 had diffuse but aberrant distribution patterns within the nucleoli. To evaluate the interaction between GFP-tagged fibrillarin mutants with other box C/D snoRNP components and UBF, we

performed immunoprecipitation experiments using anti-GFP antibodies in transfected HeLa cells. The efficiency of each transfection was determined using a fluorometer and normalized to the lowest value when prepared for the experiment. Immunoprecipitation of full-length GFP-fibrillarin from transiently transfected HeLa cell lysates precipitates other well-characterized U3 snoRNP proteins, Nop58 and Imp4 (Figure 2B; second lane, FL). These data also show that GFP-fibrillarin functions similarly to endogenous fibrillarin, and GFP tagging does not significantly impact the biochemical characteristics of the protein, including its ability to form a snoRNP. Immunoprecipitation of the full-length GFP-fibrillarin fusion protein as well as GFP-Fib5 were able to effectively pull down the transcription factor UBF (Figure 2B; FL and 5) as expected, because both constructs have localization patterns similar to endogenous fibrillarin. Additionally, UBF was detected in the immunoprecipitations of a subset of the GFP-fibrillarin deletion mutant constructs, GFP-Fib1, GFP-Fib2, and GFP-Fib6 as determined by Western blot (Figure 2B; 1, 2, and 6, respectively). GFP-fibrillarin mutant constructs missing any part of amino acids 133-222, namely mutants GFP-Fib3, GFP-Fib3.5, and GFP-Fib4, did not immunoprecipitate detectable quantities of UBF on a Western blot. The expression of each GFP-tagged construct was detected by Western blot (Figure 2B; third panel) to confirm that our results are not due to the lack of expression of the constructs after transfection. Nontransfected cell lysates were used as a negative control for the immunoprecipitation reaction (Figure 2B; first lane, –), as were lysates from cells that had been transfected with the pEGFP-C1 vector that only expresses GFP (Figure 2B; G).

To evaluate whether the ability of GFP-fibrillarin to immunoprecipitate UBF coincides with its capacity to form snoRNPs, the same blot was probed with an antibody against Nop58, another core component of box C/D snoRNPs (Figure 2B; second panel). Nop58 was chosen because immunoprecipitation studies in yeast show that fibrillarin binds to box C/D RNA independent of Nop58 (Gautier *et al.*, 1997; Lafontaine and Tollervy, 1999, 2000) and additional site-specific cross-linking analyses confirm and map the independent association of Nop58 and fibrillarin with their respective box C/D elements (Cahill *et al.*, 2002). Therefore, fibrillarin can only be coprecipitated with Nop58 through being a part of snoRNPs. Our results show that a similar pattern of Nop58 bands was detected when compared with UBF (Figure 2B; top panel), demonstrating that the coprecipitation of UBF correlates with the ability of fibrillarin to be a part of snoRNPs. To further evaluate whether the early pre-rRNA processing complex containing U3 snoRNPs is associated with UBF, we examined the association between UBF, fibrillarin, and Imp4, a noncore U3 snoRNP-specific protein (Lee and Baserga, 1999; Wehner and Baserga, 2002). Immunoprecipitations of GFP-tagged fibrillarin mutants were repeated and followed by Western blots with antibodies against UBF and Imp4 (Figure 2B, bottom panel). The same GFP-tagged fibrillarin mutants that interact with UBF and Nop58 were also able to immunoprecipitate the Imp4 protein (Figure 2B; FL, 1, 2, 5, and 6). Furthermore, GFP-fibrillarin mutant constructs missing any part of amino acids 133-222, GFP-Fib3, GFP-Fib3.5, and GFP-Fib4, that were not able to pull down UBF and Nop58, were also unable to pull down Imp4 (Figure 2B; 3, 3.5, and 4). Amino acids 133-222 of fibrillarin contain two of the three strictly conserved positive residues surrounding the functional methyltransferase binding pocket (Lys143, Arg195, and Lys265) of fibrillarin (Deng *et al.*, 2004). It is likely that these positive residues interact with the negatively charged

Figure 3. The interaction between UBF and fibrillarin is dependent on the integrity of RNA. Immunoprecipitation reactions of HeLa cell lysates were performed using anti-UBF antibodies. Western blots were performed using two processing factors, fibrillarin and Nop58, as indicated. Beads alone were incubated with HeLa cell lysate as a negative control (first lane, beads alone), and HeLa cell lysate was loaded as a positive control (last lane, lysate). Anti-UBF immunoprecipitations were treated with RNase A (third lane, RNase), or the transcription inhibitor, actinomycin D (fourth lane, ActD). Band intensities were normalized against anti-UBF immunoprecipitation reactions that were untreated (second lane, untreated).

phosphate backbone of the box C/D RNA or the box C/D RNA/rRNA duplex and are necessary to form functional snoRNPs (Deng *et al.*, 2004). Therefore, the deletion of two or more of these residues, as occurs with our mutants, might interfere with the nucleic acid binding capabilities of fibrillarin and consequently disrupt the formation of a snoRNP. Together, our results suggest that the interaction between UBF and fibrillarin is most likely dependent on the ability of fibrillarin to be assembled into snoRNPs, one of which is U3 snoRNP, thereby supporting the idea that UBF interacts with snoRNP complexes rather than with free fibrillarin. These data are consistent with reports that pol I and pre-rRNA processing factors (U3 snoRNP) are in the same complex in yeast (Fath *et al.*, 2000).

RNase A and Actinomycin D Disrupt Complex Formation between rRNA Transcription and Processing Factors

The association between pol I transcription factors such as UBF and pre-rRNA processing factors like fibrillarin could occur by at least two different mechanisms. These factors could be corecruited to the rDNA promoter in one complex, or they could be recruited separately, possibly based on the need for processing newly synthesized rRNA. To begin to distinguish between the two possibilities, we evaluated the necessity for RNA and rDNA transcription in the association between fibrillarin and UBF. HeLa cell lysates were treated with nucleases before and/or post immunoprecipitations using an anti-UBF antibody (Figure 3; RNase). The results of these experiments were quantified using Kodak 1D image analysis software to measure the intensity of fibrillarin bands on several different Western blots (Figure 3). The value of the band intensity for an experimental lane (Figure 3; beads alone, RNase or ActD) was normalized against the intensity of the untreated sample (Figure 3; untreated), and our results show a significant decrease in the amount of endogenous fibrillarin that could be precipitated. Band intensity recorded for fibrillarin when immunoprecipitation reactions were treated with RNase A was 53.1% (Figure 3; RNase) that of the untreated reactions (Figure 3; untreated), suggesting that the integrity of RNAs is important for the UBF-fibrillarin containing complex. RNase A treatment impacts two types of RNAs that could be associated with the complex, snoRNAs, and nascent pre-rRNA. To determine the role of nascent pre-rRNA in this complex, we performed

Figure 4. rRNA transcription factors can be recruited to an injected plasmid that contains the rDNA promoter only. (A) A schematic drawing of the three plasmids used in our experiments. (B) pHr-BES Δ rRNA contains the rDNA promoter and no rRNA coding sequence. (C) Immunofluorescence for control, un.injected cells shows the normal nucleolar localization of the transcription factor, UBF, and pol I subunit, RPA39. (D) Both UBF and RPA39 colocalize with pHr-BES Δ rRNA outside of the nucleolus 2 h after injection. (E) Injection of pHr-BES Δ rRNA does not alter the localization of fibrillarin and Nop58, which remain within the nucleolus. Scale bar, 10 μ m.

immunoprecipitations using anti-UBF antibodies on lysates from cells treated with the transcription inhibitor actinomycin D (ActD; Perry, 1963). The amount of fibrillarin that could be detected by Western blot from immunoprecipitates derived from ActD-treated cells (Figure 3; ActD) was 37.2% of those derived from untreated cells (Figure 3; untreated). However, treatment with RNase or ActD both failed to completely abolish the interactions between either UBF and fibrillarin or Nop58. The partial disruption of complex formation is most likely due to incomplete transcription inhibition or digestion, or it is possible that complexes that are already formed provide some protective resistance to nucleases and/or are stable during transcription inhibition. Nevertheless, the significant decrease in the amount of coprecipitated proteins suggests that the interaction between the pol I transcription factor UBF and the pre-rRNA processing factor fibrillarin could be mediated by nascent rRNA transcripts. To further test this hypothesis, we designed the following experiments using cell biological approaches.

An Ectopically Introduced Plasmid Containing Only the rDNA Promoter Recruits pol I Transcription But Not pre-rRNA Processing Factors

To further investigate whether the association between pol I transcription and pre-rRNA processing factors is mediated through active rDNA transcription, we devised a series of experiments to examine the recruitment of these factors to sites of active pol I transcription. Because both transcription and processing factors are localized within the nucleolus throughout interphase, it is difficult to dissect the recruitment of either group of factors to actively transcribing rDNA loci. To circumvent this problem, we have microinjected a series of pol I promoter-containing plasmids with varying lengths of rDNA coding sequences into HeLa cell nuclei (Figure 4A) and examined the factors that are recruited to the ectopically expressed vectors.

First, we created pHr-BES Δ rRNA, a derivative of pHr-BES (Grummt *et al.*, 1982; Miesfeld and Arnheim, 1982), that contains the ~600-base pair human rDNA promoter but has

had all rRNA sequences removed and thus produces no pre-rRNA (Figure 4B). Active transcription of the vector DNA directed by the pol I promoter was confirmed in transfected cells by RT-PCR using primers specific to the pBR322 backbone (Supplementary Figure 1). If pol I transcription and pre-rRNA processing factors are recruited to the rDNA as a complex, both types of factors should colocalize with the injected pHr-BES Δ rRNA plasmid. Conversely, if the processing factors are recruited based on the production of pre-rRNA, they should not be recruited to a plasmid that does not produce pre-rRNA.

Plasmids were microinjected into HeLa nuclei, and the cells were fixed for in situ hybridization using a plasmid-specific probe to determine the localization of the exogenous plasmids. Pol I transcription and pre-rRNA processing factors were detected by immunofluorescence in the same cells. The factors tested include pol I transcription factor UBF and a pol I subunit, RPA39/40, as well as pre-rRNA processing factors, fibrillarin and Nop58, both of which associate with boxC/D snoRNAs including U3 snoRNP. Two hours after injection, in situ hybridization and immunofluorescence analyses revealed that UBF and the pol I subunit RPA39/40 colocalized with pHr-BES Δ rRNA DNA (Figure 4D). The localization patterns of injected cells were compared with the un.injected cells on the same coverslip, which served as internal controls that did not show any foci labeling outside of nucleoli (Figure 4C). These results demonstrate that exogenously expressing plasmids containing the pol I promoter can recruit the pol I transcription machinery. We then asked whether pre-rRNA processing factors could also be recruited to the plasmid. Simultaneous detection of the injected plasmid DNA by in situ hybridization and early processing factors fibrillarin and Nop58 by indirect immunofluorescence showed that the processing factors failed to be recruited to the pHr-BES Δ rRNA foci in the nucleoplasm (Figures 4E). These results demonstrate that although pol I transcription and processing factors can be found in the same biochemical complex (Figures 1–3), their recruitment to the rDNA promoter can be visualized separately; thus, it

Figure 5. rRNA transcription factors colocalize with an exogenously expressed rDNA plasmid that contains the rDNA promoter and 5' rDNA coding sequence. (A) pHr-BES contains the rDNA promoter and 5' rDNA coding sequence. (B) pHr-BES microinjected into HeLa nuclei colocalizes with UBF within the nucleoplasm 2 h after injection. (C) RPA39/40 is also found outside of nucleoli and colocalizes with injected pHr-BES within the nucleoplasm. Scale bar, 10 μ m.

is unlikely that the two groups of factors are corecruited as a single complex to the rDNA promoter. These observations support a model in which the coordination of pol I transcription and pre-rRNA processing factors is mediated by the synthesis of pre-rRNA, and not nonspecific RNA, such as that produced from the pBR322 vector sequence.

A Plasmid Containing the rDNA Promoter and 5' rDNA Coding Sequences Recruits Both pol I Transcription and pre-rRNA Processing Factors

To determine whether the association of pre-rRNA processing machinery is dependent on the production of pre-rRNA, we injected pHr-BES (Grummt *et al.*, 1982; Miesfeld and Arnheim, 1982) into HeLa nuclei. Plasmid pHr-BES has a 1.2-kb insert containing the human rDNA promoter and ~700 bps of 5' external transcribed sequence (ETS) that includes the A₀ cleavage site (Figures 4A and 5A). This plasmid was shown to produce the appropriate rRNA in vitro (Grummt *et al.*, 1982; Miesfeld and Arnheim, 1982) and RT-PCR product when transfected into HeLa cells (Supplementary Figure 1). In addition to UBF, RPA39/40, fibrillarin and Nop58 used in the previous experiments, we tested for the recruitment of other pre-rRNA processing factors: U3 snoRNA, Sof1, and B23. Sof1, a U3-specific component of the SSU processome, can serve as an indicator for the later stages of SSU formation and 18S rRNA processing (Jansen *et al.*, 1993; Dragon *et al.*, 2002). B23 was chosen as an additional processing factor for our experiments because of its role as an internal transcribed sequence (ITS)2-specific endoribonuclease (Savkur and Olson, 1998; Itahana *et al.*, 2003). However, the biphasic nature of pre-rRNA processing (Liang and Fournier, 1997; Grandi *et al.*, 2002), which physically separates the 40S and 60S processing pathways does not favor the association of B23 with pre-rRNA cotranscriptionally and therefore B23 served as a negative control for the association with pol I transcription machinery.

Plasmids were injected into HeLa nuclei and the cells were fixed for in situ hybridization and immunofluores-

cence as described below. Immunofluorescence done on uninjected control HeLa nuclei on the same coverslips showed that each of the factors tested localizes primarily to the nucleolus (Figures 4C and 6, A and E). Two hours after injection, the localization of the pHr-BES plasmid as detected by in situ hybridization showed a punctate distribution within the nucleoplasm (Figures 5, B and C, and 6, B, D, and F). Antibodies against pol I transcription factors UBF and RPA39/40 colocalized with the plasmid DNA (Figure 5, B and C), demonstrating the recruitment of pol I transcription factors to the sites of plasmids containing the rDNA promoter, similarly to that of pHr-BES Δ rRNA. Next, we performed in situ hybridizations to the plasmid DNA and immunofluorescence for early processing factors fibrillarin and Nop58 on cells injected with pHr-BES plasmid. Contrary to our observations of pHr-BES Δ rRNA injected cells, these early processing factors were recruited to the nucleoplasmic pHr-BES DNA foci in injected nuclei (Figure 6B). To clarify whether U3 snoRNP was recruited, we performed double labeling of both the plasmid DNA and U3 RNA after injection. To clearly separate the signals, in situ hybridization with a specific oligo probe for U3 was performed first, and the nuclear distribution of U3 snoRNA was imaged (Figure 6C). Subsequently, the same cells underwent DNA in situ hybridization using a probe specific for the plasmid backbone (Figure 6D). The distribution of the injected DNA was imaged and aligned with the results of the U3 snoRNA distribution from the exact same cells (Figure 6, C and D). The aligned images show that both the plasmid DNA and U3 snoRNA are localized in the same subnuclear area. These findings indicate that an exogenous plasmid containing the pol I promoter and 5' rDNA coding sequence (including A₀ site) is able to recruit both pol I transcription machinery and early processing factors, including the U3 snoRNP. This observation further supports the hypothesis that the recruitment of transcription factors and processing factors to the site of rRNA transcription are separate events and that processing factor recruitment is mediated by the production of pre-rRNA.

Figure 6. Core U3 pre-rRNA processing factors colocalize to pHR-BES foci, whereas others do not. (A) Core U3 snoRNP-processing factors, fibrillarin and Nop58, localize to nucleoli in un.injected cells. (B) Fibrillarin and Nop58 are found outside nucleoli, in the nucleoplasm, 2 h after nuclei are injected with pHR-BES. The merged image was compiled without the DAPI layer and shows that fibrillarin and Nop58 colocalize with the pHR-BES speckles. (C) U3 snoRNA is recruited to the same subnuclear (D) areas as injected pHR-BES. (E) Pre-rRNA processing factors Sof1 and B23 localize to nucleoli in control cells not injected with plasmids. (F) The localization patterns of Sof1 and B23 do not change 2 h after the nuclei were injected with the plasmid. Neither Sof1 nor B23 are recruited to the pHRBES foci. Scale bar, 10 μ m.

In contrast to the core U3 processing factors tested, Sof1, a U3-specific component of the SSU processome (Jansen *et al.*, 1993; Dragon *et al.*, 2002), and B23, an ITS2-specific endoribonuclease (Savkur and Olson, 1998; Itahana *et al.*, 2003), did not colocalize with pHR-BES foci (Figure 6F). Although B23 is not expected to be recruited in the processing of 18S rRNA (Liang and Fournier, 1997; Grandi *et al.*, 2002), the lack of Sof1 recruitment could be because the synthesized RNA was not long enough to include processing sites required for the association of Sof1 and for the formation of SSU processome. These results suggest that the recruitment of pre-rRNA processing factors may be based on the availability of the target substrates.

Recruitment of Some pre-rRNA Processing Factors to Active Sites of Transcription May Be on a Sequential, As-needed Basis

To test the possibility that longer pre-rRNA could recruit more pre-rRNA processing factors, we injected HeLa nuclei with plasmid pHRB that contains the same Pol I promoter, but also

has an rDNA sequence that extends through the entire 3700-base pair 5' ETS and 1639 base pairs of the 18S rRNA (Wilson, 1982; Maden *et al.*, 1987). As a result, pHRB includes the A₀ and A₁ processing sites (Figures 4A and 7A). Active transcription of the vector DNA was confirmed by RT-PCR on RNA harvested from HeLa cells that had been transfected with the pHRB construct (Supplementary Figure 1). Two hours after injection, we found that pHRB not only recruited both UBF and Nop58 as expected (data not shown), but also Sof1 (Figure 7B). However, immunofluorescence did not find B23 near any of the pHRB foci (Figure 7B). These results further support our hypothesis that the recruitment of rDNA transcription and pre-rRNA processing factors to rDNA transcription sites is sequential and is on an as-needed basis.

DISCUSSION

Our observations demonstrate for the first time that a pol I transcription factor (UBF) and snoRNP pre-rRNA process-

Figure 7. The U3-associated, noncore rRNA processing factor Sof1 is recruited to a plasmid that expresses the entire 5' ETS and a majority of the 18S gene. (A) pHrB contains the rDNA promoter, the entire 3700-base pair external transcribed spacer (ETS), and the first 1639 base pairs of the 18S rRNA. (B) The U3-associated, noncore processing factor Sof1 is recruited from nucleoli to colocalize with the injected pHrB plasmid in the nucleoplasm at 2 h after injection. By contrast, the late processing factor B23 remains entirely in the nucleoli.

ing factors can be detected in the same complex in mammalian cells. The association of these factors is dependent on pre-rRNA synthesis. In addition, we visualized the coordination of these factors by injecting into HeLa nuclei, a plasmid DNA that contained the pol I promoter with or without rDNA coding sequences. We found that plasmids with the rDNA promoter alone only recruit the pol I transcription factors, and none of the pre-rRNA processing factors tested. In comparison, plasmids containing the pol I promoter and ~700 base pairs of 5' ETS rDNA, including the A₀ cleavage site, recruit pol I transcription factors and core U3 snoRNPs, but not Sof1 and B23. Furthermore, plasmids that contain the pol I promoter, the entire 5' ETS plus an additional 1639 bps of 18S rDNA that includes both the A₀ and A₁ processing sites, recruit pol I transcription factors, core U3 snoRNPs, and an additional U3-specific component of the SSU processome, Sof1. These observations indicate that transcription factors can be mobilized to the site of transcription separately from pre-rRNA processing factors, which are recruited only in the presence of the specific substrate. This suggests that the association of pre-rRNA processing factors to the newly transcribed rRNA is sequential and is on an as-needed basis.

Our findings that pol I transcription factors interact with pre-rRNA processing factors are consistent with recent studies in nonmammalian systems that found transcription factors in the same complex with pre-rRNA processing factors (Fath *et al.*, 2000; Gallagher *et al.*, 2004) and coordination between the two processes (Veinot-Drebot *et al.*, 1988; Caparros-Ruiz *et al.*, 1997; Dragon *et al.*, 2002; Gallagher *et al.*, 2004; Osheim *et al.*, 2004; Saez-Vasquez *et al.*, 2004). Studies in yeast showed that most nascent pre-rRNA transcripts no longer contain 5' external transcribed spacer sequences (Veinot-Drebot *et al.*, 1988) and that the initial steps of pre-rRNA processing take place before the completion of pre-rRNA transcription (Dragon *et al.*, 2002; Osheim *et al.*, 2004). Although these studies have elegantly shown an association of pre-rRNA processing factors with pre-rRNA elongation, the temporal coordination of the two processes were yet to be elucidated. Specifically, it is not clear whether the pol I transcription and pre-rRNA processing factors are corecruited in the same complex to the promoter or if the processing factors are only recruited in the presence of nascent pre-rRNA. Studies in plants show that a complex containing a snoRNP that includes fibrillarin and nucleolin-like proteins is competent for both rDNA binding (in the absence of transcription) and pre-rRNA processing *in vitro*, suggesting that they are in the same complex before rDNA binding (Caparros-Ruiz *et al.*, 1997; Saez-Vasquez *et al.*, 2004). Additionally, a nucleolar RNP complex containing pol I transcription and pre-rRNA processing factors has been isolated in

yeast cells that were pol I deficient or transcriptionally incompetent (Fath *et al.*, 2000). These studies suggest the existence of a preassembled complex with transcription and processing factors that binds rDNA independently of transcription. However, it is not clear whether the same is true in mammalian cells. Our studies using two complementary approaches show that transcription and processing factors are recruited separately in mammalian cells.

The results from immunoprecipitation experiments show that transcription and processing factors can be in the same complex in mammalian cells. However, the integrity of the complex is sensitive to either RNase A treatment or transcription inhibition, suggesting that formation and/or maintenance of the complex is mediated by the newly synthesized pre-rRNA. To test this possibility, we used alternative cell biological approaches. We found that a plasmid containing the pol I promoter alone (pHr-BESΔRNA) without rDNA coding sequences recruits pol I transcription factors, but none of the processing factors tested upon injection into HeLa nuclei. In contrast, a plasmid that contains both the pol I promoter and a partial rDNA coding sequence (pHr-BES) (that includes the A₀ cleavage site) recruits both pol I transcription factors, and the U3 snoRNP. These findings demonstrate that the core U3 snoRNP, a complex involved in the earliest step of pre-rRNA processing, is not corecruited with the pol I transcription machinery in the absence of nascent pre-rRNA, confirming the results of the immunoprecipitations. Additionally, Sof1, an SSU component that specifically interacts with the U3 snoRNA in yeast (Jansen *et al.*, 1993), is not recruited to the pHr-BES plasmid. These results suggest that not all factors involved in pre-rRNA processing and assembly are recruited at the same time and that a pre-rRNA fragment containing only the A₀ cleavage site is not sufficient to assemble the SSU processome. Indeed, Sof1 is recruited to a plasmid expressing the entire ETS and 18S rRNA containing both A₀ and A₁ cleavage sites. B23, an ITS2-specific endoribonuclease also involved in the assembly of pre-ribosomal particles, is not recruited to any of the plasmids tested. Together, these findings suggest that not only is the association of pol I transcription factors and early stage pre-rRNA processing factors sequential, but the recruitment of subsequent processing factors is also ordered in such a manner as to suggest that the formation of a SSU processome is a stepwise process initiated by the binding of U3 snoRNP monoparticles to the pre-rRNA.

The apparent recruitment of pre-rRNA processing factors to the site of rDNA transcription on an as-needed basis could be explained by studies from the McStay lab and our laboratory that showed pol I transcription machinery can be assembled in the absence of RNA synthesis (Chen *et al.*, 2004; Mais *et al.*, 2005). The transcription apparatus literally

coats entire active rDNA clusters, including the nontranscribed intergenic sequences and thus may also play a role in maintaining the chromatin structure of rDNA. As-needed recruitment of pre-rRNA processing factors could be used to prevent the inefficient association of processing factors with unproductive transcription machinery. A second reason for sequential recruitment could be attributed to the structural differences between pol I and pol II as described above. The CTD of the pol II large subunit plays a critical role in complex assembly by directly binding pre-mRNA processing factors (Buratowski, 2003; Bentley, 2005; Meinhardt *et al.*, 2005). Thus, the lack of the CTD domain in the related subunit of pol I may explain the absence of direct interaction between the pol I transcription machinery and processing machinery.

In summary, our observations demonstrated that pol I transcription factors and early pre-rRNA processing factors can be in the same complex in mammalian cells, consistent with findings from several groups working with yeast and plants. Additionally, we show that coordination between pol I transcription machinery and pre-rRNA processing factors is mediated by newly synthesized pre-rRNA, indicating a sequential recruitment of these factors based on the availability of substrates. These findings together with studies from other groups lead to a working model in which transcription factors coating the rDNA can be induced to transcribe pre-rRNA and the pre-rRNA processing factors, including U3 snoRNP (Fabrizio *et al.*, 1994), are recruited to the elongating pre-rRNA as the binding sites become available. The pre-rRNA (associated with a U3 snoRNP) becomes associated with other SSU factors to form a functional SSU processome where the 40S pre-rRNA is cleaved and released for assembly and export out of the nucleus.

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