All Small Nuclear RNAs (snRNAs) of the [U4/U6.U5] Tri-snRNP Localize to Nucleoli; Identification of the Nucleolar Localization Element of U6 snRNA

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Previously, we showed that spliceosomal U6 small nuclear RNA (snRNA) transiently passes through the nucleolus. Herein, we report that all individual snRNAs of the [U4/U6.U5] tri-snRNP localize to nucleoli, demonstrated by fluorescence microscopy of nucleolar preparations after injection of fluorescein-labeled snRNA into *Xenopus* oocyte nuclei. Nucleolar localization of U6 is independent from [U4/U6] snRNP formation since sites of direct interaction of U6 snRNA with U4 snRNA are not nucleolar localization elements. Among all regions in U6, the only one required for nucleolar localization is its 3' end, which associates with the La protein and subsequently during maturation of U6 is bound by Lsm proteins. This 3'-nucleolar localization element of U6 is both essential and sufficient for nucleolar localization and also required for localization to Cajal bodies. Conversion of the 3' hydroxyl of U6 snRNA to a 3' phosphate prevents association with the La protein but does not affect U6 localization to nucleoli or Cajal bodies.

INTRODUCTION

The nucleolus is the site of ribosome biogenesis (for review, see Gerbi *et al.*, 2001). However, the nucleolus seems to be plurifunctional and contains RNA used for other events, such as the RNA component of RNase P, which catalyzes the 5' processing of pre-tRNA (Jacobson *et al.*, 1997; Bertrand *et al.*, 1998; Jarrous *et al.*, 1999), signal recognition particle RNA that assembles with proteins in the nucleolus (Jacobson and Pederson, 1998; Politz *et al.*, 2000) and telomerase RNA (Mitchell *et al.*, 1999; Narayanan *et al.*, 1999b).

Recently, it has been reported that several of the small nuclear RNAs (snRNAs) pass through the nucleolus before their nucleoplasmic destination where splicing occurs. U6 snRNA transiently localizes to nucleoli (Lange and Gerbi, 2000) where it seems to undergo 2'-O-methylation and pseudouridylation of defined nucleotides, guided by small nucleolar RNAs (snoRNAs) (Tycowski *et al.*, 1998; Ganot *et al.*, 1999). Similarly, U2 snRNA is found in nucleoli (Lange and Gerbi, 2000) where it seems to be modified by guide snoRNAs, probably after reimport to the nucleus from the cytoplasm (Yu *et al.*, 2001). In addition, guide snoRNAs for modification of several spliceosomal snRNAs have also been identified (Hüttenhofer *et al.*, 2001). In this report, we present direct evidence that two of the targets of modification, U4 and U5 snRNAs, localize to nucleoli. Therefore, the list of snRNAs associated with nucleoli is expanded to U6

(Lange and Gerbi, 2000), U2 (Lange and Gerbi, 2000; Yu *et al.*, 2001), and U4 and U5 snRNAs (this report).

The observation of nucleolar localization of U6 snRNA raises the question at which point of its life cycle U6 enters the nucleolus. Upon transcription by RNA polymerase III (Dahlberg and Lund, 1991), the first protein to associate with U6 snRNA is La (Rinke and Steitz, 1985; Kunkel *et al.*, 1986), which generally binds to the 3' termini of nascent RNA polymerase III transcripts and a number of viral RNAs (Chang *et al.*, 1994; Simons *et al.*, 1996). Subsequently, the 3'-poly U end of U6 elongates during maturation and then is trimmed to approximately five uridines with conversion of the 3' end of U6 snRNA from a hydroxyl group to a 2',3'-cyclic phosphate (Lund and Dahlberg, 1992; Terns *et al.*, 1992). At this point, La is replaced by the evolutionarily conserved Sm-like (Lsm) proteins Lsm 2–8 (Cooper *et al.*, 1995; Séraphin, 1995; Pannone *et al.*, 1998; Achsel *et al.*, 1999; Mayes *et al.*, 1999; Salgado-Garrido *et al.*, 1999), which facilitate the formation of the [U4/U6] di-snRNP (Achsel *et al.*, 1999). The Lsm proteins are specific in their binding to U6 snRNA and do not associate with U1, U2, U4, or U5 snRNAs that instead are bound by Sm proteins (Achsel *et al.*, 1999; Stevens and Abelson, 1999). Then, the [U4/U6.U5] tri-snRNP forms to which Lsm2-Lsm8 and other proteins are associated (Achsel *et al.*, 1999; Gottschalk *et al.*, 1999; Mayes *et al.*, 1999; Salgado-Garrido *et al.*, 1999; Stevens and Abelson, 1999), and the use of U6 snRNA in the nucleoplasm for splicing ensues.

The experiments presented herein demonstrate that the 3' end of U6, which in vivo associates with the La protein and

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subsequently with the Lsm protein complex to form the [U4/U6.U5] tri-snRNP, is essential for nucleolar localization as well as localization to Cajal (coiled) bodies but not for assembly with U4 snRNA. Furthermore, by alteration of the 3' end of U6 snRNA, we can exclude the possibility that La plays a role in localization of U6 to the nucleolus or to Cajal bodies. In addition, we demonstrate that all snRNAs of the [U4/U6.U5] tri-snRNP localize to nucleoli.

MATERIALS AND METHODS

In Vitro Transcription and Labeling of RNA

In vitro transcription reactions using polymerase chain reaction (PCR)-generated DNA templates produced the labeled RNAs used in the present study. The templates and primers used for PCR are given below.

Templates. The starting material for the template for in vitro transcription of U6 snRNA was the human U6 clone pT7U6 (Tycowski *et al.*, 1998), which carries a U6 gene that is identical in sequence to *Xenopus tropicalis* (Krol *et al.*, 1987) except for a one base difference at nucleotide (nt) 6. An appropriate 5' primer was used to give a PCR product identical to the *Xenopus* U6 snRNA gene sequence, as described previously (Lange and Gerbi, 2000); this was subcloned into pCR3.1 (Invitrogen, Carlsbad, CA) and its sequence was confirmed. The template and primers for wild-type (WT) U6 snRNA have been described in Lange and Gerbi (2000). The primers to generate templates by PCR for in vitro transcription of mutant U6 snRNA and of wild-type U4 or U5 snRNA are listed below. Clones containing the genes for *Xenopus laevis* U5 snRNA (Kazmaier *et al.*, 1987) and chicken U4B snRNA (Hoffman *et al.*, 1986) were kindly provided by I.W. Mattaj (European Molecular Biology Laboratory, Heidelberg, Germany) in the pUC9 plasmid; the corresponding snRNAs were used in this study because their structure-function relationships were previously extensively characterized in *Xenopus* oocytes (Vankan *et al.*, 1990, 1992). Transcripts of U3 snoRNA were prepared as described previously (Lange *et al.*, 1998c).

A triple repeat of the 3' end of U6 snRNA (nt 87–107, called 3'-end nucleolar localization element [NoLE]) was generated by PCR and its sequence confirmed. This NoLE construct was generated using 5' and 3' primers listed below. The minus strand template was 5'-TTG CCG AGG AGC TT(A AAA ATA TGG AAC GCT TCA CG)(A AAA ATA TGG AAC GCT TCA CG)(A AAA ATA TGG AAC GCT TCA CG) C CCT ATA GTG AGT CGT ATT A-3'. The first 14 nucleotides of this oligonucleotide are derived from the genomic sequence (Krol *et al.*, 1987) that follows the 3' end of the U6 snRNA coding region of *X. tropicalis*. The 21 nt shown in parentheses and repeated three times are complementary to nt 87–107 at the 3' end of U6 snRNA. The nt in italics are the first 18 nucleotides of the T7 promoter. Restriction of the 3'-end PCR template with *MseI* (site is underlined above) removed the genomic sequence flanking the gene; subsequent in vitro transcription produced the 65-nt 3'-end RNA construct (triple repeat preceded by GG from the T7 promoter).

The 3'-end NoLE construct just described was also tested for nucleolar localization in a heterologous context by coupling it to the 3' end of a synthetic control RNA (Lange and Gerbi, 2000). The template for this construct (control RNA/3' end) was created by annealing (complementary sequences are underlined) and PCR extension of the following two oligonucleotides: 5'-TAA TAC GAC TCA CTA TAG GGT CCT GTC GAC TCC TCC TCC TCC TCC TCC GCG GAT TTA CCT CGG CAA CGT-3' and 5'-TAA ATG AGG AGC TT(A AAA ATA TGG AAC GCT TCA CG)(A AAA ATA TGG AAC GCT TCA CG)(A AAA ATA TGG AAC GCT TCA CG)T TGC CCA GGT AAA TCC GCG GA-3'; the T7 promoter is shown in italics and the 3'-end NoLE (nt 87–107) repeated three times are enclosed by parentheses. The synthetic control RNA by itself was created by PCR by using the template and primers described by

Lange and Gerbi (2000). U2 snRNA was used as a control for immunoprecipitation experiments and stability assays and was derived as described previously (Lange *et al.*, 1998a).

5' End Primers (T7 Promoter Shown in Italics). U4 WT 5'-TAA TAC GAC TCA CTA TAG GGA GCT TTG CGC AGT GGC AGT ATC-3'; U5 WT 5'-TAA TAC GAC TCA CTA TAG GGA TAC TCT GGT TTC TCT TCA AAT TCG AAT AAA TC-3'; U6 Δ 1–19 5'-TAA TAC GAC TCA CTA TAG GGA TAT ACT AAA ATT GGA-3'; U6 Δ 20–25 5'-TAA TAC GAC TCA CTA TAG GGT GCT TGC TTC GGC AGC ACT AAA ATT GG-3'; U6 sub20–25 (substitution is underlined) 5'-TAA TAC GAC TCA CTA TAG GGT GCT TGC TTC GGC AGC ACG AGC GGT AAA ATT GG-3'; U6 Δ 26–42 5'-TAA TAC GAC TCA CTA TAG GGT GCT TGC TTC GGC AGC ACA TAT ACA GAG AAG AT-3'; and T7 promoter for 3'-end construct 5'-TAA TAC GAC TCA CTA TAG GG-3'.

3' End Primers. U4 WT 5'-CAG TCT CCG TAG AGA CTG TCA-3'; U5 WT 5'-TAC CTG GTG TGA ACC AGG CTT C-3'; U6 Δ 43–56 5'-AAA AAT ATG GAA CGC TTC ACG AAT TTG CGT GTC ATC CTT GCG CAG GGG CCA GTA TCG TTC C-3'; U6 Δ 57–81 5'-AAA AAT ATG GAA CGC TTC ACG AAT TTT GCT AAT CTT-3'; U6 Δ 43–81 5'-AAA AAT ATG GAA CGC TTC ACG AAT TTG TAT CGT TCC-3'; U6 Δ 82–95 5'-AAA AAT ATG GAA GCG TGT CAT CCT TGC-3'; U6 Δ 96–99 5'-AAA AAT ATC GCT TCA CGA ATT T-3'; U6 Δ 100–102 5'-AAA AAG GAA CGC TTC ACG AAT TTG CGT GTC ATC CTT G-3'; U6 Δ 103–107 5'-TAT GGA ACG CTT CAC GAA TTT GCG TGT CAT CCT TG-3'; U6 Δ 100–107 5'-GGA ACG CTT CAC GAA TTT GCG TGT CAT CCT TG-3'; U6 Δ 82–107 5'-GCG TGT CAT CCT TGC GCA GGG GCC-3'; and U6 3'-end 5'-TTG CCG AGG AGC TTA AA-3' (genomic sequence downstream of U6).

In vitro transcripts of RNA were made with either fluorescein-12-UTP (PerkinElmer Life Sciences, Boston, MA) and/or α -[32 P]UTP (PerkinElmer Life Sciences) label that was added to a T7 megascript in vitro transcription kit (Ambion, Austin, TX). The T7 transcripts were purified according to Lange *et al.* (1999). They all contained GG at their 5' ends from the T7 promoter and were capped with m⁷G(5')ppp(5')G (Ambion) to improve stability.

Oocyte Microinjection

Stage V–VI oocytes from *X. laevis* were obtained as described previously (Lange *et al.*, 1998a). For fluorescence analysis of nucleolar localization as well as for stability assays, oocyte nuclei were injected with 0.8 ng of wild-type U4, U5, and U6 snRNA or U3 snoRNA in 9.2 nl of H₂O. For the U6 snRNA mutants that were shorter than the wild-type and for the 3'-end construct, amounts equimolar to the wild-type U6 RNA (0.8 ng = ~23 fmol of in vitro-transcribed WT U6 snRNA) were injected. The concentration of U6 used for injection was determined by titration to be the lowest possible that still gave specific labeling of nucleoli (our unpublished data) and is in the range of the concentration of endogenous U6 (~7.5 fmol/stage V–VI oocyte as determined by Northern blot analysis), whereas there is nuclear retention of up to ~500–600 fmol of U6 per *Xenopus* oocyte (Boelens *et al.*, 1995; Terns *et al.*, 1995). The concentration used for injection of our transcripts is also in the range of those used by Gall *et al.* (1999) for oocyte injection of U1, U2, and U5 snRNA. For the 40-nt negative control RNA, 0.8 ng/oocyte was injected, which is equivalent to ~62 fmol/oocyte. A further control was the injection of an excess of fluorescein-labeled UTP at 5 pmol/oocyte. We confirmed that microinjected U4 and U6 snRNA transcripts could participate in their normal functional pathway and form a di-snRNP. This was achieved by coimmunoprecipitation (see section below) with an anti-Sm antibody as described previously (Vankan *et al.*, 1990, 1992). Endogenous U6 and U4 snRNA were disrupted through RNase H-mediated destruction by two nuclear injections spaced 4 h apart of 9.2 nl of each of the following antisense oligonucleotides at a concentration of 3 μ g/ μ l (28 ng/oocyte):

a combination of two oligonucleotides complementary to nt 20–53 (5'-TAA TCT TCT CTG TAT CGT TCC AAT TTT AGT ATA T-3') and nt 75–102 (5'-TAT GGA ACG CTT CAC GAA TTT GCG TGT C-3') was used for U6 depletion. U4 depletion was carried out with an oligonucleotide complementary to nt 51–83 (5'-GGG TAT TGG GAA AAG TTT TCA ATT AGC AAT A-3').

Nucleolar Localization Assay

After incubation of the oocytes for a stipulated time (1.0–1.5 h unless specified otherwise), nuclear spreads were made as described previously (Lange *et al.*, 1999) using a method for preparation of lampbrush chromosomes (Gall *et al.*, 1991). For Cajal body immunostaining, the slides were fixed for 1 h in 2% paraformaldehyde in phosphate-buffered saline (PBS) (137 mM NaCl, 3 mM KCl, 6.4 mM Na₂HPO₄, and 1.5 mM KH₂PO₄, pH 7.0), washed in PBS, and unspecific binding sites saturated with 10% bovine serum albumin in PBS for 20 min. Subsequently, rabbit polyclonal serum against a synthetic 21 amino-acid fragment of *Xenopus* coilin (kindly provided by J.G. Gall, Carnegie Institution, Baltimore, MD) was applied as a primary antibody for immunostaining of Cajal bodies at a dilution of 1:1000 in PBS for 20 min at 4°C. The preparations were rinsed 3× for 5 min each in PBS and incubated for 20 min at 4°C with the secondary antibody (goat anti-rabbit; Molecular Probes, Eugene, OR) coupled to the dye Alexa 594. Subsequently, slides were washed 3× with PBS for 5 min each and the DNA stained with 200 ng/ml 4'-6-diamidino-2-Phenylindole (DAPI) in PBS for 5 min. Fluorescence microscopy was performed as described previously (Lange and Gerbi, 2000) with the exception that ProLong mounting medium (Molecular Probes) was used. Nucleolar preparations were analyzed with an Axiophot epifluorescence microscope (Carl Zeiss, Thornwood, NY) equipped with a 100× Neofluar Ph 3 objective and a 100-W mercury lamp. Pictures were taken with constant exposures for each filter set (for DAPI, Alexa 594, or fluorescein) by using Ektachrome 400× professional film (Eastman Kodak, Rochester, NY). Therefore, any difference in signal strength between various samples is directly visualized without the interference of software or automatic camera settings.

snoRNA Stability Assay

To determine the stability of the various in vitro transcripts after injection into oocyte nuclei, U2 snRNA was coinjected and served as an internal control to normalize for any differences in injection or recovery of the samples. At defined time points after injection of the oocytes with [α -³²P]UTP-labeled RNAs, the RNA of four nuclei per sample was recovered and analyzed as described previously (Lange and Gerbi, 2000).

Immunoprecipitation of U6 snRNA from Oocytes

For immunoprecipitation experiments, 0.8 ng/oocyte of the purified U6 snRNA (colabeled with [α -³²P]UTP and with fluorescein-12-UTP) and either 0.8 ng/oocyte fluorescein-12-UTP-labeled U4 snRNA or [α -³²P]UTP-labeled U3 snoRNA were coinjected into *Xenopus* oocytes and incubated for 1 or 4 h (depending on the experiment) at 20°C. For each sample, 10 nuclei were homogenized on ice in 50 μ l of isolation buffer (50 mM NaCl, 10 mM Tris pH 8.0, 1 mM dithiothreitol, 100 U/ml RNase inhibitor [Roche Applied Science, Mannheim, Germany], one tablet of protease inhibitor cocktail [Roche Applied Science] per 10 ml of isolation buffer), and spun for 1 min in a microcentrifuge at 10,000 rpm. The supernatant was removed, spun again twice, and then added to 240 μ l of IP 150 (150 mM NaCl, 10 mM Tris pH 8.0, 0.1% NP-40, 1 mM dithiothreitol, 10 U/ml RNase inhibitor [Roche Applied Science], and one tablet of protease inhibitor cocktail [Roche Applied Science] per 10 ml of isolation buffer), and 20 μ l of protein A-Sepharose beads.

The beads had been coupled either to rabbit-anti-*Xenopus* La antibody (immune serum 79, provided by S. Clarkson, University

Medical Center-CMU, Geneva, Switzerland; Lin-Marq and Clarkson, 1998) or to preimmune serum as a control, or in other cases to monoclonal Y12 mouse anti-Sm antibody (Lerner *et al.*, 1981) or mouse IgG as a control by incubation of 150 μ l of preswollen beads with 150 μ l of IP 500 (500 mM NaCl, 10 mM Tris pH 8.0, 0.1% NP-40, and 0.1% sodium azide) and 200 μ l of antibody for 4 h at 4°C with end over end rotation before they were spun and washed 3× in IP 150 in a microcentrifuge at 1000 rpm. The mixture of nuclear extract and antibody-coupled beads was rotated end over end for 90 min (anti-La antibody) or 8 h (anti-Sm antibody) at 4°C before the beads were spun and washed 5× in IP 150 in a microcentrifuge at 1000 rpm. Then the RNA was isolated and purified. Precipitated RNA and supernatant were analyzed on a denaturing 7 M urea, 8% polyacrylamide gel (1 mm in thickness, 35 cm in length). It is important to note that although U4 transcript association with Sm proteins and U6 is initiated upon injection into the oocyte, further association can also occur during coincubation of nuclear lysate with antibody-coupled beads. Therefore, accurate kinetics of in vivo association of U4/U6 cannot be carried out.

For alteration of the 3'-hydroxyl end of U6 to a 3'-phosphate group, 10 μ l with the in vitro transcript (~1 μ g) was incubated in Whitfield's reagent (25 mM NaIO₄ and 1 M lysine, pH 8.5) for 2 h at 45°C (Lund and Dahlberg, 1992; Terns *et al.*, 1992). This treatment of oxidation- β elimination also shortens the input RNA by 1 nt (Terns *et al.*, 1992).

RESULTS

Nucleolar Localization of U4 and U5 snRNA

U4 snRNA and subsequently U5 snRNA and U2 snRNA associate with U6 to form the [U4/U6] di-snRNP, [U4/U6.U5] tri-snRNP, and [U2.U4/U6.U5] tetra-snRNP (Konarska and Sharp, 1988; Hall and Konarska, 1992; Wassarman and Steitz, 1992; Raghunathan and Guthrie, 1998b). To complete the picture, we analyzed whether U4 and U5 snRNAs associate with nucleoli, like U6 (Lange and Gerbi, 2000) and U2 (Lange and Gerbi, 2000; Yu *et al.* 2001). This was monitored by a technique used previously to define the nucleolar localization elements (NoLEs) of snoRNAs from various families (Lange *et al.*, 1998a,b,c, 1999; Narayanan *et al.*, 1999a,b) as well as nucleolar localization of U6 snRNA (Lange and Gerbi, 2000). Fluorescein-labeled in vitro transcripts were injected into *Xenopus* oocyte nuclei to allow direct visualization of the labeled RNA in nucleolar preparations made subsequently.

In the present study, fluorescein-labeled in vitro transcripts of U4 snRNA and U5 snRNA were injected into *Xenopus* oocyte nuclei. Controls included injection of nucleolar U3 snoRNA as a positive control, and injection of a 40-nt synthetic RNA or fluorescein-UTP as negative controls to rule out nonspecific nucleolar staining (e.g., by diffusion of excess material). After 8 min, 1 h, and 24 h (the longest time point feasible to be studied), the oocytes were manually dissected and the nuclear contents, including nucleoli, were centrifuged onto a microscope slide. As shown in Figure 1a, strong fluorescent signals depicting nucleolar localization of U4 and U5 snRNA were detected 8 min and 1 h after injection of 0.8 ng of transcript per oocyte nucleus. Likewise, nucleoli were stained by a positive control (U3 snoRNA), injected at the same concentration that had been previously optimized for the nucleolar localization assay (Lange *et al.*, 1998c). In contrast, negative controls (40-nt synthetic RNA = control RNA, or unincorporated fluorescein-UTP), both injected in excess (see MATERIALS AND METHODS) did not

Figure 1. Nucleolar localization of U4 and U5 snRNA. (a) Fluorescein-labeled U4 snRNA or U5 snRNA were injected into the nuclei of *X. laevis* oocytes to analyze nucleolar localization. Controls included injection of nucleolar U3 snoRNA as well as synthetic RNA or fluorescein-UTP to rule out nonspecific nucleolar staining. After 8 min, 1 h, or 24 h, nuclear spreads were prepared and analyzed by phase contrast (PC) or fluorescence microscopy. Nucleoli can be distinguished from other nonchromosomal nuclear bodies by staining of the rDNA (DAPI, blue). Strong nucleolar labeling (FL, green) is seen as early as 8 min after nuclear injection of U4 or U5 snRNA and continues at 1 h after injection. Likewise, nucleoli were stained by a positive control (U3 snoRNA) at these time points, whereas negative controls (control RNA or unincorporated fluorescein-UTP), both injected in excess (see MATERIALS AND METHODS), did not stain nucleoli. Twenty-four hours after incubation only U3 snoRNA retains strong signals in nucleoli, whereas U4 snRNA labeling is reduced and U5 snRNA signals are close to background. Stronger nucleolar staining by U4 snRNA compared with U5 snRNA or U6 snRNA was generally observed at all time points. Bar, 10 μ m. (b) 32 P-Labeled transcripts of U4 and U5 snRNA were injected into oocyte nuclei to determine their stability; the RNAs were isolated and analyzed by gel electrophoresis as described previously (Lange *et al.*, 1999). U2 snRNA transcripts were coinjected and served as an internal control to normalize for any differences in injection or recovery of the samples. U4 and U5 snRNA are stable 24 h after oocyte injection, compared to the 0-h control. Similarly, as published previously (Lange and Gerbi, 2000) U3 snoRNA, U6 snRNA, or the control RNA were stable through 24 h.

stain nucleoli. Twenty-four hours after incubation, only U3 snoRNA retained strong signals in nucleoli, whereas U4 snRNA labeling was reduced and U5 snRNA signals were close to background (Figure 1a). This observation is consistent with the nucleolus being the functional compartment for U3 snoRNA and with the predicted transient nature of snRNA localization to nucleoli, as has been already shown for U6 snRNA in the same system (Lange and Gerbi, 2000).

The nucleolar localization of fluorescent U4 and U5 snRNA was specific, because injection of an unrelated control RNA, even at ~3 times the molar amount of U4 or U5, did not stain nucleoli. Additional controls demonstrated that the observed fluorescent signals were not due to degradation of fluorescent snRNA and subsequent reutilization of the label by other nuclear components. First, injection of a ~75-fold molar excess of fluorescein-UTP alone did not label the nucleoli (Figure 1a). Second, stability assays using ³²P-labeled transcripts demonstrated that U4 and U5 snRNA transcripts were stable at the times the nucleolar localization assay was performed (Figure 1b). Similarly, as previously published for U3 snoRNA, U6 snRNA, or the control RNA (Lange and Gerbi, 2000), U4 and U5 snRNAs were stable over 24 h.

Sequences of U6 snRNA Essential for Nucleolar Localization

To define *cis*-acting elements of U6 snRNA necessary for nucleolar localization, the localization of mutant transcripts was compared with that of wild type. The scheme in Figure 2 (modified from Vankan *et al.*, 1990 and Tycowski *et al.*, 1998) points out areas of functional interest in wild-type U6 snRNA and also gives an overview of the various U6 mutants designed for the present study. Sequences of mature wild-type U6 snRNA and of the mutants are listed in detail below the scheme.

There is no previous information at which stage of its life cycle U6 enters nucleoli. It might enter nucleoli as the individual snRNP or in a complex as a di-snRNP or tri-snRNP directly bound to U4 and in conjunction with U5 snRNA, both of which are also found in nucleoli (see above; Figure 1a). Therefore, it was of particular interest to analyze whether the sites in U6 (nt 49–74) that base pair with U4 before association with the spliceosome and that are important for snRNP assembly (Vankan *et al.*, 1990) might play a role in U6 nucleolar localization. However, deletions within that sequence (Δ 43–56, Δ 57–81; Figure 3) or total deletion of the entire middle part of the molecule, including the entire U4 binding site (Δ 43–81; Figure 3), do not appreciably influence nucleolar localization of U6 snRNA compared with the wild type. This area also contains all sites for nucleolar posttranscriptional methylation of U6 as well as a region that base pairs with U2 snRNA in the spliceosome (Figure 2). Similarly, U6 snRNA carrying a deletion of the 5' stem (Δ 1–19) or a deletion of nt 26–42 containing a U2-binding site retained the ability to localize to nucleoli (Figure 3). Therefore, areas of U6 snRNA that will base pair with U4 snRNA or later with U2 snRNA during splicing are not required for nucleolar localization. Interestingly, a deletion or substitution of nt 20–25 does not affect U6 nucleolar localization (Figure 3). This sequence element is responsible for nuclear import of U6 snRNA after injection into the cytoplasm (Hamm and Mattaj, 1989). Although U6 during

maturation normally does not travel to the cytoplasm, a mutation of this region was used previously to study the effect of additional mutations on the natural nuclear retention of U6 by preventing nuclear reimport of molecules leaking into the cytoplasm (Boelens *et al.*, 1995).

In contrast to the mutations described above, nucleolar localization of U6 could be completely abolished by a deletion of nt 82–107 of U6 snRNA (Figure 3). This area contains the 3' terminus of U6, which binds the La protein and subsequently the Lsm proteins (see INTRODUCTION). The 3'-terminal U residues also may somewhat assist nuclear retention of U6, although after deletion of the 3' end the majority of U6 injected into *Xenopus* oocytes still remained in the nucleus even after long incubation times (Boelens *et al.*, 1995). The present study now demonstrates that the 3' end of U6 snRNA is essential for nucleolar localization, whereas the remainder of the molecule representing more than three-quarters of the U6 snRNA sequence, including sites absolutely required for [U4/U6] di-snRNP formation, splicing complex assembly, and splicing activity (Vankan *et al.*, 1990, 1992), lacks elements important for nucleolar localization.

In additional control experiments (Figure 4), we confirmed that nucleolar localization is indeed mediated independently from [U4/U6] snRNP assembly because 1) the synthetic U6 snRNA and U4 snRNA transcripts generated here by *in vitro* transcription retain their functional activity to form a [U4/U6] snRNP, 2) the mutant U6 Δ 43–81 still localizes to nucleoli even though it lacks the U4 base-pairing sites and cannot interact with U4, and 3) the 3'-NoLE U6 mutant that does not localize to nucleoli (Δ 82–107) is still able to assemble in a [U4/U6] snRNP. The experiments leading to these observations involved coinjection of U6 snRNA (colabeled with [³²P]UTP and fluorescein-UTP) with U4 snRNA transcripts (labeled with fluorescein-UTP) into U6- and U4-depleted *Xenopus* oocytes and subsequent coimmunoprecipitation of these snRNAs with an anti-Sm antibody. Because the Sm proteins are bound to U4 and not to U6 snRNA, precipitation of U6 occurs only if it is associated with U4 snRNA; this technique was used by Vankan *et al.*, (1990) to identify domains of U4 and U6 required for snRNP assembly. Figure 4 shows that U6 WT and the NoLE 3'-mutant (Δ 82–107) but not U6 mutated in nt 43–81 were coprecipitated with U4 when using an anti-Sm antibody. Moreover, in the converse experiment, U4 carrying a deletion of the base-pairing sites for U6 (Δ 1–18/56–63) failed to precipitate wild-type U6 snRNA.

Although not the primary focus of the present study, we also analyzed association of U6 with Cajal bodies, identified by their immunostaining for coilin (Figure 5). One hour after nuclear injection of fluorescein-labeled wild-type U6 snRNA, the Cajal bodies are stained, although not as strongly as nucleoli (Figure 5). Four hours after injection, consistent with the previously reported kinetics of nucleolar localization of U6 snRNA (Lange and Gerbi, 2000), the nucleoli are weakly stained. At this time point, Cajal body staining by U6 snRNA remains at low levels (our unpublished data). It has been reported that snoRNA lacking the box C/D NoLE accumulates in Cajal bodies (Narayanan *et al.*, 1999a), and so we investigated whether a similar phenomenon occurred for U6 snRNA. However, U6 snRNA lacking its NoLE no longer localized to Cajal bodies or nucleoli (Figure 5). Therefore, the 3'-end of U6 snRNA is

Figure 2. Sequence and mutations of U6 snRNA. The sequence of *Xenopus* U6 snRNA (Krol *et al.*, 1987), which is highly conserved across the eukaryotic kingdom (Brow and Guthrie, 1988), is shown. The figure has been modified from Vankan *et al.* (1990) and Tycowski *et al.* (1998). Shaded areas are sites of base pairing with U2 snRNA in the spliceosome and nt 49–74 base pairs with U4 snRNA before association with the spliceosome. Several of the modifications (ψ , pseudouridine; m, methylated nucleotides) occur in areas of U6 snRNA involved in intermolecular base pairing during splicing (Vankan *et al.*, 1990, 1992). Most mutations designed for this study were deletions covering the nucleotides indicated by lines. Δ 1–19 was designed to remove the 5'-terminal stem. Nucleotides used for substitution of nt 20–25, which is a sequence needed for nuclear import after injection of U6 into the cytoplasm (Hamm and Mattaj, 1989), are shown in a box. In various mutations of the middle part of the molecule, sites of base pairing with U2 and U4 snRNA were removed. In addition various deletions were introduced between nt 82 and 107, which includes regions that base pair in the spliceosome with U2 snRNA as well as the 3' terminus of U6 that binds the La protein and subsequently the Lsm proteins (see text). Furthermore, a triple repeat of nt 87–107 of the U6 3' end (3' end) was designed (open box at top of diagram). The lower portion of this figure lists the sequences of mutant U6 snRNAs. Nucleotides that are the same as in wild-type U6 are shown by dots in the sequence alignment, and deletions are indicated by dashes.

Figure 3. Nucleolar localization of U6 snRNA mutated in various positions throughout the molecule. Fluorescein-labeled U6 snRNA mutants were injected into the nuclei of *X. laevis* oocytes and 1.5 h later the localization assay performed. U6 snRNA carrying a deletion of the 5' stem (Δ 1–19) or a deletion or substitution of nt 20–25 localized as well to nucleoli as the wild-type (WT) molecule (FL, green). Similarly, various mutants with deletions throughout the middle part of the molecule (Δ 26–42, Δ 43–56, Δ 57–81, and Δ 43–81) retained full ability to localize to nucleoli. In contrast, a deletion of nt 82–107 completely abolished nucleolar localization. Other details as in Figure 1a.

essential not only for localization to nucleoli but also to Cajal bodies.

Further mutational analysis was carried out to reveal whether specific sequences of a few nucleotides within the 3' part of U6 are essential for nucleolar localization, similar to the discrete NoLEs of snoRNAs. Four mutations were designed that spanned the region of interest in U6 snRNA

(Δ 82–95, Δ 96–99, Δ 100–102, and Δ 103–107) (Figure 2). In addition, a mutation spanning the last 8 nt (Δ 100–107) was used, which drastically decreased the interaction of U6 with the La protein in *Xenopus* oocytes (Boelens *et al.*, 1995). All five mutations of the 3' end of U6 (Δ 82–95, Δ 96–99, Δ 100–102, Δ 103–107, and Δ 100–107) significantly impaired localization (Figure 6). However, weak fluorescent signals were

Figure 4. [U4/U6] snRNP assembly after nuclear injection. U6 snRNA (colabeled with [³²P]UTP and fluorescein-UTP) and U4 snRNA transcripts (labeled with fluorescein-UTP) were coinjected into *Xenopus* oocytes depleted of endogenous U4 and U6 snRNA. After 4 h of incubation the functional ability of the in vitro transcripts to form a [U4/U6] snRNP was analyzed by immunoprecipitation from the nuclear lysate with an anti-Sm protein antibody. The equivalents of 10 nuclei/sample of the precipitated RNA (pellet) and 0.1 nuclei/sample of the supernatant (control for equal amounts injected) were analyzed on a denaturing 8% polyacrylamide, 8 M urea gel and by autoradiography. [U4/U6] snRNP assembly occurs between wild-type U6 snRNA (U6 WT)

and wild-type U4 snRNA (U4 WT), also with the U6 NoLE mutant (Δ82–107), and even with the truncated 3' end mutant (Δ82–107), consistent with Vidal *et al.* (1999). Ability of the transcripts to form a snRNP was disrupted by using either mutant U6 (Δ43–81) or mutant U4 (Δ1–18/Δ56–63) that lack the sites to base pair with each other and that are important for snRNP assembly before association with the spliceosome (Vankan *et al.*, 1990). It is notable that this U6 mutant (Δ43–81) is still capable of localizing to nucleoli (see Figure 3). None of the samples were precipitated by beads coupled to control antibody.

still obtained by all five U6 mutants and localization was not entirely abolished. This is in contrast to the U6 mutant, which lacks the entire 3' end (Δ82–107) and which was entirely incapable of localization to nucleoli (Figure 3). Therefore, nucleolar localization of U6 snRNA relies on many nucleotides in the 3' end region, rather than on just a few nucleotides.

It was important to ascertain the stability of each mutant U6 snRNA transcript, to guard against the possibility that failure of some mutants to localize to nucleoli was simply due to their degradation. Stability assays using ³²P-labeled transcripts demonstrated that all transcripts were sufficiently stable 1.5 h after injection into oocyte nuclei (the time when localization assays were carried out) (Figure 7). This included U6 mutant Δ82–

107 that failed to localize to nucleoli, as well as the control RNA. In addition, the use of U6 wild-type as well as U6 mutants (Δ43–81 and Δ82–107) in immunoprecipitation experiments confirmed their stability when labeled with fluorescein-12-UTP and [^α-³²P]UTP. Although well beyond the time frame of the localization assay, we were also interested to see whether any of the U6 mutants would show significant instability after longer incubation periods in oocytes. By assaying the long-term stability of transcripts 24 h after injection, we found that several U6 mutants of the 3' end as well as the 3' end NoLE constructs (3' end, control RNA/3' end) were significantly less stable than wild-type U6 snRNA (our unpublished data). This precluded us from carrying out long-term localization studies.

Figure 5. The 3' end of U6 is essential for localization to Cajal bodies. Fluorescein-labeled wild-type U6 snRNA as well as the 3' mutant (Δ82–107) were injected into the nuclei of *Xenopus* oocytes and the localization assay performed. Immunostaining of Cajal bodies (CB, red) was carried out in addition to DNA staining of nucleoli (DAPI, blue) to distinguish between these two sites of U6 snRNA localization (FL, green). Injection of wild-type U6 snRNA results in strong labeling of nucleoli and weaker labeling of Cajal bodies 1 h after injection. In contrast, deletion of the U6 3' end (Δ82–107) completely abolished localization to nucleoli as well as to Cajal bodies, comparable with background levels seen in uninjected control oocytes (no injection). Other details as in Figure 1a.

Figure 6. Sequences within the 3' end of U6 snRNA that mediate nucleolar localization. Mutational analysis of the 3' portion of U6 snRNA was carried out to analyze whether specific sequences within this region are essential for nucleolar localization. Various deletion mutants of the 3' end of U6 ($\Delta 82-95$, $\Delta 96-99$, $\Delta 100-102$, $\Delta 103-107$, and $\Delta 100-107$) were impaired in their nucleolar localization 1.5 h after injection. However, they still labeled nucleoli weakly, unlike the larger deletion of the 3' end of U6 ($\Delta 82-107$; Figure 3) that had no nucleolar signal. Therefore, nucleolar localization relies on many nucleotides in the 3' region. In addition, a triple repeat of the NoLE (3'-end) was tested in the nucleolar localization assay and revealed that nt 87-107 are not only important but also sufficient for nucleolar localization because the construct was able to localize to nucleoli. Similarly, this 3'-end NoLE also localized to nucleoli when it was coupled to a synthetic control RNA (control RNA/3'-end), whereas the control RNA by itself did not. Other details as in Figure 1a.

For some snoRNAs it has been shown that the NoLEs not only are essential but also are sufficient for nucleolar localization because just the box C/D core structure of C/D snoRNAs can target synthetic RNA sequences to the nucleolus (Lange *et al.*, 1998a). Therefore, we analyzed if the 3' sequence of U6 snRNA by itself is not only essential but also sufficient for nucleolar targeting. As shown in Figure 6, a transcript of just the 3' end of U6 is sufficient for nucleolar localization. Similarly, this 3'-end NoLE construct also localized to nucleoli when it was coupled to a synthetic control RNA (control RNA/3'-end), whereas the 40-nt control RNA by itself did not (Figure 6). Thus, the nucleotides that are both essential and sufficient for nucleolar localization are nt 87-107, which represent the NoLE for U6 snRNA.

Does the 3' End of U6 snRNA Function as a NoLE by Binding to the La Protein?

It has been hypothesized that NoLEs of snoRNAs act by binding protein(s) that either transport the snoRNA from the nucleoplasm to the nucleolus and/or anchor it within the nucleolus. Similarly, binding of specific protein(s) to the NoLE of U6 might initiate nucleolar localization. Deletion of the 8 nt of the U6 NoLE at the 3' end drastically decreases the interaction of U6 with the La protein in *Xenopus* oocytes (Boelens *et al.*, 1995). La is the first protein to associate with U6 snRNA, and later it is replaced by the evolutionarily conserved Lsm proteins, which leads to formation of the di- and tri-snRNPs (see INTRODUC-

Figure 7. Stability of wild-type and mutated U6 snRNA. 32 P-labeled U6 snRNAs (mutants or wild type or the 3'-end NoLE constructs) were injected into oocyte nuclei, and the RNAs were isolated and analyzed by 8% polyacrylamide, 8 M urea gel electrophoresis. The top panel shows the 0-h controls and the bottom panel shows the short-term stability at 1.5 h (the time when localization assays were carried out) after injection into oocyte nuclei. To determine the stability of the various RNAs after nuclear injection, U2 snRNA transcripts were coinjected and served as an internal control to normalize for any differences in injection or recovery of the samples. All the U6 mutants as well as the control RNA were stable at the time point used for analysis of nucleolar localization.

TION). It has been shown that a 3'-end alteration modulates the binding of La protein to U6 snRNA (Lund and Dahlberg, 1992; Terns *et al.*, 1992). This alteration allowed us to find out whether interaction of U6 with La might be involved in U6 nucleolar localization.

The La protein binds the stretch of uridylates at the 3'-hydroxyl end of newly synthesized U6 snRNA both in vivo and in vitro but is unable to bind to U6 snRNA that lacks a 3' hydroxyl, and conversion of the U6 3' end in vivo to a 2',3'-cyclic phosphate leads to a replacement of La by Lsm proteins (Lund and Dahlberg, 1992; Terns *et al.*, 1992). Consequently, we generated an in vitro transcript of U6 with an altered 3' end that was unable to bind La but would allow the nucleolar localization assay to be carried out to determine whether La plays a role in nucleolar localization. For alteration of the 3'-hydroxyl end, U6 snRNA was incubated with Whitfield's reagent; this treatment via oxidation- β elimination converts the 3' hydroxyl to a 3'-phosphate group, resulting in a loss of La binding (Lund and Dahlberg, 1992; Terns *et al.*, 1992). Figure 8a shows migration on a polyacrylamide gel of U6 transcripts (with or without a 3'-hydroxyl group) that were colabeled with 32 P for immunoprecipitation and with fluorescein for further studies in a nucleolar localization assay. The transcripts were coinjected into *Xenopus* oocytes, and binding to the La protein was analyzed by immunoprecipitation (Figure 8b) to confirm that only U6 snRNA with a 3'-hydroxyl group but not a 3'-phosphate group effectively binds to La and can be precipitated by anti-La antibodies. The same antibodies precipitated only traces of U3 snoRNA transcripts that were coinjected as a control, and the control serum did not precipitate any of the samples. The gel also shows that the transcripts remain stable over the time of incubation in the oocyte, which is equivalent to the time of incubation for the localization assay (1.5 h). Regardless of the alteration, both the 3'-OH and 3'-phosphate forms of U6 localized strongly to nucleoli and, although weaker, to Cajal bodies as well (Figure 8c), indicating that the mechanism of U6 localization to

either nuclear subcompartment does not require a 3'-hydroxyl terminus nor binding of the La protein to the 3' NoLE.

DISCUSSION

All snRNA Components of [U4/U6.U5] Tri-snRNP Localize to Nucleoli

There are significant differences in the maturation and trafficking of U6 snRNA compared with other spliceosomal snRNAs, including U4 and U5 of the [U4/U6.U5] tri-snRNP, and it was unknown whether these snRNAs localize to nucleoli, similar to U6 snRNA (Lange and Gerbi, 2000). U6 is transcribed by RNA polymerase III and retained in the nucleus (Vankan *et al.*, 1990; Terns and Dahlberg, 1994; Boelens *et al.*, 1995; Terns *et al.*, 1995). In contrast, the other spliceosomal snRNAs are transcribed by RNA polymerase II and are exported to the cytoplasm where the 5' cap is converted from a monomethyl G (7mGpppG) to a trimethyl G (2,2,7mGpppG) and Sm proteins are bound; after these events these snRNAs are reimported back into the nucleus to function in splicing (for review, see Izaurralde and Mattaj, 1995). The data presented herein demonstrate that U4 and U5 snRNAs, like U2 snRNA (Lange and Gerbi, 2000; Yu *et al.*, 2001) and U6 snRNA (Lange and Gerbi, 2000), associate with nucleoli. These conclusions are based on the nucleolar localization of injected synthetic T7 polymerase U4 and U5 snRNA transcripts with a monomethyl G cap like their newly synthesized in vivo counterparts. Previous studies showed that such transcripts microinjected into *Xenopus* oocytes exhibit normal nucleo/cytoplasmic traffic as their endogenous counterparts (Fischer *et al.*, 1991). In addition, functionality of the synthetic RNAs was shown by the specificity of their nucleolar localization and also confirmed by the ability of U5 (our unpublished data) and U4 snRNA to associate with Sm proteins and, moreover, by the capacity of U4 to form the [U4/U6] snRNP via base pairing.

Figure 8. U6 localization to nucleoli and Cajal bodies does not require a 3'-hydroxyl terminus nor binding of the La protein. (a) U6 snRNA in vitro transcripts with either a 3'-hydroxyl (3'-OH) or a 3'-phosphate (3'-P) group (colabeled with [³²P]UTP and fluorescein-UTP [FL]) were run on a denaturing 8% polyacrylamide, 8 M urea gel and analyzed by autoradiography (left two lanes) and under UV light (right two lanes). Because the 3' modification via oxidation-β elimination also results in shortening of the RNA by one nucleotide, the transcripts can be distinguished by their different migration. (b) Colabeled U6 snRNA (³²P; FL) with either a 3'-OH or 3'-P group and a control (³²P-labeled U3 snoRNA) were coinjected into *Xenopus* oocytes. One hour after injection, binding of the injected RNA to the La protein was analyzed by immunoprecipitation with anti-*Xenopus* La (La) or preimmune serum as a control (Ctrl). The equivalents of five nuclei per sample of the precipitated RNA (pellet) and 0.2 nuclei per sample of the supernatant were analyzed by PAGE as in (a). Only U6 snRNA with a 3'-OH group but not a 3'-P group effectively binds to La and can be found in the precipitate. The control preimmune serum did not precipitate any of the samples. (c) Colabeled U6 snRNA transcripts with either a 3'-OH or 3'-P group were injected into the nuclei of *X. laevis* oocytes and the localization assay carried out. Both transcripts (FL, green) localized strongly to nucleoli (DAPI, blue) and with weaker signals to Cajal bodies (CB, red), indicating that the conversion of the 3'-OH group to a 3'-P group, which blocks La-binding does not affect localization to nucleoli or to Cajal bodies. Other details as in Figure 1a.

Injection of synthetic transcripts allows the visualization of RNA that transiently passes through the nucleolus. In contrast, detection by in situ hybridization of endogenous RNA can only detect steady state levels, which for U4 and U5 are weak at background levels for nucleoli in contrast to snurposomes (Gall *et al.*, 1999). Injection of synthetic transcripts also allows a kinetic analysis of nucleolar localization, which showed that U4 snRNA labeling was reduced and U5 snRNA signals were close to background 24 h after incubation. This observation is consistent with the predicted transient nature of snRNA localization to nucleoli, as has been already shown for U6 snRNA in the same system (Lange and Gerbi, 2000).

Furthermore, the kinetic analysis in the present study suggests that nucleolar localization of U4 snRNA and U5

snRNA can occur early during their maturation and before export to the cytoplasm. It seems that nuclear import after microinjection of various snRNAs, including U4 and U5 snRNA, into *Xenopus* oocytes is a time-limiting factor and consumes several hours (Fischer *et al.*, 1991), whereas efficient nuclear export occurs within 2 h (Terns and Goldfarb, 1998, and references therein). Even when U5 snRNA was equipped with a mature trimethyl G cap instead of a monomethyl G cap, efficient nuclear import still required 4 h of incubation (Fischer *et al.*, 1991). In the present study, nucleolar localization of injected U4 and U5 snRNA with a monomethyl G cap (same as after synthesis in vivo), however, took place within 8 min after nuclear injection. Thus, it is unlikely that the snRNAs traveled to the cytoplasm and were reimported into the nucleus within that short time

frame. It is probable that the injected snRNAs localized to nucleoli before export to the cytoplasm.

Because U4 and U5 snRNAs localize to nucleoli similar to U6 snRNA (Lange and Gerbi, 2000), it was possible that U6 snRNA might passively be carried to the nucleolus as part of the [U4/U6] di-snRNP or [U4/U6.U5] tri-snRNP. The present study rules out this possibility. First, we have shown herein that deletions of the proposed [U4/U6] interaction domain as well as the sequences flanking the interaction domain, which are essential for [U4/U6] snRNP assembly (Vankan *et al.*, 1990), do not affect nucleolar localization. Second, the 3' end of U6 is identified herein as the NoLE; it is not only essential but also sufficient for nucleolar localization and can be targeted to nucleoli by itself. The U6 3' end alone, however, is unable to assemble into a [U4/U6.U5] tri-snRNP (Vankan *et al.*, 1990, 1992). In contrast, the 3'-NoLE U6 mutant that does not localize to nucleoli is still able to assemble into a [U4/U6] snRNP (Figure 4); similarly, 3'-truncated U6 can form a tri-snRNP (Vidal *et al.*, 1999).

Although [U4/U6] snRNP formation is one prerequisite for splicing, recent studies have shown that regions of U6 essential for splicing exceed those essential for [U4/U6] snRNP assembly (Vankan *et al.*, 1990) and cover most of the molecule, including its middle part and 3' terminus. An important conclusion of the present study is that the 3' sequence of U6, while equally important for further function in splicing, enjoys a role as a NoLE fundamentally distinct from internal sequences in U6 that are used for [U4/U6] snRNP formation. Similarly, functionally important regions of box C/D snoRNAs involved in pre-rRNA processing are distinct from the box C/D NoLEs needed for nucleolar targeting of these RNAs (Lange *et al.*, 1998a,b,c). Thus, formation of a [U4/U6] snRNP is not a qualifying event for nucleolar localization because U6 snRNA seems fully capable of localizing to nucleoli independent of an association with U4 snRNA. Nevertheless, this capacity might be retained when U6 is part of the di-snRNP or tri-snRNP.

Alternatively, it could be hypothesized that nucleolar localization of U6 snRNA requires association with the snoRNAs guiding its modification; they might anchor U6 snRNA in the nucleolus. Just as in the scenario discussed above, U6 snRNA would passively localize in nucleoli due to interactions with other molecules that themselves contained the NoLEs. We have ruled out this possibility because the U6 3' construct of nt 87–107 (3' end) by itself can localize to nucleoli, although it does not include any of the sites to be methylated or pseudouridylated. Thus, the interaction of guide snoRNAs with regions to be modified in U6 snRNA cannot be essential for nucleolar localization of U6.

In line with previous reports (Carmo-Fonseca *et al.*, 1992; Matera and Ward, 1993; Bauer *et al.*, 1994), we have also shown here that U6 snRNA localizes to Cajal bodies. The relationship between Cajal bodies and nucleoli is not yet fully understood. However, data from yeast suggest that box C/D snoRNAs pass through Cajal bodies when trafficking to nucleoli (Verheggen *et al.*, 2001). Moreover, it has been reported that disruption of the snoRNA C/D motif seemed to block transfer from Cajal bodies to nucleoli (Narayanan *et al.*, 1999a). Those results suggest that the sequence essential for nucleolar localization of box C/D snoRNAs is not involved in localization to Cajal bodies. To date, only one sequence essential for RNA localization to Cajal bodies has

been identified, namely, the Sm site in U7 (Wu *et al.*, 1996), an snRNA that is not found in nucleoli. The present study shows that U6 snRNA after mutation of the 3' NoLE loses its ability to localize to both nucleoli and Cajal bodies, suggesting that this sequence may be both a Cajal body localization element (CaBLE) and a NoLE.

It seems that the nucleolus is an organelle where specific internal posttranscriptional modifications of snRNAs (2'-O-methylation and ψ) may be carried out by snoRNAs. Three box C/D snoRNAs have already been identified as guide RNAs for the 2'-O-methylation of U6, and other results suggest that factors needed to form 2'-O-methylation and ψ of U6 snRNA function in the nucleolus (Tycowski *et al.*, 1998; Ganot *et al.*, 1999). In addition, correlative evidence suggests that U2 snRNA modifications occur in the nucleolus rather than the Cajal bodies (Yu *et al.*, 2001). Because the 3' end of U6 snRNA is required for localization to both nucleoli and Cajal bodies, it cannot be discerned whether snRNA modification occurs in just one or both of these organelles.

Candidate Proteins That May Interact with the 3' NoLE of U6 snRNA

NoLEs of snoRNAs are recognized by proteins that might transport the snoRNA from the nucleoplasm to the nucleolus and/or anchor it within the nucleolus. Recent evidence supports this idea, because all four proteins associated with the box C/D motif are needed for efficient nucleolar localization of U14 snoRNA (Verheggen *et al.*, 2001). Yeast box C/D snoRNAs interact with Srp40p in the Cajal body and then seem to be delivered by Nsr1p to the nucleolus (Verheggen *et al.*, 2001).

Similarly, the nucleolar localization of U6 might be mediated by proteins that assemble on its NoLE before engagement in a di- or tri-snRNP. Although, several proteins make direct contact with U6 snRNA during conversion from free U6 to the tri-snRNP (Vidal *et al.*, 1999), only the La protein and the Lsm protein complex bind to the 3' end of U6 snRNA (see INTRODUCTION), which is the NoLE essential for U6 nucleolar localization as shown in the present report. La is the first protein to associate with U6 snRNA upon transcription (Rinke and Steitz, 1985; Kunkel *et al.*, 1986), and it also has been shown that deletion of the 8 nt at the U6 3' end decreases the interaction of U6 with the La protein (Boelens *et al.*, 1995) and significantly reduces nucleolar localization ($\Delta 100$ –107; this study). Although La is found predominantly in nuclear speckles (Bachmann *et al.*, 1989), it might transiently and/or in low amounts appear in nucleoli where it has been observed (Deng and Tan, 1985; Graus *et al.*, 1985). Various RNA polymerase III transcripts, which require binding of La to their precursors to guarantee a normal pathway of maturation (Yoo and Wolin, 1997), are found in nucleoli (Maraia, 2001), including pre-tRNAs (Bertrand *et al.*, 1998) and U6 (Lange and Gerbi, 2000). All these observations suggest that La could be involved in nucleolar localization of RNAs. It has even been proposed that La is needed to stabilize some RNA polymerase II transcripts like U4 (Xue *et al.*, 2000), shown here to localize to nucleoli and also binds U3 snoRNA (Kufel *et al.*, 2000). Intriguing although the idea may be, in the present report we have ruled out a role of La for U6 snRNA nucleolar localization. We show herein that U6 snRNA that lacks a 3'-hydroxyl group and therefore cannot associate with the La protein localizes

efficiently to nucleoli nonetheless. Therefore, we conclude that it is not La but instead is another factor that mediates nucleolar localization by binding to the 3' NoLE of U6. Similarly, it seems unlikely that La mediates nucleolar localization of certain other RNA polymerase III transcripts (e.g., SRP RNA, RNase P RNA; for review, see Maraia, 2001).

In the *in vivo* situation, during the maturation of U6 snRNA, La is replaced with the Sm-like protein complex Lsm2–8, which is a likely candidate to play an important role in nucleolar localization of U6 snRNA. Interestingly, Lsm proteins also associate with pre-RNase P RNA (Salgado-Garrido *et al.*, 1999), which is found in nucleoli (Jacobson *et al.*, 1997; Bertrand *et al.*, 1998; Jarrous *et al.*, 1999). The Lsm complex binds to the 3' end of U6 snRNA with a 3'-hydroxyl end and to mature U6 snRNA (Achse *et al.*, 1999) whose 3' end is a 2',3'-cyclic phosphate (Lund and Dahlberg, 1992; Terns *et al.*, 1992). Similarly, we found that U6 snRNA with either a 3' hydroxyl or a 3' phosphate end localized to nucleoli. Moreover, no other part than the U6 3' end associates with the Lsm proteins stably enough to be coprecipitated with an anti-Lsm4 antibodies (Achse *et al.*, 1999; Stevens and Abelson, 1999). Our finding that the 3' end of U6 is the NoLE is consistent with the hypothesis that the Lsm complex might mediate U6 nucleolar localization. Although the five uridine residues at the extreme 3' end are required for Lsm binding (Achse *et al.*, 1999), they are not sufficient. Similarly, we found that U6 mutants with deletion of areas within nt 82–107 showed weak although greatly diminished nucleolar localization, suggesting that the NoLE cannot be subdivided.

Another protein, Prp24, interacts with Lsm proteins and binds to U6 snRNA, promoting formation of the [U4/U6] di-snRNP (Ghetti *et al.*, 1995; Jandrositz and Guthrie, 1995; Raghunathan and Guthrie, 1998a; Vidal *et al.*, 1999; Fromont-Racine *et al.*, 2000), but it cannot play an independent role in nucleolar localization of U6 because it binds to U6 at positions 39–56 and 64–76 (Ghetti *et al.*, 1995) that are not NoLEs as we have demonstrated here.

Nucleolar localization of U6 snRNA via the 3' NoLE might occur after U6 engagement in a di- or tri-snRNP. However, in the more likely scenario, nucleolar localization is initiated for noncomplexed U6 before formation of the di- or tri-snRNP, because we have shown herein that association with U4 snRNA is not a prerequisite for localization. Therefore, the present report suggests the nucleolar entry of U6 snRNA at a point during its life cycle when Lsm proteins replace La in binding to the NoLE of U6 and before association of U6 with U4 in the di-snRNP.

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