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U4 snRNA nucleolar localization requires the NHPX/15.5-kD protein binding site but not Sm protein or U6 snRNA association

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All small nuclear RNAs (snRNAs) of the [U4/U6.U5] tri-snRNP localize transiently to nucleoli, as visualized by microscopy after injection of fluorescein-labeled transcripts into *Xenopus laevis* oocyte nuclei. Here, we demonstrate that these RNAs traffic to nucleoli independently of one another, because U4 snRNA deleted in the U6 base-pairing region still localizes to nucleoli. Furthermore, depletion of endogenous U6 snRNA does not affect nucleolar localization of injected U4 or U5. The wild-type U4 transcripts used here are functional: they exhibit normal nucleocytoplasmic traffic, associate with Sm proteins, form the [U4/U6] di-snRNP, and localize to nucleoli and Cajal bodies. The nucleolar localization element (NoLE) of U4 snRNA was mapped by mutagenesis. Neither the 5′-cap nor the 3′-region of U4, which includes the Sm protein binding site, are essential for nucleolar localization. The only region in U4 snRNA required for nucleolar localization is the 5′-proximal stem loop, which contains the binding site for the NHPX/15.5-kD protein. Even mutation of just five nucleotides, essential for binding this protein, impaired U4 nucleolar localization. Intriguingly, the NHPX/15.5-kD protein also binds the nucleolar localization element of box C/D small nucleolar RNAs, suggesting that this protein might mediate nucleolar localization of several small RNAs.

Introduction

A fascinating feature of the nucleolus is the multitude of RNA localizing or transiting through this plurifunctional organelle in the nucleus of eukaryotic cells (Bertrand et al., 1998; Pederson, 1998; Jarrous et al., 1999; Mitchell et al., 1999; Gerbi et al., 2001). Recently, it has been demonstrated that even spliceosomal small nuclear RNAs (snRNAs) and small nuclear RNP (snRNP) components can transit through the nucleolus (Sleeman and Lamond, 1999; Lange and Gerbi, 2000; Yu et al., 2001; Gerbi and Lange, 2002). Nucleolar localization of U4 snRNA is the subject of this paper.

For RNA polymerase (pol) III–transcribed U6 snRNA, nucleolar localization appears to occur early during its maturation and might be mediated by Sm-like (Lsm) proteins, which assemble on the 3′-nucleolar localization element (NoLE) of U6 (Gerbi and Lange, 2002). In contrast to U6, which remains in the nucleus, the RNA pol II–transcribed snRNAs of the spliceosome include a cytoplasmic phase during their maturation. After transcription, they are exported to the cytoplasm where they assemble with the Sm protein complex and their 5′–methylguanosine cap is hypermethylated (for reviews see Will and Lührmann, 2001; Gerbi et al., 2003). Although Sm proteins are essential for subsequent nuclear re-import, the requirement for a hypermethylated cap seems to depend on the cell type (Fischer et al., 1994).

The spliceosome forms by the ordered interaction of the U1 and U2 snRNPs, the [U4/U6.U5] tri-snRNP particle, and assistance by non-snRNP splicing factors (Konarska and Sharp, 1988; Behrens and Lührmann, 1991; Wassarman and Steitz, 1992; Will and Lührmann, 1997, 2001). U4 snRNA base pairs with U6 snRNA to form the [U4/U6] di-snRNP; this process is assisted by Prp24p in yeast (Raghunathan and Guthrie, 1998; Rader and Guthrie, 2002) or the human homologue SART3/p110 (Bell et al., 2002; Rader and Guthrie, 2002). The di-snRNP then associates with U5 to form the [U4/U6.U5] tri-snRNP and SART3/p110 is released. Subsequent rearrangements of snRNAs in the spliceosome result in the dissociation of U4 from U6 when U6 enters a new base-pairing interaction with U2 snRNA (Staley and Guthrie, 1998). Splicing of pre-

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Abbreviations used in this paper: NoLE, nucleolar localization element; pol, polymerase; scaRNA, small Cajal body–specific RNA. snRNA, small nuclear RNA; snRNP; small nuclear RNP; snoRNA, small nucleolar RNA.

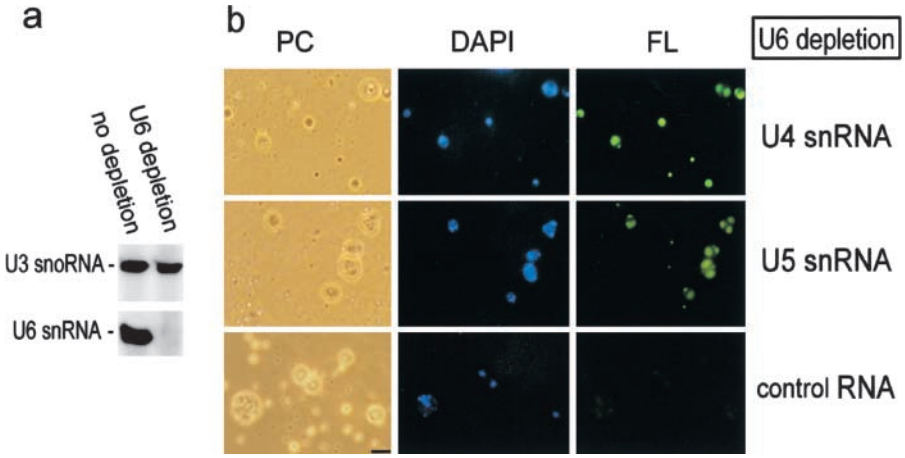

Figure 1. **Nucleolar localization of U4 and U5 snRNAs does not depend on U6 snRNA.** Fluorescein-labeled U4 or U5 snRNA were injected into the nuclei of *Xenopus* oocytes that were depleted of endogenous U6 snRNA using antisense oligonucleotides. (a) Northern blot analysis of RNA from U6-depleted oocyte nuclei demonstrated the absence of endogenous U6 snRNA, in contrast to the presence of endogenous U3 snoRNA (control). (b) Injection of U4 or U5 snRNAs into U6-depleted oocyte nuclei resulted in nucleolar labeling (FL, green) 1.5 h later, showing that U4 and U5 nucleolar localization is independent from U6. The multiple nucleoli present in *Xenopus* oocyte nuclei are visualized in nucleolar preparations by phase contrast (PC) and can be distinguished from other nuclear bodies by the staining of rDNA (DAPI, blue) located only in nucleoli. Bar, 10 μM.

mRNA occurs in the nucleoplasm, and upon its completion, the snRNPs are released and recycled in the nucleus for another round of splicing. Regeneration of the spliceosome requires reformation of the di- and tri-snRNP (Raghunathan and Guthrie, 1998; Staley and Guthrie, 1998). Little is known about the cellular location of di- and tri-snRNP formation.

There is a paucity of information about when in their life cycle U4, U5, and U6 snRNAs traffic through the nucleolus. Here, we identify the NoLE of U4 snRNA and analyze if certain steps of cytoplasmic maturation of snRNA, namely assembly with Sm proteins or trimethylation of the 5′-cap, are required for U4 nucleolar localization.

The experiments presented here demonstrate that all snRNA components of the spliceosomal [U4/U6.U5] tri-snRNP particle localize independently to nucleoli. The base-pairing interaction of U4 snRNA with U6 snRNA is not essential for nucleolar localization of U4 snRNA, and the presence of U6 snRNA is not important for nucleolar localization of U4 or U5 snRNA. Moreover, nucleolar localization of U4 and U5 does not require the Sm protein binding site nor a hypermethylated 5′–G cap. These observations are consistent with the possibility that U4 snRNA can traffic through the nucleolus before nuclear export in its maturation pathway. U4 snRNA localizes to nucleoli and Cajal bodies; nucleolar localization is more pronounced after nuclear injection of U4 transcripts and Cajal body localization predominates after cytoplasmic injection. U4 snRNA does not use its 5′ end or any sequences in the 3′ half of the molecule for nucleolar association. The only region in U4 snRNA required for nucleolar localization is the 5′-proximal stem loop, which includes the binding site for the NHPX/15.5-kD protein; mutation of just five nucleotides essential for binding this protein impaired U4 nucleolar localization.

Results

The RNA components of the [U4/U6.U5] tri-snRNP localize to nucleoli independent of one another

Previously, we reported that U4, U5, and U6 transiently localize to nucleoli of *Xenopus laevis* oocytes (Lange and Gerbi, 2000; Gerbi and Lange, 2002). To address if U4 and U5 have their own NoLEs and do not depend on U6 snRNA as a carrier, fluorescein-labeled in vitro transcripts of U4 snRNA, U5 snRNA, or a control RNA were injected into *Xenopus* oocyte nuclei that were depleted of endogenous U6 snRNA (Fig. 1 a). This assay allows direct visualization of the labeled RNA in nucleolar preparations (Fig. 1 b) and has been used in nondepleted oocytes to monitor nucleolar localization of small nucleolar RNAs (snoRNAs) from various families as well as snRNAs (for review see Lange, 2003). Here, the assay was combined with disruption of endogenous U6 snRNA through RNase H–mediated degradation by nuclear injections of anti-U6 snRNA antisense oligonucleotides (Vankan et al., 1990, 1992; Gerbi and Lange, 2002).

As shown in Fig. 1 b, injection of U4 or U5 transcripts into oocyte nuclei results in specific fluorescent nucleolar signals 1.5 h later, despite the depletion of endogenous U6 snRNA (Fig. 1 a), whereas injection of a control RNA does not label nucleoli (Fig. 1 b). The signal strength is strong for U4 and moderate for U5 snRNA, similar to nondepleted oocytes (compare with Figs. 3 and 8 and Gerbi and Lange, 2002), indicating that the nucleolar localization of U4 and U5 snRNAs is not impaired by the absence of U6 snRNA. Therefore, the data presented in Fig. 1 demonstrate that U4 and U5 snRNAs localize to nucleoli independently of U6 snRNA.

This result was also supported by mutational analysis of U4 snRNA that demonstrated that sites of base pairing between U4 and U6 snRNA are not essential for nucleolar localization. Fig. 2 depicts all the U4 snRNA mutations studied here. Deletion of nt 1–18 and 56–63 removed the sequences of U6 snRNA that base pair with U4 snRNA; nevertheless, fluorescent transcripts of Δ1–18/56–63 U4 still localized to nucleoli as efficiently as wild-type U4 (Fig. 3).

The 5′-proximal stem loop containing the NHPX/15.5-kD protein binding site of U4 snRNA is a NoLE

To define cis-acting NoLEs of U4 snRNA necessary for nucleolar localization, the localization of mutant transcripts

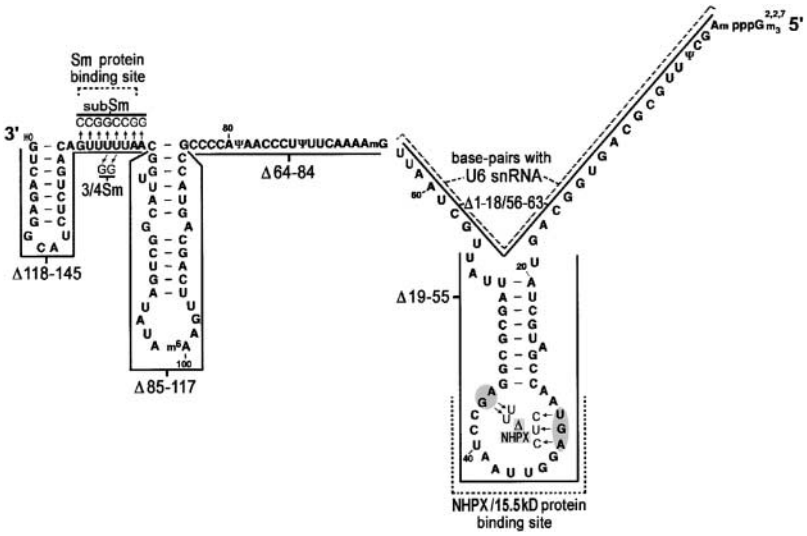

Figure 2. **Sequence and mutations of U4 snRNA.** The structure of chicken UB4 snRNA is shown with sites of 2′-*O*-methylation and pseudouridylation indicated (modified after Tycowski et al., 1998 for human U4A). The 3′-end is extended by three nucleotides not removed by processing from some U4 isoforms (Hoffman et al., 1986). Most mutations designed for this work were deletions covering the nucleotides indicated by lines. The site of base pairing with U6 in the di-snRNP (dashed line) was deleted in mutant Δ1–18/56–63. Nucleotides within the binding domain (dotted) for the NHPX/15.5-kD protein that are essential for NHPX/15.5-kD protein interaction (Nottrott et al., 1999) are shaded and were substituted in the present work (ΔNHPX/15.5 kD) as indicated. The Sm protein-binding site was mutated either by substitution of two nucleotides (3/4Sm) or the entire sequence (subSm).

(mutations shown in Fig. 2) was compared with that of wild-type U4. Such NoLEs were defined recently for various families of snoRNAs (for review see Lange, 2003) as well as U2 snRNA (Yu et al., 2001) and U6 snRNA (Gerbi and Lange, 2002).

As noted above, deletion of sites in U4 (Δ1–18/56–63) that base pair with U6 and are important for snRNP assembly (Vankan et al., 1990) did not affect nucleolar localization of U4. Similarly, U4 snRNA carrying deletions of nt 64–84 or 85–117 localizes to nucleoli. Interestingly, a deletion of nt 118–145 of U4 that lacks both the 3′-terminal stem and also the Sm protein binding site (discussed further in the next section) did not affect U4 nucleolar localization (Fig. 3). In contrast, nucleolar localization of U4 was completely abolished by a deletion of the 5′-proximal stem loop (nt 19–55; Fig. 3).

One outstanding feature of nt 19–55 required for nucleolar localization of U4 is the presence of the binding site for

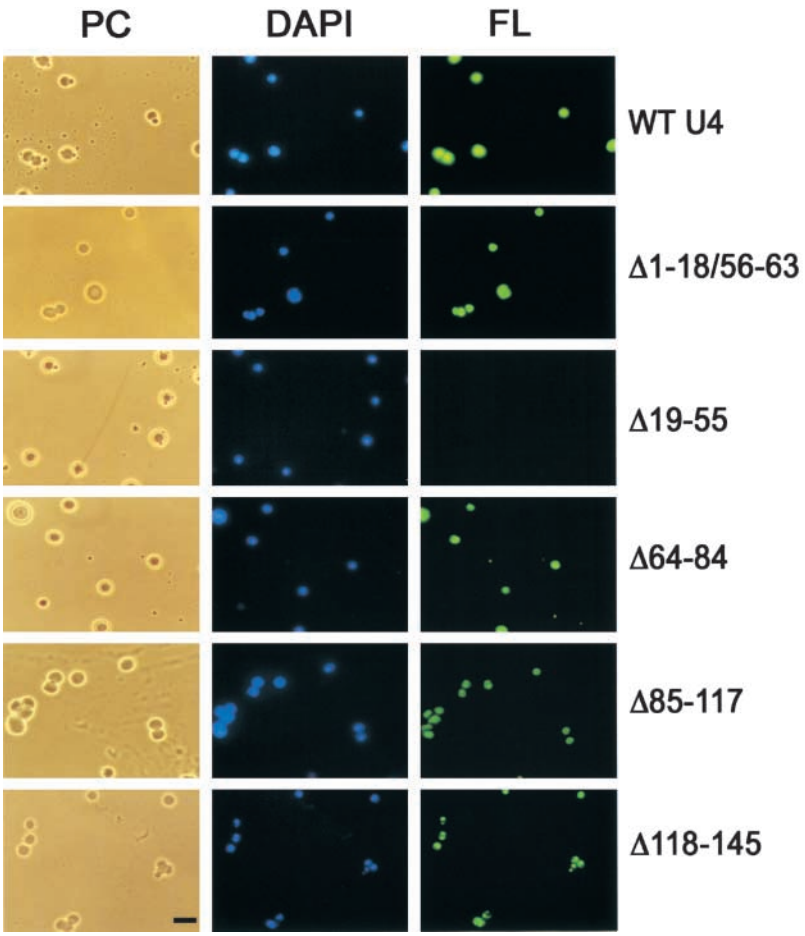

Figure 3. **Nucleolar localization of U4 snRNA mutated in various positions throughout the molecule.** Wild-type or mutated fluorescein-labeled U4 snRNA transcripts were injected into the nuclei of *Xenopus* oocytes. U4 snRNA deleted in the sites for base pairing with U6 (Δ1–18/56–63), carrying other deletions (Δ64–84, Δ85–117), or deleted in the 3′-area containing the Sm protein binding site (Δ118–145), retained the ability to localize to nucleoli (FL-green). In contrast, a deletion of the 5′-proximal stem loop (Δ19–55) completely abolished nucleolar localization, indicating that this sequence included a nucleolar localization element (NoLE). Bar, 10 μM. Other details as in Fig. 1.

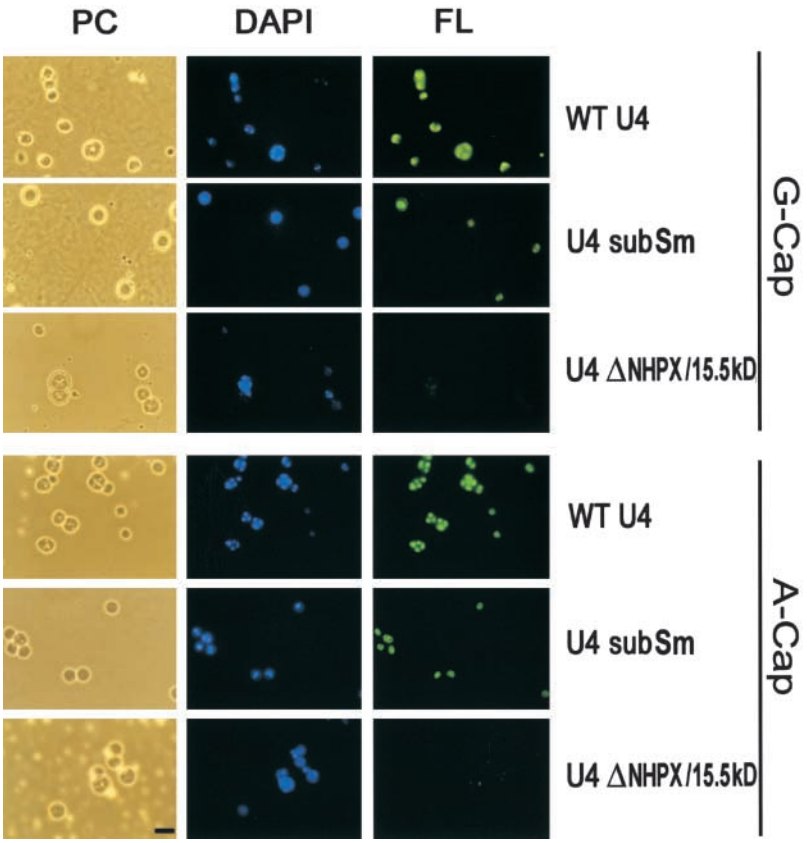

Figure 4. **Nucleolar localization of U4 snRNA requires the NHPX/15.5-kD protein binding region but not the Sm protein binding site or the 5′-cap structure.** The nucleolar localization assay was performed to compare nucleolar localization of U4 mutated in the Sm binding site (U4 subSm) with U4 substituted in nucleotides essential for binding the NHPX/15.5-kD protein (U4 ΔNHPX/15.5 kD). U4 association with Sm proteins is not required for nucleolar localization. In contrast, mutation of the NHPX/15.5-kD protein binding site impaired nucleolar localization of U4 snRNA. Nucleolar localization is independent of the 5′-cap structure because capping of U4 with a synthetic–A cap instead of a G cap still allowed nucleolar localization of wild-type or mutant U4 snRNA. Bar, 10 μM. Other details as in Fig. 1.

the NHPX/15.5-kD (= yeast Snu13p) protein (Nottrott et al., 1999). We dissected this region further by mutating just the NHPX/15.5-kD protein binding site of U4 by substitution of five nucleotides (Fig. 2), which are essential for binding this protein (Nottrott et al., 1999). The nucleolar localization assay shows that mutation of the NHPX/15.5-kD protein binding site impaired nucleolar localization of U4 snRNA (Fig. 4). In contrast to mutant Δ19–55 (Fig. 3) with a deletion of the entire 5′-proximal stem loop, which completely abolished nucleolar localization, the ΔNHPX/15.5-kD mutant substituted in only five nucleotides exhibited some variability with signals ranging from weak to background staining of nucleoli. In comparison to wild-type U4, however, the ΔNHPX/15.5-kD mutant is clearly impaired in nucleolar localization.

Several control experiments were performed to confirm that the binding site for the NHPX/15.5-kD protein to U4 is required for U4 nucleolar localization and that the remainder of the U4 snRNA molecule 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.

To guard against the possibility that failure of a mutant to localize to nucleoli might simply be due to its degradation, stability assays using ^{32}P-labeled transcripts were performed. All transcripts were stable 1.5 h after injection into oocyte nuclei, the time when the localization assays were performed (Fig. 5). This included U4 mutants Δ19–55 and ΔNHPX/15.5 kD that failed to localize to nucleoli.

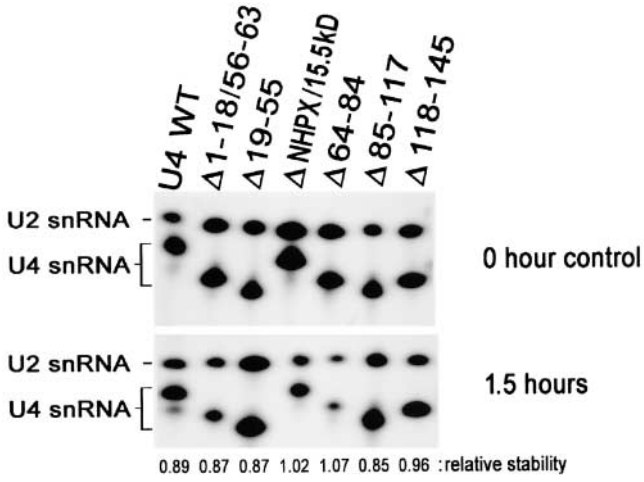

Figure 5. **Stability of wild-type and mutated U4 snRNA.** ^{32}P-labeled U4 snRNA (mutants or wild type) were injected into oocyte nuclei; nuclear RNA was isolated and analyzed by 8% polyacrylamide, 8 M urea gel electrophoresis (PAGE). The top panel shows the controls (sample recovery immediately after injection, 0 h), the bottom panel shows the short-term stability at 1.5 h (the time when localization assays were performed). To determine the stability of the various RNAs after nuclear injection, ^{32}P-labeled U2 snRNA was coinjected and served as an internal control to normalize for any differences in injection or recovery of the samples. The relative RNA stability is the ratio [(U4 RNA transcript/U2 after incubation)/(U4 RNA transcript/U2 at 0 h)]. All the mutants are stable at the 1.5-h time point used for analysis of nucleolar localization.

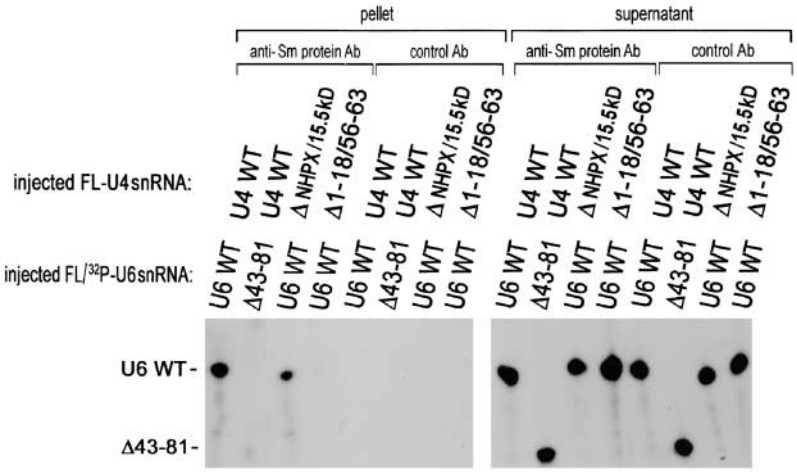

Figure 6. **The NoLE mutant of U4 snRNA can form a [U4/U6] snRNP.** U4 snRNA transcripts (labeled with fluorescein-UTP) and U6 snRNA (co-labeled with [^{32}P]UTP and fluorescein-UTP) were coinjected into *Xenopus* oocyte nuclei depleted of endogenous U4 and U6 snRNA. After 4 h of incubation, the ability of the in vitro transcripts to form a [U4/U6] snRNP was analyzed by immunoprecipitation from nuclear lysate with anti-Sm protein antibody. 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 by PAGE. U4/U6 snRNP assembly occurs between wild-type U4 snRNA (U4 WT) and wild-type U6 snRNA (U6 WT), and even the U4 NoLE mutant (ΔNHPX/15.5 kD) retains the ability to coprecipitate U6 snRNA. Di-snRNP formation was disrupted in mutant U4 (Δ1–18/56–63) or mutant U6 (Δ43–81) that lack the sites for U4-U6 base pairing. No immunoprecipitation occurred when using beads coupled to control antibody. The supernatant lanes demonstrate that equal amounts of U6 transcript were used in the various immunoprecipitations.

Control experiments confirmed that in vitro transcripts of U4 and U6 snRNAs retained their functional activity to form a [U4/U6] di-snRNP. U6 snRNA (co-labeled with [^{32}P]UTP and fluorescein-UTP) was coinjected together with U4 snRNA transcripts (labeled with fluorescein-UTP) into U6- and U4-depleted *Xenopus* oocytes, and subsequently immunoprecipitated from the nuclear extract by an anti-Sm antibody. Because Sm proteins are bound to U4 and not to U6 snRNA, the immunoprecipitation of radioactive U6 indicates that it is associated with U4 snRNA (Vankan et al., 1990). As shown in Fig. 6, wild-type U4 and U6 in vitro transcripts are functionally able to form a [U4/U6] di-snRNP, as they are coimmunoprecipitated by the anti-Sm antibody but not by a control antibody. Mutation of the base-pairing site in either U4 (Δ1–18/56–63) or U6 (Δ43–81) prevented coimmunoprecipitation (Fig. 6); nonetheless, both the U4 and U6 base-pairing mutants could still localize to nucleoli (Fig. 3; Gerbi and Lange, 2002). Importantly, the NoLE mutant of U4 (ΔNHPX/15.5 kD) still retained the ability to base pair with U4 and form a di-snRNP, even though it was unable to localize to nucleoli. Therefore, nucleolar localization and di-snRNP formation are separable properties of U4 and U6 snRNAs.

Immunoprecipitation assays confirmed that the NoLE mutants of U4 snRNA cannot associate with the NHPX/15.5-kD protein anymore, supporting the implication of this protein in nucleolar localization. The functional ability of U4 to bind to the NHPX/15.5-kD protein was first analyzed using HeLa cell nuclear lysate incubated with various U4 transcripts and anti–human NHPX/15.5-kD protein antiserum coupled to protein A–Sepharose beads (Fig. 7 a). The NHPX/15.5-kD protein can bind to wild-type U4 snRNA (U4 WT) and with a slightly lesser efficiency to U4 mutated in the Sm site (subSm), but not to NoLE mutants with either deletion of the entire 5′-proximal stem loop (Δ19–55) or substituted in the five conserved nucleotides (ΔNHPX/15.5 kD) known to be essential for binding to human NHPX/15.5-kD protein (Nottrott et al., 1999).

Similarly, the NHPX/15.5-kD antiserum precipitated wild-type U4, and U4 mutated in the Sm site (subSm), but not U4 transcripts with the five-nucleotide substitution in the 5′-proximal stem loop (ΔNHPX/15.5 kD) from *Xenopus* oocyte nuclear lysate (Fig. 7 b). Moreover, U4 snRNA carrying a deletion of sequences needed to base pair with U6 snRNA (Δ1–18/56–63), but still containing the 5′-proximal stem loop, could be precipitated (Fig. 7 b, bottom) although the signals obtained, and, thus, the protein–RNA interaction, were somewhat reduced. Only background signals were observed with control beads coupled with preimmune serum (Fig. 7 b).

In summary, the antiserum used precipitated all tested U4 transcripts other than the NoLE mutants and, thus, specifically recognized the endogenous counterpart of the human NHPX/15.5-kD protein in *Xenopus*. By immunostaining of a nuclear preparation of *Xenopus* oocyte nuclei, we investigated the nuclear location of the NHPX/15.5-kD protein. Cajal bodies and also nucleoli were specifically stained (Fig. 7 c). Thus, the endogenous counterpart of the human NHPX/15.5-kD protein can be detected in both *Xenopus* Cajal bodies and nucleoli. In contrast, anticoilin antiserum (as a control for detection of Cajal bodies; Gall et al., 1999) strongly stained Cajal bodies but not nucleoli. Rabbit preimmune serum (as a negative control) did not stain any structure in the nuclear spreads (Fig. 7 c).

The Sm protein binding site and the 5′-cap structure are not essential for nucleolar localization of U4 or U5 snRNAs

Recently, it has been be suggested that both the internal modification, as well as the nucleolar localization of U2 snRNA, is dependent on the Sm protein binding site (Yu et al., 2001) and consequently the Sm binding site could be a NoLE, which enables all snRNAs, other than U6, to individually localize to nucleoli. As shown the preceding section for U4 snRNA, we observed that a deletion of nt 118–145

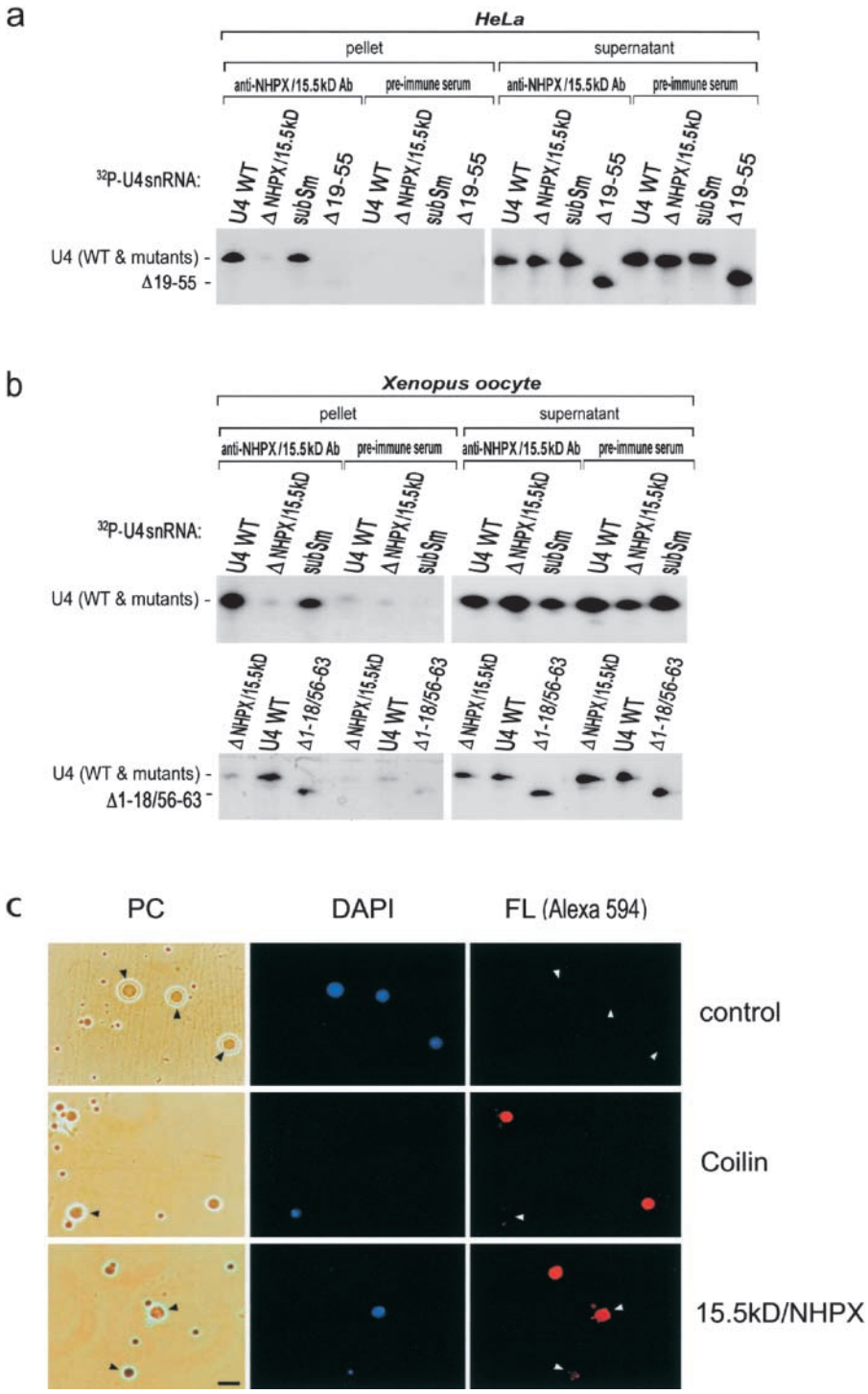

Figure 7. **U4 wild-type snRNA but not the NoLE mutants associate with the endogenous counterpart of the human NHPX/15.5-kD protein in *Xenopus*.** The ability of U4 transcripts (labeled with [^{32}P]UTP) to bind to the NHPX/15.5-kD protein was analyzed by immunoprecipitation from either (a) HeLa cell nuclear lysate or (b) *Xenopus* oocyte nuclear lysate using an anti–human NHPX/15.5-kD protein antiserum. After immunoprecipitation, either the total precipitate or 1/100 of the supernatant (control for equal amounts injected) were analyzed by PAGE. The NHPX/15.5-kD protein from Hela cells as well as from *Xenopus* can bind to wild-type U4 snRNA (U4 WT) in vitro transcripts and with slightly less efficiency to U4 mutated in the Sm site (subSm). In contrast, the NoLE mutants with a deletion of the entire 5′-proximal stem loop (Δ19–55) or with substitution in five conserved nucleotides (ΔNHPX/15.5 kD) shown to be essential for binding the NHPX/15.5-kD protein (Nottrott et al., 1999) are not precipitated. U4 snRNA after deletion of sequences that base pair with U6 snRNA (Δ1–18/56–63), which still carried the 5′-proximal stem loop, could still be recognized and precipitated by the antibody although the signals obtained were slightly reduced (b, bottom). Only background signals were observed with control beads coupled with preimmune serum. The supernatant lanes show that equal amounts of U4 were used in the various immunoprecipitations. (c) Immunostaining of a nuclear preparation of *Xenopus* oocyte nuclei was performed using rabbit anti-NHPX/15.5-kD antiserum, rabbit anticoilin antiserum (as a control for staining of Cajal bodies) or rabbit preimmune serum (as a negative control). The goat anti–rabbit secondary antibody was coupled to Alexa 594 (red signals). Nucleoli contain rDNA (DAPI, blue) and can be distinguished from Cajal bodies that lack rDNA and, thus, are not stained by DAPI. Moreover, Cajal bodies are often associated with B-snurposomes (Gall et al., 1999 and references therein). Coilin was detected in Cajal bodies, whereas the anti–NHPX/15.5-kD antiserum stained Cajal bodies uniformly and also stained nucleoli (arrowheads in phase contrast [PC] and immunofluorescence [FL] panels) in a more spotted manner, suggesting that the endogenous counterpart of the human NHPX/15.5-kD protein is located in *Xenopus* Cajal bodies and nucleoli. The control serum did not stain any structure in the nuclear spread. Bar, 10 μm.

that includes the Sm protein binding site does not appreciably affect U4 nucleolar localization (Fig. 3). Thus, we studied the role of the Sm site in nucleolar localization in more detail by designing mutants of the Sm site of U4 as well as U5 snRNA. The Sm site of these two snRNAs was fully substituted by a stretch of unrelated nucleotides (subSm; Fig. 2). When the ability of the U4 mutant subSm to localize to nucleoli was compared with wild-type U4 (positive control) or mutant ΔNHPX/15.5 kD (negative control), it could be observed that the Sm site is not an essential NoLE (Fig. 4). Signals for the U4 subSm mutant were generally close to signals obtained with the wild-type snRNA, though with somewhat more variability. Signals for the U4 NoLE mutant ΔNHPX/15.5 kD were weak or at background levels.

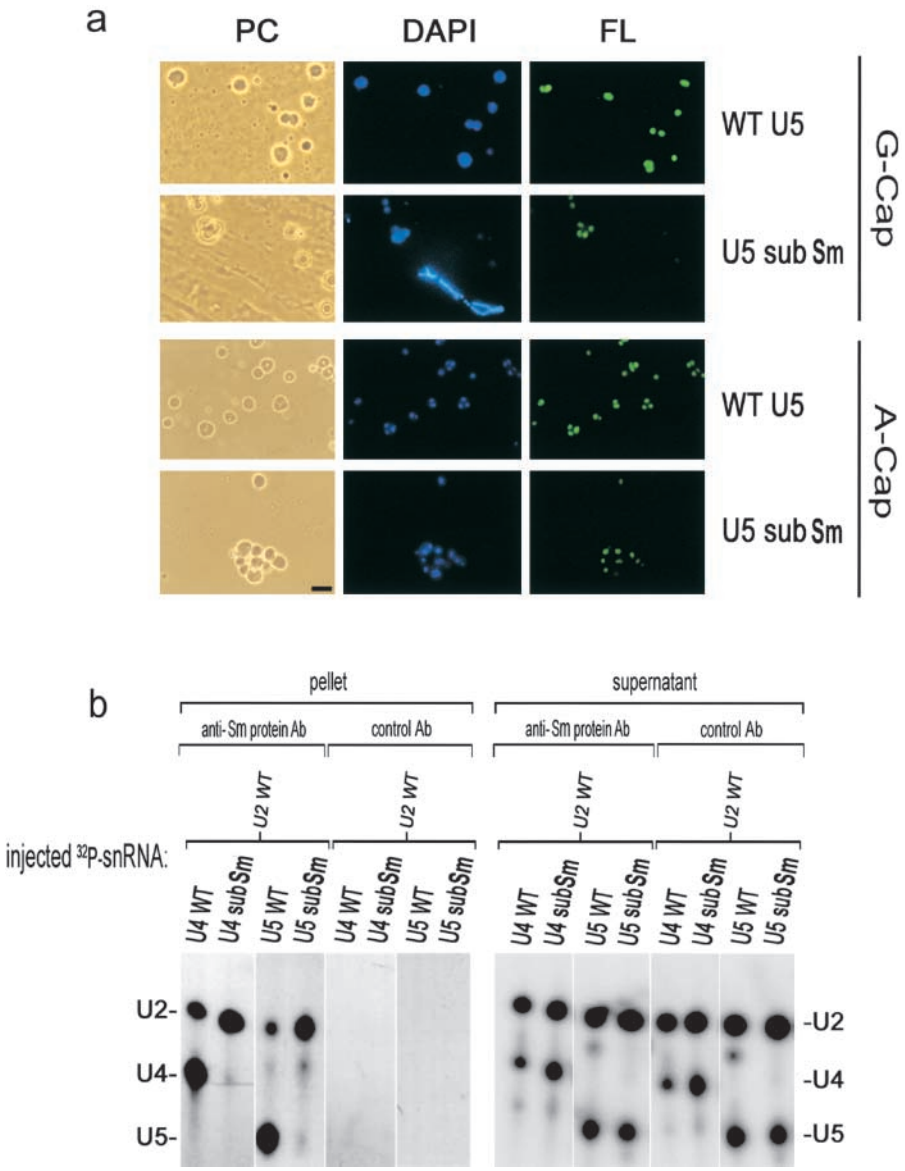

Figure 8. **Nucleolar localization of U5 snRNA does not depend on the 5′-cap structure and can occur after mutation of the Sm protein binding site.** (a) Mutational analysis of U5 snRNA was performed to determine if the Sm protein binding site is essential for nucleolar localization. No major differences in nucleolar signals from wild-type U5 were observed for U5 subSm, carrying a substitution of the entire Sm binding site. Nucleolar localization was comparable for wild-type and subSm U5 snRNA capped with an A cap instead of a G cap. Thus, the Sm site and nature of the 5′-cap are not essential NoLEs for U5 snRNA, consistent with data for U4 snRNA (Fig. 4). Bar, 10 μM. Other details as in Fig. 1. (b) The ability of the synthetic RNA transcripts used here to associate with Sm proteins was analyzed. U4 or U5 snRNAs (co-labeled with [^{32}P]UTP and fluorescein-UTP) were injected into *Xenopus* oocyte nuclei. After 4 h of incubation, immunoprecipitation from nuclear lysates was performed with an anti-Sm protein antibody. Coinjection of labeled wild-type U2 snRNA served as an internal control. The equivalent of five nuclei/sample of the immunoprecipitated RNA (pellet) and 0.2 nuclei/sample of the supernatant (control for equal amounts injected) were analyzed on a denaturing gel. Wild-type U2, U4, and U5 snRNAs can be immunoprecipitated with an anti-Sm antibody, unlike U4 and U5 mutants with substitution (subSm) of the Sm site. No immunoprecipitation occurred when using beads coupled to control antibody.

As shown in Fig. 8 a, a similar result was obtained for U5 snRNA when the Sm site was substituted (U5 subSm), suggesting that the Sm protein binding site is not essential for U4 nor U5 snRNA nucleolar localization. Similarly, nucleolar localization was still observed when nucleotide positions three and four of the Sm binding site in U4 and U5 snRNAs were substituted with GG (unpublished data); this is a comparable mutation to that used by Yu et al. (2001) for U2 snRNA that was reported to impair U2 nucleolar localization.

We performed a series of control experiments with the Sm mutants of U4 and U5 to confirm a loss of function after mutation of the Sm site. Accordingly, after injection into oocyte nuclei and before export to the cytoplasm all of these mutant snRNAs showed a high stability and presence in the nucleus just like the wild type. However, after injection into the cytoplasm, in contrast to wild-type transcripts, none of the four Sm mutants of U4 or U5 was able to travel to the nucleus (unpublished data). Moreover, as shown in Fig. 8 b, the Sm mutant transcripts fail to bind to endogenous Sm proteins of *Xenopus* oocytes, whereas the wild-type snRNAs do. In these experiments wild-type or subSm transcripts of U4 or U5 snRNAs, co-labeled with [^{32}P]UTP and fluorescein-UTP, were coinjected together with wild-type U2 snRNA (as an internal control for recovery of material) into oocyte nuclei. Thus, subsequent immunoprecipitation of these snRNAs from the nuclear lysate with an anti-Sm antibody and PAGE allows visualization of transcripts bound by Sm proteins. Fig. 8 b confirms that the U4 and U5 synthetic wild-type transcripts were able to functionally associate with endogenous Sm proteins whereas the Sm mutants were not. Thus, nucleolar localization occurs even though the Sm site is dysfunctional and cannot bind Sm proteins (Fig. 4 and Fig. 8 a).

We also ruled out that the nature of the 5′-cap on the injected U4 or U5 snRNAs is important for nucleolar localization. In vivo, the spliceosomal snRNAs transcribed by RNA pol II are exported to the cytoplasm where the 5′-cap is converted from a monomethyl G (m^7GpppG) to a trimethyl G ($m^{2,2,7}$GpppG) and Sm proteins are bound; subsequently, the snRNAs are re-imported back into the nucleus to func-

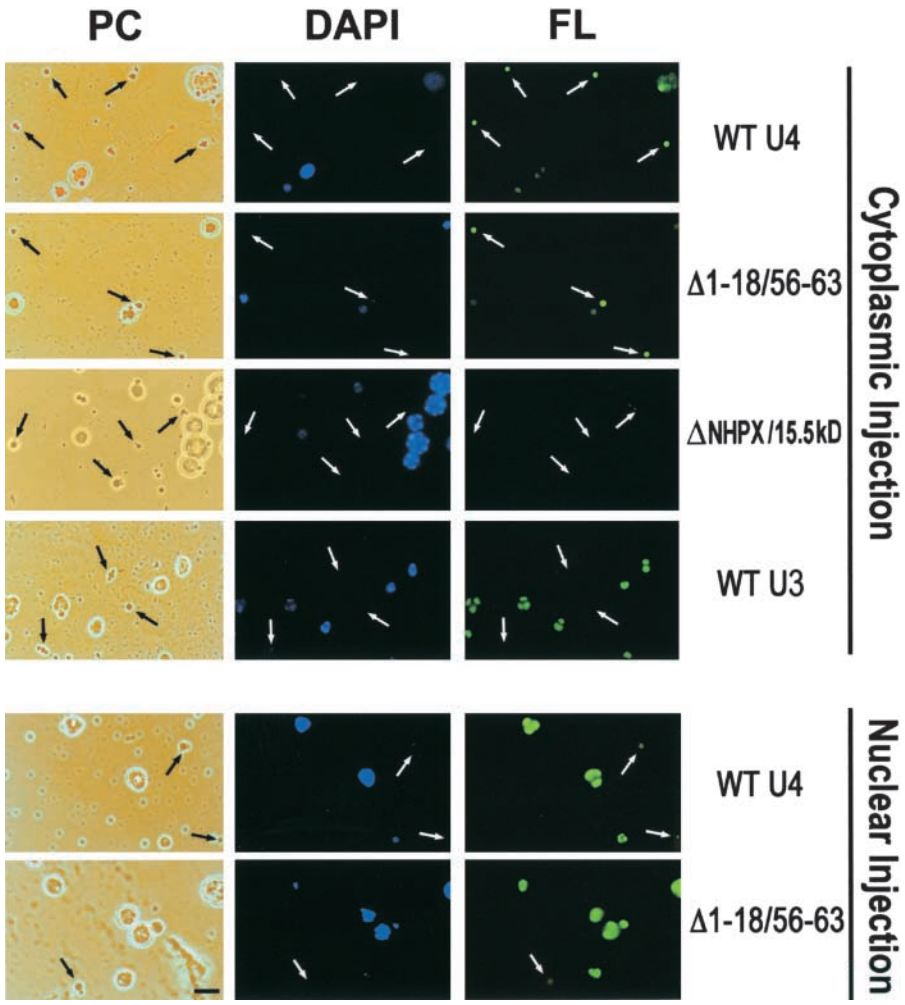

Figure 9. **Localization of U4 snRNA to nucleoli and Cajal bodies after cytoplasmic and nuclear injections.** Fluorescein-labeled synthetic transcripts of wild-type U4 snRNA (WT U4), a U4 mutant unable to base pair with U6 snRNA (Δ1–18/56–63), the NoLE mutant of U4 (ΔNHPX/15.5 kD), or wild-type U3 snoRNA (WT U3) were injected into *Xenopus* oocytes. Either 5 h after cytoplasmic injection or 1.5 h after nuclear injection, nuclear spreads were prepared and analyzed by phase contrast (PC) or fluorescence microscopy (FL, green). Nucleoli can be distinguished from other nonchromosomal nuclear bodies because they contain rDNA visualized by staining (DAPI, blue). Cajal bodies are indicated by arrows; they do not contain rDNA (DAPI negative) and often are associated with B-snurposomes (Gall et al., 1999 and references therein). After cytoplasmic injection, both wild-type U4 as well as mutant Δ1–18/56–63 localize weakly to nucleoli (compare DAPI with FL) and exhibit a much stronger preference to localize to Cajal bodies (arrows). The same observations were made for U5 snRNA (Lange, 2003). In contrast, U4 NoLE mutant ΔNHPX/15.5 kD does not reveal any signals in either nuclear compartment. U3 snoRNA after cytoplasmic injection strongly stained nucleoli but not Cajal bodies, unlike the pattern for U4 or U5. After nuclear injection, as shown before (Fig. 3), wild-type U4 and mutant Δ1–18/56–63 preferentially localized to nucleoli which are stained strongly (compare DAPI with FL); Cajal bodies are stained weakly and indicated by arrows (no DAPI signal). U3 snoRNA, similar to U4 snRNA after injection into *Xenopus* oocyte nuclei, can weakly stain Cajal bodies but strongly localizes to nucleoli (Lange and Gerbi, 2000). A synthetic negative control RNA after nuclear or cytoplasmic injection did not stain either nucleoli or Cajal bodies (Lange and Gerbi, 2000; Lange, 2003). Bar, 10 μm.

tion in splicing (Izaurralde and Mattaj, 1995; Will and Lührmann, 2001). The data presented here demonstrate that injected synthetic T7 pol U4 and U5 snRNA transcripts localize to nucleoli when injected with the same monomethyl G cap as their in vivo counterparts, thus, emulating the in vivo situation as closely as possible. Previous studies showed that such transcripts injected into *Xenopus* oocytes exhibit normal nucleo/cytoplasmic traffic, comparable to their endogenous counterparts (Fischer et al., 1991). However, as shown in Figs. 4 and 8, U4 or U5 could still localize to nucleoli even when the monomethyl–G cap was replaced by a synthetic–A cap, which cannot be trimethylated and which impairs the nucleo/cytoplasmic traffic of both snRNAs when they are injected into the nucleus (Fischer et al., 1991). This holds true for either wild-type transcripts or Sm mutants and, thus, neither the nature of the cap nor the Sm site provide signals essential for nucleolar localization of U4 or U5 snRNA.

This observation and other data (see Discussion) suggest that U4 and U5 snRNAs can localize to nucleoli without cytoplasmic passage. Accordingly, we compared the localization of fluorescein-labeled synthetic U4 transcripts after injection into either *Xenopus* oocyte nuclei (and incubation for 1 h) or the cytoplasm (and incubation for over 5 h). Interestingly, after nuclear injection wild-type U4 stains nucleoli strongly and Cajal bodies weakly, but after cytoplasmic injection the pattern is reversed and U4 exhibits a stronger preference to localize to Cajal bodies than to nucleoli, which are weakly labeled (Fig. 9). The same observations were made for U5 snRNA (Lange, 2003). Moreover, U4 NoLE mutant NHPX/15.5 kD cannot localize to Cajal bodies or nucleoli after cytoplasmic injection.

In contrast, the U4 mutant (Δ1–18/56–63) that cannot base pair with U6 snRNA behaved like wild-type U4. After nuclear injection it strongly localizes to nucleoli (and weakly to Cajal bodies), and after cytoplasmic injection localizes strongly to Cajal bodies (and weakly to nucleoli; Fig. 9). Thus, prior di-snRNP formation is not a prerequisite for either nucleolar or Cajal body localization. Moreover, this result suggests that association into a [U4/U6] di-snRNP is not mandatory for import from the cytoplasm to the nucleus.

The specificity of the observed signals was shown by various controls. Nuclear injection of U3 snoRNA into *Xenopus* oocytes can weakly stain Cajal bodies but strongly localizes

to nucleoli (Lange and Gerbi, 2000), similar to the observations here for U4 and U5 snRNAs. However, cytoplasmic injection of U3 snoRNA at the same concentration as U4 or U5, strongly stained nucleoli but not Cajal bodies (Fig. 9), unlike the pattern for U4 or U5. Moreover, a synthetic control RNA in both scenarios did not stain either nucleoli or Cajal bodies (Lange and Gerbi, 2000; Lange, 2003). Controls such as these, and the lack of signals for mutant small RNAs in contrast to their wild-type counterparts, suggest that nucleolar localization is specific. For U4, it appears to occur primarily before cytoplasmic passage and to specifically rely on the presence of the NHPX/15.5-kD protein binding site in the 5′-proximal stem loop.

Discussion

A cytoplasmic phase is not a prerequisite for nucleolar localization of U4 and U5 snRNAs

It is important to understand the intracellular traffic of snRNA components, which eventually form the spliceosome. The results presented here leads us to conclude that nucleolar localization of U4 or U5 spliceosomal snRNAs can occur independent of certain steps of maturation that occur in the cytoplasm, and, therefore, does not rely on passage through the cytoplasm.

We show that nucleolar localization of U4 and U5 snRNAs does not require 5′-cap trimethylation nor association with Sm proteins. Specifically, mutants of U4 or U5 that were incapable of binding Sm proteins still localized to nucleoli after nuclear injection. Because association with Sm proteins is a prerequisite for nuclear import, we conclude that the nucleolar signals observed for U4 and U5 must reflect traffic without a cytoplasmic step. Moreover, U4 and U5 snRNAs equipped with an artificial 5′–A cap, which cannot be hypermethylated (Fischer et al., 1991), still localized efficiently to nucleoli. The 5′–A cap also deprives these snRNA of efficient export and re-import into the nucleus (Mattaj, 1986; Fischer et al., 1991). However, as shown here, the 5′-cap structure had no effect on nucleolar localization of U4 and U5 after nuclear injection, suggesting that a cytoplasmic phase is not required before nucleolar localization. In addition, kinetic analysis in *Xenopus* oocytes (Gerbi and Lange, 2002) indicated that nucleolar localization of U4 and U5 snRNAs occurs within minutes after injection, and, thus, before export and re-import to the nucleus which in *Xenopus* oocytes takes hours (Fischer et al., 1991).

Thus, all components of the tri-snRNP can localize to nucleoli without cytoplasmic passage. However, U4 and U5 retain their functional ability to localize to nucleoli even when injected into the cytoplasm though the fluorescein-labeled U4 and U5 snRNA transcripts preferentially stain Cajal bodies stronger than nucleoli (Fig. 9; Lange, 2003).

Nucleolar localization of U4, U5, and U6 snRNA does not require di- or tri-snRNP formation

Previously, we determined the NoLE of U6 snRNA (Gerbi and Lange, 2002). The possibility existed that U4 and U5 snRNAs do not travel independently to nucleoli, but piggyback along with U6 as part of the tri-snRNP. The data presented here show that each of the tri-snRNP components can localize to nucleoli independently of one another. This has been demonstrated in several ways. First, mutant U4 that lacks the U6 base-pairing sites still localizes to nucleoli. Similarly, mutant U6 that lacks the U4 base-pairing sites is still able to localize to nucleoli (Gerbi and Lange, 2002). Therefore, U4 and U6 snRNA can both localize to nucleoli individually without being part of a di-snRNP. Moreover, data presented here demonstrate that nucleolar localization of U4 or U5 snRNA transcripts can occur even after depletion of endogenous U6 snRNA. Finally, the 3′-end of U6 snRNA is essential and sufficient for nucleolar localization and can be targeted to nucleoli by itself (Gerbi and Lange, 2002), but the 3′-end of U6 by itself is unable to assemble into a [U4/U6.U5] tri-snRNP (Vankan et al., 1990, 1992).

Formation of the functional spliceosome requires some posttranscriptional modifications of snRNAs (Yu et al., 1998). Because certain of these modifications appear to occur in nucleoli (see Role of the nucleolus in maturation of spliceosomal RNAs section), it seems likely that nucleolar localization occurs before splicing, and as shown here, can occur even before cytoplasmic passage. Recycling of snRNPs after a round of splicing requires reformation of the [U4/U6] di-snRNP and the [U4/U6.U5] tri-snRNP (Raghunathan and Guthrie, 1998; Staley and Guthrie, 1998), but the subcellular site where this occurs is unknown.

It has been proposed that Cajal bodies are sites of RNP assembly (Gall et al., 1999). Moreover, it has been suggested that di-snRNP formation occurs in Cajal bodies (Stanek et al., 2003). As shown here, the region of U4 snRNA involved in base pairing with U6 is not required for localization to Cajal bodies (Fig. 9), supporting the hypothesis that [U4/U6] di-snRNP formation may occur in Cajal bodies.

Candidate proteins that may interact with the NoLEs of snRNAs

NoLEs of the various snRNAs of the spliceosome bind to different proteins. Nucleolar localization of U6 snRNA is mediated by its 3′-terminal NoLE, probably by binding to the Lsm protein complex (Gerbi and Lange, 2002). It has been suggested that the nucleolar localization of U2 snRNA depends on the Sm protein binding site (Yu et al., 2001). In contrast, data presented here show that nucleolar localization of U4 and U5 does not require the Sm protein binding site. An explanation for this difference between U2 snRNA as compared with U4 or U5 is not at hand. However, it is known that structural features unique to U1 and U5 snRNAs individually influence the otherwise conserved Sm binding site (Jarmolowski and Mattaj, 1993). Moreover, the Sm site seems not to enable another pol II–transcribed snRNA, U7, to localize to nucleoli but instead is essential for localization of U7 snRNA exclusively to Cajal bodies (Wu et al., 1996). In the case of U7, there are some protein differences in the heteroheptameric Sm complex as compared with other snRNPs, which might explain the different function of the Sm site, as well as different subnuclear localization (Pillai et al., 2001).

Here, we show that the NHPX/15.5-kD protein binding motif is the only site essential for nucleolar localization of

U4 snRNA in *Xenopus* oocytes. In the case of U14 snoRNA, it has recently been shown that localization to nucleolar bodies is mediated by four proteins commonly associated with the box C/D motif (Verheggen et al., 2001), which is known to be the NoLE of this snoRNA class (for review see Lange, 2003). Interestingly, one of these NoLE-binding proteins is NHPX/15.5 (yeast homologue Snu13p) that binds not only to the snoRNA box C/D motif but also to a similar motif in U4 snRNA (Nottrott et al., 1999; Vidovic et al., 2000; Watkins et al., 2000). Thus, this protein might mediate nucleolar localization of several classes of small RNA.

As discussed in the preceding section, formation of the [U4/U6] di-snRNP is not a qualifying event for U4 localization to nucleoli or Cajal bodies. Therefore, any candidate factor, which may transport U4 snRNA from the nucleoplasm to the nucleolus, likely recognizes U4 before its engagement with other snRNAs. This is the case for the NHPX/15.5-kD protein, which interacts in vivo with U4 snRNA that is not yet associated with U6 snRNA (Leung and Lamond, 2002). Moreover, as shown here, U4 mutants of the NHPX/15.5-kD binding site do not localize to nucleoli anymore. Therefore, we propose that the NHPX/15.5-kD protein by binding to the NoLE of U4 snRNA is specifically able to mediate nucleolar localization of an individual U4 snRNA before formation of a di- or tri-snRNP.

Recent data showed that the NHPX/15.5-kD protein itself after in vivo expression in various cell lines can be found in nucleoli and Cajal bodies (Leung and Lamond, 2002) and a *Xenopus* NHPX/15.5-kD protein homologue can be detected in both nuclear compartments (Fig. 7 c). Depending on the cell system, snRNAs and snRNP components can accumulate in nucleoli and/or Cajal bodies (Carmo-Fonseca et al., 1992; Gall et al., 1999; Sleeman and Lamond, 1999; Lange and Gerbi, 2000; Sleeman et al., 2001; Yu et al., 2001; Gerbi and Lange, 2002; Leung and Lamond, 2002; Mouaikel et al., 2002). U5 transcripts injected into *Xenopus* oocytes associated with Cajal bodies as well as nucleoli (Lange, 2003). This is also the case for U4 snRNA (Fig. 9). If the NHPX/15.5-kD protein has a role in such intranuclear shuttling of various small RNAs including U4 snRNA, then it may be rather complex. Curiously, the unidirectional movement of this protein seems to be reciprocal to the route of nuclear maturation of snRNPs. SnRNPs seem to transiently localize to nucleoli and Cajal bodies before they eventually accumulate in nuclear speckles for later function in splicing (Sleeman and Lamond, 1999; Sleeman et al., 2001), whereas labeled NHPX/15.5 kD first localized to speckles and then to Cajal bodies and to nucleoli (Leung and Lamond, 2002).

Role of the nucleolus in maturation of spliceosomal RNAs

The nucleolus is the site of ribosome biogenesis and also posttranscriptional modification of RNAs (for reviews see Gerbi et al., 2001, 2003; Kiss, 2001). The snoRNAs found in nucleoli are used for ribosomal RNA processing and/or ribosomal RNA modifications involving ribose methylation or pseudouridylation. Some of the snoRNAs are also used for modifications of snRNAs. The nucleolus is the place where 2′-*O*-methylation of eight nucleotides and pseudouridylation of three nucleotides of the RNA pol III–synthesized U6 snRNA are performed (Tycowski et al., 1998; Ganot et al., 1999). Less is known about modification of the pol II–transcribed snRNAs. Recently, it has been suggested that modification of pol II–transcribed snRNAs takes place in the Cajal body rather than the nucleolus, mediated by a novel class of small nuclear RNAs called the small Cajal body–specific RNAs (scaRNAs; Darzacq et al., 2002). So far, scaRNAs have been linked with the synthesis of 12 2′-*O*-methylated nucleotides and two pseudouridines in the U1, U2, U4, and U5 snRNAs (Darzacq et al., 2002), but modification in U4 and U5 at other sites for which a guide scaRNA has not been identified could still be performed in the nucleolus rather than the Cajal body. The nucleolus is favored as the place where at least some modifications of U2 snRNA occur (Yu et al., 2001).

In addition to modification of snRNAs, another function of nucleoli during maturation of snRNPs might be participation in certain steps during protein assembly. We have shown here, as previously reported for mammalian tissue culture cells (Leung and Lamond, 2002), that the NHPX/15.5-kD protein, which binds to the NoLE of U4 snRNA, is located in both nucleoli and Cajal bodies of *Xenopus* oocytes. This protein helps to nucleate the assembly of the [U4/U6] snRNP before splicing catalysis (Nottrott et al., 1999; Vidovic et al., 2000; Will and Lührmann, 2001). These observations support the idea that some steps in snRNP protein assembly may occur in the nucleolus.

Concluding remarks

Nucleolar traffic is mediated by proteins that transport the snRNA to and/or anchor it within the nucleolus by binding to NoLEs. It has been suggested that the NHPX/15.5-kD protein by binding to U4 snRNA, the LSm protein complex by binding to U6 snRNA, and the Sm protein complex by binding to U2 snRNA play such a role (for reviews see Lange, 2003; data in Results). Localization of snRNA components of the spliceosomal [U4/U6.U5] tri-snRNP can take place independent of cytoplasmic steps of snRNP maturation and independent of association with one another before formation of the spliceosome or during recycling after splicing. Further studies on the mechanism and role of intranuclear sorting of spliceosomal snRNAs are underway, focusing on the relationship between nucleoli and the maturation pathway.

Materials and methods

In vitro transcription and labeling of RNA

All transcripts were obtained from DNA templates constructed by PCR using a T7 megascript in vitro transcription kit (Ambion) according to Lange et al. (1999) and were labeled either with fluorescein-12–UTP (DuPont) or α-[^{32}P]UTP (DuPont) and purified according to Lange et al. (1999). Their 5′ ends contained GG from the T7 promoter and were capped with m^{7}G(5′)ppp(5′)G (Ambion) like the in vivo counterparts of newly synthesized U4 and U5 snRNAs. In some experiment, U4 or U5 snRNA transcripts were capped with m^{7}G(5′)ppp(5′)A cap to prevent cap hypermethylation and hinder nucleo–cytoplasmic traffic (Fischer et al., 1991).

DNA templates, as well as primers that were used in PCR reactions, are listed in Lange and Gerbi (2000) for U6 snRNA, U2 snRNA, and U3

snoRNA, or in Gerbi and Lange (2002) for U4 and U5 snRNAs, or listed as follows: 5′-end primers (T7 promoter shown in italics; substituted nucleotide in bold font): U4 ΔNHPX/15.5 kD 5′-*TAA TAC GAC TCA CTA TAG GG*A GCT TTG CGC AGT GGC AGT ATC GTA GCC AA**C UC**G GTT AAT CC**T** **T**GG CGC GAT TAT-3′ U4 Δ19–55 5′-*TAA TAC GAC TCA CTA TAG GG*A GCT TTG CGC AGT GGC AG/T GCT AAT TGA AAA CTT TTC CCA-3′ U4 Δ1–18/56–63 5′-*tAA TAC GAC TCA CTA TAG GG*/T ATC GTA GCC AAT GAG GTT AAT CCG AGG CGC GAT TAT /GAA AAC TTT TCC CAA TAC CCC GCC-3′. 3′-end primers: U4 Δ64–84 5′-CAG TCT CCG TAG AGA CTG TCA AAA ATT GCC AAT GCC GAC TAT ATT TCA AGT CGT CAT GGC/ AAT TAG CAA TAA TCG CGC-3′ U4 Δ85–117 5′-CAG TCT CCG TAG AGA CTG TCA AAA ATT/ GGG GTA TTG GGA AAA GTT TTC AAT-3′ U4 Δ118–145 5′-GCC AAT GCC GAC TAT ATT TCA AGT-3′ U4 subSm 5′-CAG TCT CCG TAG AGA CTG T**GG CCG GCC** GCC AAT GCC gac-3′ U4 3/4Sm 5′-CAG TCT CCG TAG AGA CTG TCA A**CC** ATT GCC AAT GCC GAC-3′ U5subSm 5′-TAC CTG GTG TGA ACC AGG CTT **GGC CGG CC**T GAA CGA AAC TCA-3′ U5 3/4Sm 5′-TAC CTG GTG TGA ACC AGG CTT CAA A**CC** ATT GAA CGA AAC TCA-3′.

Clones containing the genes for *Xenopus* U5 snRNA (Kazmaier et al., 1987) and chicken U4B snRNA (Hoffman et al., 1986) were provided by I.W. Mattaj (European Molecular Biology Laboratory, Heidelberg, Germany) in the pUC9 plasmid; the corresponding snRNAs were used here because their structure–function relationships were previously extensively characterized in *Xenopus* oocytes (Vankan et al., 1990, 1992; Gerbi and Lange, 2002).

Oocyte microinjection

Stage V–VI oocytes from *Xenopus* were obtained as described previously (Lange et al., 1999). For fluorescence analysis of subnuclear localization of U4 snRNA or U3 snoRNA after cytoplasmic injection, oocytes were injected with 1.6 ng transcript in 18.4 nl. For nuclear injection for localization and stability assays, oocyte nuclei were injected with 0.8 ng U4, U5, or U6 snRNA wild-type and/or mutant transcripts in 9.2 nl H_20. The concentration of transcript was chosen to optimize the visualization of the differences between snRNA transcripts that localize to nucleoli and snRNA mutants and control transcripts that do not associate with nucleoli. The wild-type U4 snRNA transcripts used here are functional in that they exhibit normal nucleo/cytoplasmic traffic, associate with Sm proteins, form [U4/U6] snRNP via base pairing, and localize to Cajal bodies and nucleoli. In addition, the U4 transcripts were able to stain nucleoli even at concentrations equal to their cellular counterparts. We titrated fluorescent U4 snRNA could be titrated down in concentration and injected amounts as low as 0.1 ng per oocyte, which still resulted in signals above background (unpublished data). This is in the range of endogenous U4, which by Northern blot analysis of stage IV–V oocytes in independent experiments was determined to be ~2 fmol/oocyte (~0.095 ng/oocyte.

In various experiments, endogenous U6 and U4 snRNA were disrupted through RNase H–mediated destruction by two nuclear injections spaced 4 h apart of 9.2 nl each of the following oligonucleotides at a concentration of 3 μg/μl (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 performed 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 (1.0–1.5 h unless specified otherwise), nuclear spreads were prepared and fluorescence microscopy was performed as described previously (Lange and Gerbi, 2000) with the exception that ProLong mounting medium (Molecular Probes) was used.

Immunostaining of nucleoli and/or Cajal bodies was performed as described previously (Gerbi and Lange, 2002). Either rabbit polyclonal serum against a synthetic 21–amino acid fragment of *Xenopus* coilin (provided by J.G. Gall, Carnegie Institution of Washington, Baltimore, MD) or a rabbit anti–human NHPX/15.5-kD antiserum (provided by T. Achsel and R. Lührmann, Max Planck Institute, Göttingen, Germany, or A.I. Lamond, University of Dundee, Dundee, UK) was applied as a primary antibody for immunostaining at a dilution of 1:1,000 in PBS for 20 min at 4°C and goat anti–rabbit secondary antibody (Molecular Probes) coupled to the dye Alexa 594 was used.

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-[^{32}P]UTP–labeled RNAs, the RNA of four nuclei per sample was recovered and analyzed as described previously (Lange and Gerbi, 2000).

Immunoprecipitation

For assay of assembly with Sm proteins (Fig. 8 b), 0.8 ng/oocyte of purified U4 or U5 snRNAs (both co-labeled with α-[^{32}P]UTP and fluorescein-12–UTP) was injected into *Xenopus* oocyte nuclei and incubated for 4 h at 20°C. For assay of [U4/U6] snRNP assembly (Fig. 6), 0.8 ng/oocyte of purified U6 snRNA (co-labeled with α-[^{32}P]UTP and fluorescein-12–UTP) and 0.8 ng/oocyte (fluorescein-12–UTP-labeled) U4 snRNA were coinjected into *Xenopus* oocytes. Immunoprecipitations were performed as described previously (Gerbi and Lange, 2002). For certain analyses, the monoclonal Y12 mouse anti-Sm antibody was used (Lerner et al., 1981).

To assay the capability of U4 snRNA transcripts to associate with the NHPX/15.5-kD protein, immunoprecipitation was performed using a rabbit anti–human NHPX/15.5-kD antiserum (provided by T. Achsel and R. Lührmann) and either HeLa cell nuclear extract or *Xenopus* nuclear extract. HeLa nuclei were isolated according to Dignam et al. (1983) and *Xenopus* oocyte nuclei were manually dissected. Nuclei were sonicated in IP 150 buffer (for buffer see Gerbi and Lange, 2002) with 100 U/ml RNase inhibitor (Roche) for preparation of nuclear extract. For each sample, 20 μl U4 snRNA (~3 ng) labeled with α-[^{32}P]UTP was added to 50 μl nuclear extract (for HeLa cells ~5 μg protein; for *Xenopus* 10 oocyte nuclei/~1 μg protein), 20 μl (~1 μg) tRNA to block unspecific binding, 240 μl IP150 buffer, and 30 μl protein A–Sepharose beads. The beads had been coupled to polyclonal rabbit anti–human NHPX/15.5-kD antiserum or preimmune serum (as a control) by incubation of 120 μl pre-swollen beads (for a total of four samples) with 240 μl IP 500 buffer (for buffer see Gerbi and Lange, 2002), one tablet of protease inhibitor cocktail (Roche) per 10 ml buffer, and 40 μl antiserum overnight at 4°C with end over end rotation before they were spun and washed three times in IP 150. The mixture of nuclear extract and antibody-coupled beads was rotated end over end 4 h at 4°C before the beads were spun and washed seven times in IP 150, and the RNA was isolated and purified. Precipitated RNA and the supernatant were analyzed as in Gerbi and Lange (2002).

This paper is dedicated to Juliette M. Lange.

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