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Transient Nucleolar Localization Of U6 Small Nuclear RNA In *Xenopus Laevis* Oocytes

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Recent studies on the 2′-O-methylation and pseudouridylation of U6 small nuclear RNA (snRNA) hypothesize that these posttranscriptional modifications might occur in the nucleolus. In this report, we present direct evidence for the nucleolar localization of U6 snRNA and analyze the kinetics of U6 nucleolar localization after injection of in vitro transcribed fluorescein-labeled transcripts into *Xenopus laevis* oocytes. In contrast to U3 small nucleolar RNA (snoRNA) which developed strong nucleolar labeling over 4 h and maintained strong nucleolar signals through 24 h, U6 snRNA localized to nucleoli immediately after injection, but nucleolar staining decreased after 4 h. By 24 h after injection of U6 snRNA, only weak nucleolar signals were observed. Unlike the time-dependent profile of strong nucleolar localization of U6 snRNA or U3 snoRNA, injection of fluorescein-labeled U2 snRNA gave weak nucleolar staining at all times throughout a 24-h period; U2 snRNA modifications are believed to occur outside of the nucleolus. The notion that the decrease of U6 signals over time was due to its trafficking out of nucleoli and not to transcript degradation was supported by the demonstration of U6 snRNA stability over time. Therefore, in contrast to snoRNAs like U3, U6 snRNA transiently passes through nucleoli.

INTRODUCTION

The nucleolus is the site of ribosome biogenesis in eukaryotic cells (reviewed by Hadjiolov, 1985; Gerbi *et al.*, 1990). This process entails the transcription, processing and modification of the rRNA precursor (pre-rRNA) and the association of ribosomal proteins with rRNA. Small nucleolar RNAs (snoRNAs) assist in rRNA processing and modification. snoRNAs of the Box C/D family act as guide RNAs for 2′-O-methylation of rRNA, whereas snoRNAs of the Box H/ACA family guide rRNA pseudouridylation; certain members of both snoRNA families are required for cleavages within pre-rRNA (reviewed by Gerbi, 1995; Maxwell and Fournier, 1995; Venema and Tollervey, 1995; Sollner-Webb *et al.*, 1996; Smith and Steitz, 1997; Tollervey and Kiss, 1997).

Recent studies have suggested that the nucleolus carries out more functions than just ribosome biogenesis. These suggestions are based on observations that the nucleolus contains molecules used for other processes, 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) and telomerase RNA (Mitchell *et al.*, 1999; Narayanan *et al.*, 1999a). Furthermore, the nucleolus is implicated as playing roles in mRNA export (reviewed by Schneiter *et al.*, 1995), signal recognition particle maturation (Jacobson and Pederson, 1998; Politz *et al.*, 2000) and perhaps even gene silencing, a meiotic checkpoint, and senescence, as well as proof-reading for the translational apparatus (Cockell and Gasser, 1999; Garcia and Pillus, 1999; Pederson and Politz, 2000). Thus, the nucleolus appears to be a plurifunctional organelle (Pederson, 1998).

In addition to nucleolar modifications of rRNA, recent reports hypothesize that posttranscriptional modifications of splicosomal U6 small nuclear RNA (snRNA) occur in the nucleolus (Tycowski *et al.*, 1998, Ganot *et al.*, 1999). U6 has eight sites of 2′-O-ribose methylation, and three pseudouridylation sites (Epstein *et al.*, 1980; Harada *et al.*, 1980; Reddy and Busch, 1988). Three Box C/D snoRNAs (mgU6-47, mgU6-53, and mgU6-77) have already been identified that act as guide RNAs for the 2′-O-methylation of U6, and results of modification of chimeric constructs are in accord with the idea that all factors needed for 2′-O-methylation and pseudouridylation of U6 snRNA reside and are functionally active in the nucleolus (Tycowski *et al.*, 1998; Ganot *et al.*, 1999). U6 snRNA is the first example of a non-rRNA molecule whose modification is guided by snoRNAs. Because mature U6 snRNA in its role in mRNA splicing shows a steady-state nucleoplasmic localization, the notion that its modification occurs in the nucleolus presupposes that U6 passes through the nucleolus.

As mentioned by others (Tycowski *et al.*, 1998; Ganot *et al.*, 1999), the evidence that U6 snRNA traffics through nucleoli for its modification is suggestive, but not absolutely conclusive. First, some U6 snRNA and its guide snoRNAs are found in a nucleolar fraction from mammalian tissue culture

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cells (Tycowski *et al.*, 1998; Ganot *et al.*, 1999), but this preparation potentially contains additional nuclear bodies, such as Cajal bodies (also known as coiled bodies, spheres or C-snurposomes; Gall *et al.*, 1999, and references therein). Second, although the subcellular location of the RNAs that guide U6 snRNA modification have not been determined cytologically, other snoRNAs (e.g., U3, U8, and U14) are found in Cajal bodies as well as in nucleoli of somatic cells (Bauer *et al.*, 1994; Jiménez-Garcia *et al.*, 1994; Samarksy *et al.*, 1998; Shaw *et al.*, 1998). In addition, the protein fibrillarin, which has been shown to immunoprecipitate two of the snoRNAs that guide U6 snRNA 2′-O-methylation, is found not only in the dense fibrillar component of nucleoli (Ochs *et al.*, 1985; Reimer *et al.*, 1987a,b), but also in Cajal bodies (Gall *et al.*, 1999). Third, U6 snRNA has been visualized in Cajal bodies by in situ hybridization (Carmo-Fonseca *et al.*, 1992; Matera and Ward, 1993; Matera, 1998; Gall *et al.*, 1999).

In light of the above, it was desirable to directly monitor whether U6 snRNA passes through the nucleolus. To that end, we have investigated the subnuclear location of U6 snRNA. The present report shows that fluorescein-labeled U6 snRNA specifically localizes to nucleoli after injection into *Xenopus* oocytes in a time-dependent manner. Immediately after injection, nucleoli are strongly stained by U6 snRNA, but the signal decreases over 24 h after which only weak nucleolar labeling is observed. This is in contrast to U3 snoRNA. Our findings support the hypothesis that U6 transiently passes through the nucleolus, where its posttranscriptional modifications may occur (Tycowski *et al.*, 1998; Ganot *et al.*, 1999).

MATERIALS AND METHODS

In Vitro Transcription and Labeling of RNA

The labeled RNAs used in the present study were produced by in vitro transcription reactions utilizing DNA templates that were constructed by PCR using the templates and primers listed 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 1-base difference at nucleotide 6. An appropriate 5′ primer (see below) was used to give a PCR product identical to the *Xenopus* U6 snRNA sequence, which was used as the template for in vitro transcription. The templates for *Xenopus laevis* U2 snRNA and U3 snoRNA were the clones pXlU2 (Mattaj and Zeller, 1983) and pXlU3A (Savino *et al.*, 1992), respectively; the 5′-end and 3′-end primers for U2 snRNA and U3 snoRNA have been described by Lange *et al.* (1998c). The template for the control oligonucleotide was 5′-TCC TGT CGA CTC CTC CTC CTC CTC CGC GGA TTT A-3′.

5′-End Primers (T7 Promotor Shown in Italics)

U6 snRNA 5′-*TAA TAC GAC TCA CTA TAG GG*T GCT TGC TTC GGC AGC AC-3′; control RNA 5′-*TAA TAC GAC TCA CTA TAG GG*T CCT GTC GAC TC-3′.

3′-End Primers

U6 snRNA 5′-AAA AAT ATG GAA CGC TTC ACG-3′; control RNA 5′-TAA ATC CGC GG-3′.

In vitro transcripts of RNA were generated and labeled either with fluorescein-12-UTP (DuPont New England Nuclear, Boston, MA) or [α-^{32}P]UTP (Du Pont New England Nuclear) using a T7 megascript in vitro transcription kit (Ambion, Austin, TX). The T7 transcripts were purified according to Lange *et al.* (1999); they all contained GGG at their 5′ ends. Stability of the transcripts was improved by capping the 5′ end with m^{7}G(5′)ppp(5′)G cap analog (Ambion).

Oocyte Microinjection and Fractionation

Stage V–VI oocytes from *Xenopus laevis* were obtained as previously described (Lange *et al.*, 1998a). For fluorescence analysis of nucleolar localization as well as for stability assays, oocyte nuclei were injected with ~23 fmol of in vitro-transcribed U6 or U2 snRNA or with ~11 fmol of U3 snoRNA, as a positive control for nucleolar localization (Lange *et al.*, 1998c), in 9.2 nl of H_2O. Thus, the injected amount per oocyte was 0.8 ng of U6 snRNA or U3 snoRNA and 1.4 ng of U2 snRNA. For the 40-nt negative control RNA, 1.4 ng/oocyte were injected which is equivalent to ~116 fmol/oocyte. A further control was the injection of an excess of fluorescein-labeled UTP at 5 pmol/oocyte. As shown by Terns *et al.* (1995), oocyte nuclear retention of U3 occurs at up to ~25 fmol/oocyte, whereas U6 nuclear retention occurs even up to ~600 fmol/oocyte. The concentration used for our transcripts is also in the range of those used by Gall *et al.* (1999) for oocyte injection of U1, U2, U3, and U5. After subsequent incubation for various times ranging from 8 min up to 24 h at 20°C, oocytes were transferred from OR2 buffer to an isolation buffer as previously described (Lange *et al.* 1999), and the nuclear envelopes were manually removed.

Nucleolar Localization Assay

After incubation of the oocytes for a stipulated time, nuclear spreads were made as described previously (Lange *et al.*, 1999) following a method for preparation of lampbrush chromosomes (Gall *et al.*, 1991). Subsequently, the DNA in the preparations was stained with 4′-6-diamidino-2-phenylindole (DAPI) and analyzed by fluorescence microscopy as described previously (Lange *et al.*, 1998a, 1998b, 1998c, 1999), with the exception that two-fold higher exposure times for pictures of fluorescein labeling were used than in previous studies.

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 *et al.*, 1998a, 1998b, 1999). After quantitative analysis using a Fuji BAS 1000 phosphorimager (Fuji Medical Systems, Stamford, CT), the ratio of a given RNA to the U2 control at 1.5, 4, or 24 h, compared with the 0-h control (sample recovery immediately after injection) was calculated to determine the relative stability of RNA.

RESULTS

Detection of U6 snRNA Localization to Nucleoli

Nucleolar localization of U6 snRNA was monitored by a technique previously used to analyze the nucleolar localization elements (NoLEs) of various snoRNAs in *Xenopus* oocytes (Lange *et al.*, 1998a,b,c, 1999; Narayanan *et al.*, 1999a,b). When we were establishing controls for those studies, we initially observed that in vitro transcripts of *Xenopus* U6 snRNA were able to localize to nucleoli. The present report systematically analyzes the nucleolar localization of U6 snRNA and its kinetics as compared with other RNAs. At defined time points after injection of fluorescein-labeled in

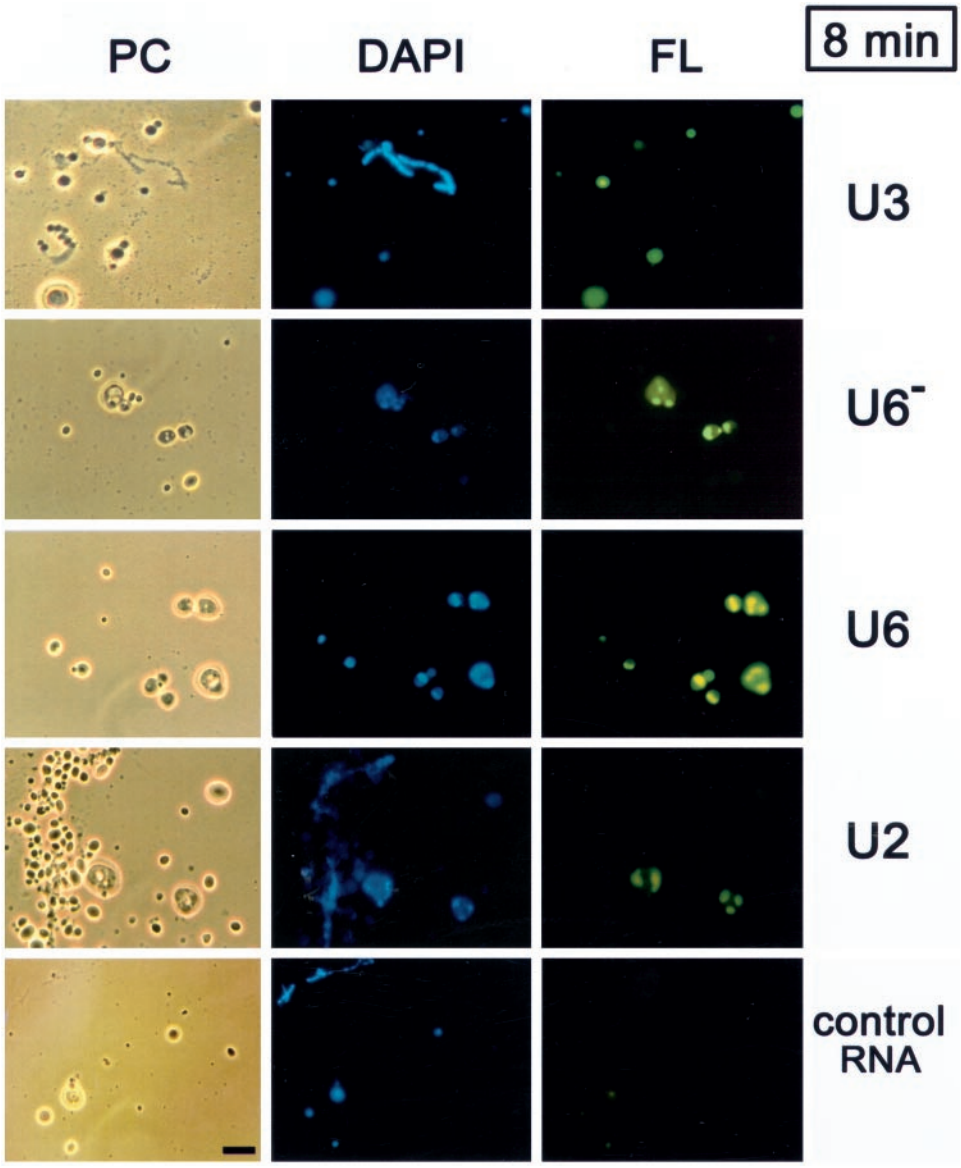

Figure 1. Short term nucleolar localization of U6 snRNA. Fluorescein-labeled U3 snoRNA, U6 snRNA, U2 snRNA, or a 40-nt control RNA were injected into the nuclei of *Xenopus laevis* oocytes. After 8 min, nuclear spreads were prepared and analyzed by phase contrast (PC) or by fluorescence microscopy (FL green). Nucleoli can be distinguished from other nonchromosomal nuclear bodies because only the nucleoli contain DNA which is visualized by staining (DAPI blue). U3 snoRNA localizes to nucleoli only modestly at this time point, whereas U6 snRNA shows strong nucleolar signals in the dense fibrillar component that surrounds the DAPI-positive rDNA. U6 snRNA that does not carry a stabilizing 5′ cap (U6$^-$) shows a higher variability of signals than transcripts with a 5′ cap (U6), but generally localizes well to nucleoli. U2 snRNA in an equimolar amount to U6 also stains nucleoli, although weakly, whereas the 40-nt control RNA even at five times the molar amount does not stain nucleoli. A lampbrush chromosome is visible in the U3 panel and also in the U2 panel (see PC and blue stain in DAPI) where it is coated with B-snurposomes, which are DAPI-negative. The snurposomes are not labeled by any of the injected RNAs after 8 min. Bar, 10 μm.

vitro transcripts of U6 snRNA, U3 snoRNA, U2 snRNA or an unrelated 40-nt synthetic RNA as a control, *Xenopus* oocyte nuclei were manually dissected and the nuclear contents were centrifuged onto a microscope slide. Using this method, soluble components of the nucleoplasm are not retained on the slide, but various structures within the nucleus were, including ca. 1500 nucleoli (which are variable in size; Wu and Gall, 1997), 50–100 Cajal bodies, a large number of B-snurposomes (with RNA polymerase II transcription and splicing components), and the lampbrush chromosomes (Gall *et al.*, 1999, and references therein).

As shown in Figure 1, strong fluorescent signals depicting nucleolar localization of U6 snRNA were detected immediately (8 min) after injection of 0.8 ng of transcript per oocyte nucleus. The injection of U3 snoRNA at the same concentration yielded only moderate nucleolar staining in this short postinjection period (Figure 1). The observed nucleolar localization of fluorescent U6 snRNA was specific, because injection of an unrelated control RNA, even at five times the molar amount of U6, did not stain nucleoli at any of the time points after injection (Figures 1–4). Additional controls demonstrated that the fluorescent signals we observed were not due to degradation of fluorescent snoRNA and subsequent reutilization of the label by other nuclear components. For example, as published previously (Lange *et al.*, 1988b,c) and also repeated in the present study, injection of a 200-fold molar excess of fluorescein-UTP alone did not label the nucleoli.

In contrast to nonintronic snoRNAs such as U3 and splicosomal snRNAs such as U2 that are transcribed by RNA polymerase II and that posttranscriptionally receive a 5′ monomethyl G cap which is subsequently converted to a trimethyl G cap (Mattaj, 1986; Terns and Dahlberg, 1994; Terns *et al.*, 1995), U6 is naturally transcribed by RNA poly-

merase III and possesses a γ-monomethylphosphoryl G cap (Singh and Reddy, 1989). However, as documented in Figures 1–4, nucleolar localization of in vitro transcripts of U6 snRNA occurred regardless of the presence or absence of a 5′ cap on the injected material (the cap status of the RNA once it has localized to the nucleolus is unknown). Because in vitro transcripts of U6 snRNA with an unprotected 5′ end are less stable in *Xenopus* oocytes than that with a 5′ cap, as will be demonstrated below, we have tested the nucleolar localization of U6 snRNA with and without a 5′ cap and found them to be comparable (Figures 1–4). In agreement with our observation that U6 snRNA nucleolar localization was independent of a 5′ cap on the injected RNA, it was shown previously that the presence or absence of a cap on injected snoRNA transcripts did not significantly affect nucleolar localization of a given snoRNA in *Xenopus* oocytes (Lange *et al.*, 1998b,c). Moreover, naturally occurring intronic snoRNAs localize to nucleoli without any 5′ cap structure at all.

In our previous studies, U2 snRNA served as a negative control in our nucleolar localization experiments, because only background levels of nucleolar staining were found 2 h after its injection into *Xenopus* oocytes (Lange *et al.*, 1998a, 1998b, 1998c, 1999). By injecting U2 snRNA at a concentration (~23 fmol/oocyte; 1.4 ng/oocyte) equivalent to that of U6 snRNA, which was higher than in most of the previous studies, and with the necessity of using two-fold higher constant exposure times to monitor background after injection of the unrelated 40-nt control RNA, we observed some nucleolar staining by U2 in the present study (Figure 1). However, unlike U6 snRNA, the nucleolar signals after injection of U2 snRNA remained weak at all time points and did not show a kinetic effect over a longer incubation period (Figures 2–4). In a recent report by Gall *et al.* (1999), very weak staining of nucleoli with a U2 snRNA probe was seen at both 2 and 22 h after injection; no nucleolar signal was seen at all for the steady-state situation analyzed by in situ hybridization (Gall *et al.*, 1999). At this point, the biological relevance of weak U2 snRNA nucleolar localization is unclear, especially as its posttranscriptional 2′-O-methylation and pseudouridylation seems to occur outside the nucleolus (see DISCUSSION), unlike the situation for U6 snRNA.

Kinetics of U6 snRNA Localization to Nucleoli

The final destination of U6 snRNA is the nucleoplasm where it functions in the spliceosome. Consequently, U6 snRNA may reside only transiently in the nucleolus. To test this prediction experimentally, we performed the nucleolar localization assay over longer periods after injection of the transcripts into *Xenopus* oocyte nuclei, including 1.5 h (Figure 2), 4 h (Figure 3) and 24 h (Figure 4). U3 snoRNA nucleolar staining increased with time (Figures 2 and 3) and remained strong even after 24 h (Figure 4). In contrast, U6 snRNA localization to nucleoli was only temporary. By comparison with the situation 8 min after injection where nucleolar labeling by U6 snRNA was stronger than labeling by U3 snoRNA (Figure 1), at 1.5 h after injection the nucleolar labeling by U6 snRNA, either with or without a stabilizing 5′ cap, was the same as the labeling by U3 (Figure 2). Over time, however (after 4 h; Figure 3), the U6 snRNA signals became weaker than those of U3 snoRNA and by 24 h after injection the U6 nucleolar staining had further decreased to just slightly above background level (Figure 4). Therefore, we conclude that U6 snRNA localization in nucleoli is transient.

The nuclear spreads prepared to analyze the localization of U6 snRNA to nucleoli also included other nuclear bodies. Among these were Cajal bodies, which are similar in size to some nucleoli but do not contain DNA and may be associated with B-snurposomes that are embedded in their matrix or attached to their surface (Gall *et al.*, 1999, and references therein). Although not the focus of the present study and therefore not systematically analyzed, Cajal bodies revealed some staining for U2, U3 and U6 at 1.5 h after injection (Figure 2, arows) whereas at longer time points they were only stained by U2 and U6 snRNA (Figure 3, arrows). Similarly, Gall *et al.* (1999) have seen Cajal body staining by injected fluorescent U1, U2 and U5 snRNAs at 2 and 22 h after injection. Moreover, others have also noticed that Cajal bodies are labeled by U3 snoRNA at short (Gall *et al.*, 1999; Narayanan *et al.*, 1999b), but not at long time points (Narayanan *et al.*, 1999b) after injection into oocytes.

In addition to nucleoli, small spherical bodies known as B-snurposomes (Gall *et al.*, 1999, and references therein), which can be distinguished from nucleoli because they lack DNA, are also present in nuclear spreads. Unlike Cajal bodies, the B-snurposomes were not labeled by any of the RNAs at 8 min, 1.5 h or 4 h after their injection (Figures 1–3). However, U2 snRNA stained B-snurposomes 24 h after injection (Figure 4). Similarly, Gall *et al.* (1999) saw B-snurposome labeling by U2 snRNA at 22 h after injection but not at earlier times. At 24 h after injection, we did not see B-snurposome staining by injected U6 snRNA, which may have required a longer time to reach the B-snurposomes than U2 snRNA. As expected, U3 snoRNA was not seen in snurposomes at any time point after injection.

Stability of Injected U6 snRNA

It was important to analyze the stability of U6 snRNA transcripts to ascertain that the reduction of fluorescent signals over the time course of the experiments was not simply due to degradation. Stability assays using ^{32}P-labeled transcripts demonstrated that all capped transcripts were sufficiently stable 1.5 (our unpublished results), 4, and 24 h after injection into oocyte nuclei (Figure 5). Only uncapped U6 showed reduced stability by 24 h after injection (relative stability of 0.5 = 50%) compared with U3 (the long-term positive control in our localization assay where 0.8 = 80% remains). The relative stability of U6 snRNA with a stabilizing 5′ cap was even higher (0.9) than that of U3 snoRNA after 24 h. These results clearly show that the failure of U6 molecules to efficiently stain nucleoli 24 h after injection was not due to degradation of the transcripts, but rather to their exit from nucleoli in a time-dependent manner.

DISCUSSION

Small RNAs Differ in Their Traffic Patterns

The three small RNAs studied here differ from one another in their intracellular traffic patterns. Both U3 snoRNA and U2 snRNA are transcribed by RNA polymerase II, whereas U6 snRNA is transcribed by RNA polymerase III (reviewed by Dahlberg and Lund, 1988).

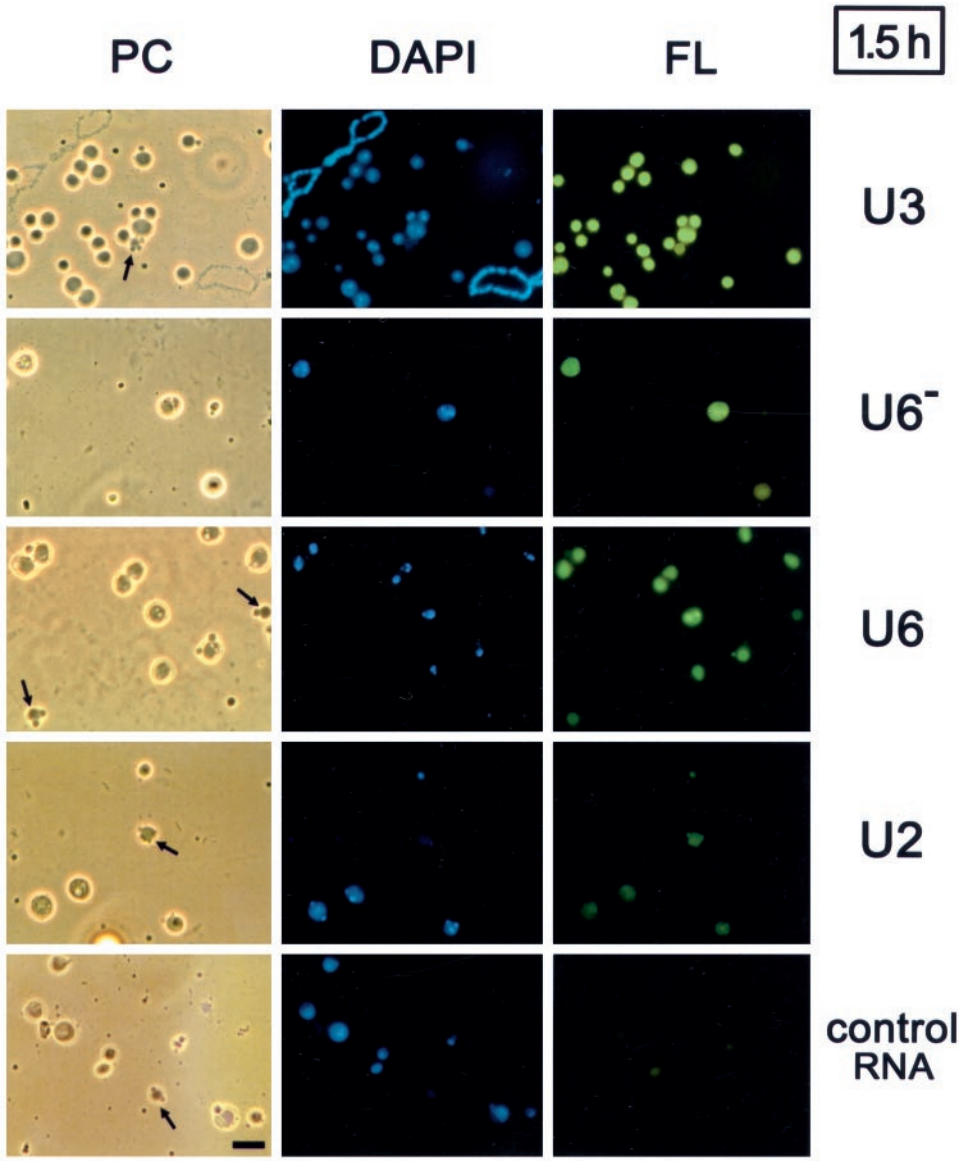

Figure 2. Nucleolar localization of U6 snRNA 1.5 h after injection into oocyte nuclei. The nucleolar localization of U3 snoRNA, U6 snRNA, U2 snRNA, or a 40-nt control RNA was analyzed 1.5 h after injection into the nuclei of *Xenopus laevis* oocytes. U3 snoRNA, which served as a positive control (see Lange *et al.*, 1998c), and U6 snRNA, either with a stabilizing 5′ cap (U6) or without cap (U6$^-$), show strong nucleolar localization. U2 snRNA stains nucleoli weakly and the control RNA fails to stain nucleoli. Cajal bodies, which do not contain DNA (DAPI-negative) and are often associated with B-snurposomes (Gall *et al.* 1999), are indicated by arrows in the phase contrast panels; they are weakly stained by fluorescein-labeled U3, U6 and U2. Lampbrush chromosomes are present in the U3 preparation shown here (see PC and DAPI panels). Other details as in Figure 1.

Subsequently, U6 snRNA obtains a γ-monomethyl phosphoryl G cap (Singh and Reddy, 1989) and is complexed with proteins. All of these events leading to its maturation occur in the nucleus, where it remains to function in splicing (Vankan *et al.*, 1990). In contrast, another component of the spliceosome, U2 snRNA, posttranscriptionally receives a monomethyl G cap in the nucleus and then is exported to the cytoplasm, where the cap is converted to trimethyl G and Sm proteins are bound; after these events, U2 snRNA is reimported back into the nucleus to function in splicing (reviewed by Izaurralde and Mattaj, 1995). Unlike U2 snRNA, cap trimethylation of U3 snoRNA occurs in the nucleus (Terns and Dahlberg, 1994 and 1995; Cheng *et al.*, 1995), where it is also complexed with proteins. Thus, all three RNAs are transcribed in the nucleoplasm, but then they diverge in their traffic patterns. U3 snoRNA moves to the nucleolus, U2 snRNA goes out to the cytoplasm and then reenters the nucleus, and U6 snRNA passes transiently through the nucleolus (see below). Early in these traffic patterns, all three RNAs can be found in Cajal bodies (Gall *et al.*, 1999, and this report), which might be important for their intracellular sorting (Gall *et al.*, 1999).

The present report demonstrates the transient nucleolar localization of U6 snRNA, thus confirming the previous hypothesis (Tycowski *et al.*, 1998; Ganot *et al.*, 1999) that U6 snRNA passes through the nucleolus en route to its final destination in the nucleoplasm where it functions in splicing. In contrast, U3 snoRNA remains in the nucleolus, for its role in rRNA processing (Kass *et al.*, 1990; Savino and Gerbi, 1990; Hughes and Ares, 1991; Hughes, 1996; Méreau *et al.*, 1997; Borovjagin and Gerbi, 1999, 2000). The two molecules are dissimilar not only in their final destination in the cell and their function, but also in

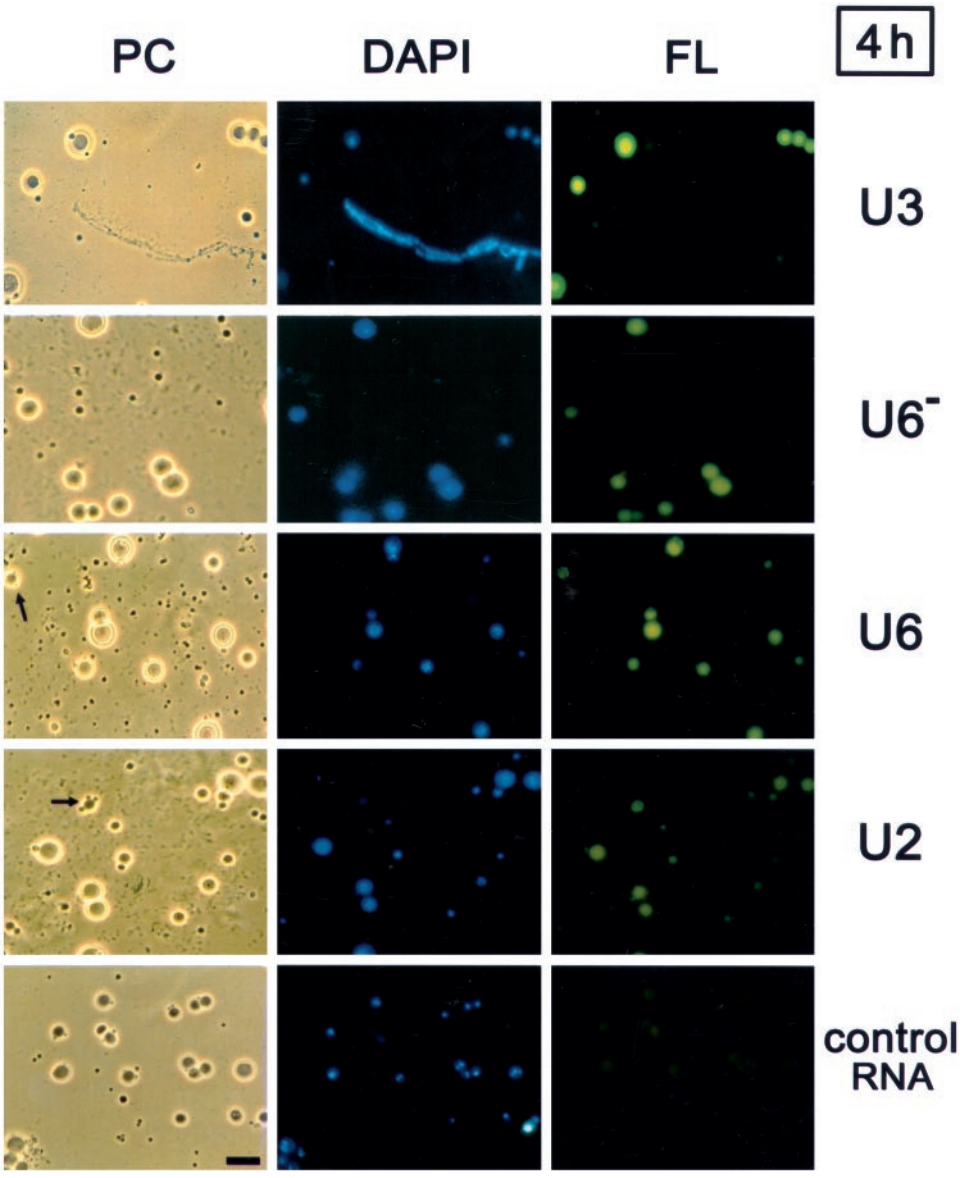

Figure 3. Nucleolar localization of U6 snRNA 4 h after injection into oocyte nuclei. The nucleolar localization of U3 snoRNA, U6 snRNA, U2 snRNA, and a 40-nt control RNA was analyzed 4 h after injection into oocyte nuclei. U3 snoRNA, as a positive control (see Lange *et al.*, 1998c), stains nucleoli strongly. U6 snRNA, either with a stabilizing 5′ cap (U6) or without a cap (U6$^-$), shows moderate nucleolar localization. Nucleolar signals are weak for U2 snRNA and at background levels for the control RNA. Cajal bodies (Gall *et al.*, 1999) are indicated by arrows in some phase contrast panels and reveal moderate staining by U6 and U2. A lampbrush chromosome is visible in the U3 preparation (see PC and DAPI panels). Other details as in Figure 1.

their kinetics of nucleolar localization, which are rapid and transient for U6 snRNA and slower but permanent at steady-state levels for U3 snoRNA (summarized in Figure 6). These kinetics suggest that U6 snRNA and U3 snoRNA do not share the same mechanism of nucleolar localization. Nonetheless, the destination within the nucleolus appears to be similar for U6 snRNA and U3 snoRNA. As for U3 snoRNA (Lange *et al.*, 1998c), U8 and U14 snoRNA (Lange *et al.*, 1998a), and U17 snoRNA (Lange *et al.*, 1999), U6 snRNA labeled the dense fibrillar component of the nucleolus, which surrounds the rDNA-containing fibrillar center (Shah *et al.*, 1996).

Signals for Intranuclear Localization

The principles governing the nucleolar localization of RNAs are beginning to be elucidated. SnoRNAs are targeted to the nucleolus by family specific motifs called NoLEs (Jacobson *et al.*, 1995, 1997; Lange *et al.*, 1998a,b,c; Samarsky *et al.*, 1998; Lange *et al.*, 1999; Narayanan *et al.*, 1999a,b). The NoLEs are believed to be recognized by proteins that either transport the snoRNA from the nucleoplasm to the nucleolus and/or anchor it within the nucleolus. The specificity of U6 snRNA nucleolar localization suggests that it may also be mediated by intrinsic features within the molecule, such as unique structures and/or defined sequences. Similarly, distinct sequences may be required for RNA traffic through Cajal bodies. For example, export from Cajal bodies requires Box D in U3 snoRNA (Narayanan *et al.*, 1999b) and sequences at the 5′ end of U1 snRNA (Gall *et al.*, 1999). It will be interesting to learn in future studies if any of the sequences required for U6 traffic within the nucleus coincide with sequences for its nuclear retention (Boelens *et al.*, 1995).

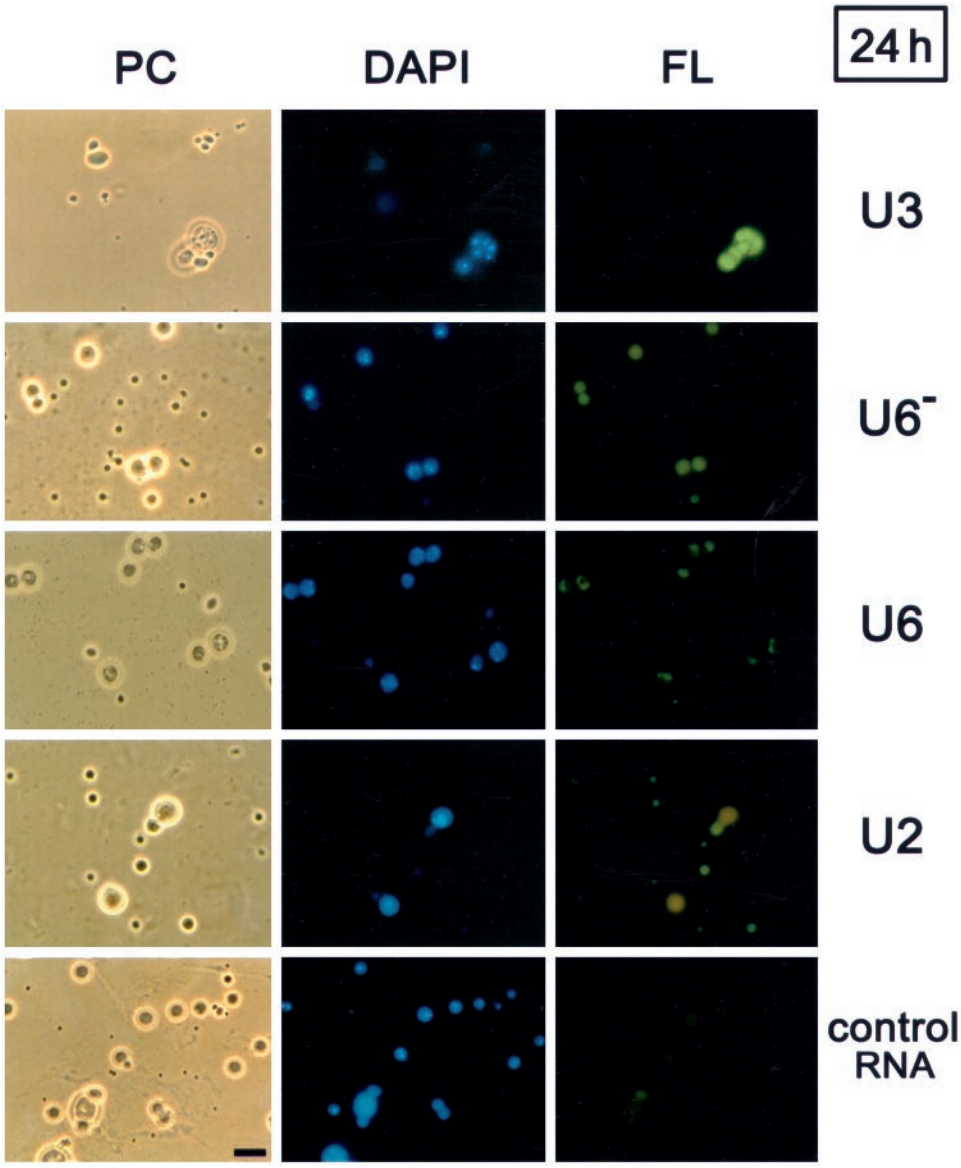

Figure 4. Long-term nucleolar localization of U6 snRNA. The nucleolar localization of U3 snoRNA, U6 snRNA, U2 snRNA, and a 40-nt control RNA was analyzed 24 h after injection into oocyte nuclei. U3 snoRNA strongly localizes to nucleoli. In contrast to earlier time points, nucleoli are only weakly stained by U6 snRNA (with capped U6 snRNA, signals were slightly more variable than with all other transcripts and occasionally moderate nucleolar staining was observed). As with earlier time points (Figures 1–3), U2 signals remain weak in nucleoli, and nucleoli are not stained by the control RNA. B-snurposomes (which are DAPI-negative) are stained by U2 snRNA at this time point. Other details as in Figure 1.

Recently, it has been reported for U3 snoRNA that there is some overlap in the sequences for its nucleolar localization and nuclear retention (Speckmann *et al.*, 1999).

Cellular Location for Small RNA Modification

The transient localization of U6 snRNA in the nucleolus concurs with the idea that it passes through this organelle to be modified (Tycowski *et al.*, 1998; Ganot *et al.*, 1999). Similarly, recent experiments suggest that U3 snoRNA is pseudouridylated in the nucleolus (Ganot *et al.*, 1999). In contrast, the posttranscriptional 2′-O-methylation and pseudouridylation of U2 snRNA seems to occur outside the nucleolus (Yu *et al.*, 1998; Ganot *et al.*, 1999). In fact, one of the enzymes for U2 snRNA modification has been found in the nucleoplasm (Simos *et al.*, 1996; Massenet *et al.*, 1999). This hypothesis is strengthened by our results, which indicate that U2 snRNA only labeled nucleoli weakly at all time points after injection and did not show the differences in nucleolar labeling over time such as that seen for U6 or U3 (summarized in Figure 6). The nucleolar localization of U6 snRNA might be directly linked to the state of its posttranscriptional modification. For example, U6 could be tethered in the nucleolus as a result of base pairing with the guide snoRNAs that modify it (Tycowski *et al.*, 1998; Ganot *et al.*, 1999), and once the 2′-O-methylations and pseudouridylations have been completed, the complex may dissociate, allowing U6 snRNA to leave the nucleolus and reenter the nucleoplasm. In this case, export might be the default state when nucleolar retention of U6 ceases.

In summary, this is the first analysis of the transient nucleolar localization of a small spliceosomal RNA, U6

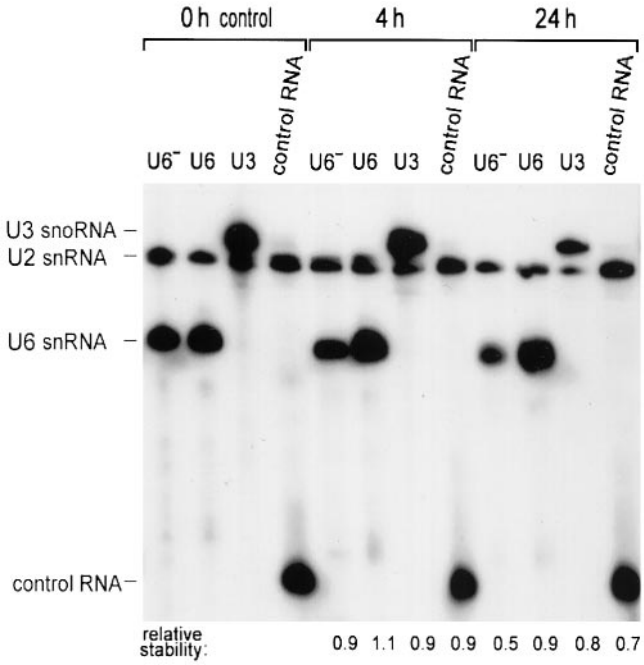

Figure 5. Stability of RNAs. ^{32}P-labeled transcripts of U3 snoRNA, U6 snRNA that was capped (U6) or uncapped (U6$^-$) or a 40-nt control RNA were injected into oocyte nuclei; the RNAs were isolated and analyzed by gel electrophoresis as previously described (Lange *et al.*, 1999). The first four lanes show the controls (0 h: sample recovery immediately after injection), the middle lanes show the RNA recovered at 4 h, and the lanes at the right show long-term stability assayed at 24 h after injection. 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. The relative RNA stability was calculated as the ratio of a given RNA transcript to the U2 control at 4 h or 24 h compared with the 0-h control ratio [(RNA transcript/U2 after incubation)/(RNA transcript/U2 at 0 h)].

snRNA. The kinetics, and therefore most likely the mechanism, for nucleolar localization of U6 snRNA differ from those of U3 snoRNA, which enters the nucleolus more slowly than U6 but subsequently remains there in its steady state. The present study lays the groundwork for future investigations on U6 snRNA traffic within the nucleus.

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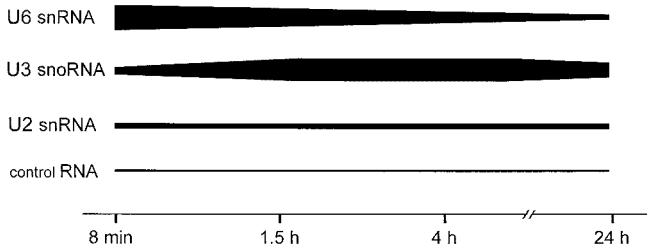

Figure 6. Schematic representation of nucleolar accumulation of injected RNA. The change in nucleolar labeling over time after injection of U3 snoRNA, U6 snRNA, U2 snRNA or a control RNA into *Xenopus* oocytes is indicated. The thickness of the black line is proportional to the strength of nucleolar labeling by the injected RNA. Nucleolar labeling by U6 snRNA is initially strong but decreases over the course of 24 h. In contrast, nucleolar labeling by U3 snoRNA increases to a strong signal seen 1.5 h after injection, which is maintained at longer time points. Nucleoli are only weakly stained by U2 snRNA at all time points and are not stained by the 40-nt control RNA.

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