Nucleolar localization elements in U8 snoRNA differ from sequences required for rRNA processing

THILO SASCHA LANGE, ANTON V. BOROVJAGIN, and SUSAN A. GERBI

Division of Biology and Medicine, Brown University, Providence, Rhode Island 02912, USA

ABSTRACT

U8 small nucleolar RNA (snoRNA) is essential for metazoan ribosomal RNA (rRNA) processing in nucleoli. The sequences and structural features in *Xenopus* U8 snoRNA that are required for its nucleolar localization were analyzed. Fluorescein-labeled U8 snoRNA was injected into *Xenopus* oocyte nuclei, and fluorescence microscopy of nucleolar preparations revealed that wild-type *Xenopus* U8 snoRNA localized to nucleoli, regardless of the presence or nature of the 5' cap on the injected U8 snoRNA. Nucleolar localization was observed when loops or stems in the 5' portion of U8 that are critical for U8 snoRNA function in rRNA processing were mutated. Therefore, sites of interaction in U8 snoRNA that potentially tether it to pre-rRNA are not essential for nucleolar localization of U8. Boxes C and D are known to be nucleolar localization elements (NoLEs) for U8 snoRNA and other snoRNAs of the Box C/D family. However, the spatial relationship of Box C to Box D was not crucial for U8 nucleolar localization, as demonstrated here by deletion of sequences in the two stems that separate them. These U8 mutants can localize to nucleoli and function in rRNA processing as well. The single-stranded Cup region in U8, adjacent to evolutionarily conserved Box C, functions as a NoLE in addition to Boxes C and D. Cup is unique to U8 snoRNA and may help bind putative protein(s) needed for nucleolar localization. Alternatively, Cup may help to retain U8 snoRNA within the nucleolus.

Keywords: nucleolar localization; U8 small nucleolar RNA (snoRNA); *Xenopus* oocyte

INTRODUCTION

The nucleolus is a prominent organelle in the nucleus of eukaryotic cells. It is the site of ribosome biogenesis, which involves ribosomal RNA (rRNA) synthesis, modification and processing, and association with ribosomal proteins. Several of these events are mediated by small nucleolar RNAs (snoRNAs).

There are about 200 snoRNA species in the nucleolus, most of which are used to guide the modifications that occur on pre-rRNA: 2'-O-ribose methylation by snoRNA members of the Box C/D family (Cavaillé et al., 1996; Kiss-László et al., 1996, 1998; Maden, 1996; Nicoloso et al., 1996; Tollervy, 1996; Tycowski et al., 1996; Maden & Hughes, 1997; Smith & Steitz, 1997), and pseudouridine formation by snoRNA members of the Box H/ACA family (Ganot et al., 1997; Ni et al., 1997; Smith & Steitz, 1997). The function of these modifications in rRNA is not yet understood,

and the snoRNAs utilized for rRNA modifications are dispensable.

In contrast, there are a handful of snoRNAs that are essential for viability of the cell, and are required for rRNA processing events that remove the external transcribed spacers and internal transcribed spacers from the rRNA precursor (summarized in Gerbi, 1995; Maxwell & Fournier, 1995; Sollner-Webb et al., 1995; Venema & Tollervy, 1995). These include U3 (Kass et al., 1990; Savino & Gerbi, 1990; Hughes & Ares, 1991; Hughes, 1996), U8 (Peculis & Steitz, 1993, 1994), U14 (Jarmolowski et al., 1990; Li et al., 1990; Li & Fournier, 1992; Liang & Fournier, 1995; Dunbar & Baserga, 1998; Lange et al., 1998), U22 (Tycowski et al., 1994), and E1 (=U17), E2, and E3 (Mishra & Elicieri, 1997). U3 and U14 are used for rRNA processing both in metazoa and in yeast, but the other snoRNAs listed above have only been identified in metazoa. There are some additional snoRNAs that are essential for rRNA processing that have only been found in yeast. Of all the snoRNAs that have been identified, U8 is the only one that is needed for 28S rRNA maturation. When intact U8 snoRNA is depleted by antisense oligonucleotide injection

Reprint requests to: Susan A. Gerbi, Division of Biology and Medicine, Brown University, Providence, Rhode Island 02912, USA; e-mail: susan_gerbi@brown.edu.

tion into *Xenopus* oocytes, the cleavages adjacent to 5.8S rRNA and the 3' end of 28S rRNA are hindered so that 32S pre-rRNA is not formed (Peculis & Steitz, 1993, 1994; Peculis, 1997). At the end of rRNA processing, 32S rRNA is cleaved internally to form 5.8S and 28S rRNA; these cleavages are also absent when U8 snoRNA is depleted (Peculis & Steitz, 1993, 1994).

Despite the wealth of knowledge that has accumulated about the nucleolus, very little is known about the transport of molecules to this organelle and their retention there, resulting in nucleolar localization. Previously, we found that evolutionarily conserved Boxes C and D are essential nucleolar localization elements for snoRNAs of the Box C/D family, such as U8 and U14 (Lange et al., 1998). However, that study did not explore whether other regions besides Boxes C and D, especially those important for rRNA processing, are also used as nucleolar localization elements for U8 snoRNA. This question is addressed in the present study, where we investigate which regions are needed for nucleolar localization of U8 snoRNA and compare them to those required for function of U8 in rRNA processing. U8 snoRNA is among the best-studied snoRNA, and is found in foci within nucleoli of tissue culture cells (Matera et al., 1994). It has been finely dissected by mutagenesis to define the regions necessary for rRNA processing (Peculis & Steitz, 1994; Peculis, 1997). We have analyzed U8 snoRNA mutants, some of which have defects in rRNA processing, by fluorescence microscopy for their nucleolar localization. This allowed us to identify *cis*-acting regions necessary for nucleolar localization, which we call **Nucleolar Localization Elements (NoLEs)**. Our results indicate that most sequences within U8 snoRNA necessary for rRNA processing are not essential for nucleolar localization. A sequence unique to U8 snoRNA (Cup) is needed for nucleolar localization, in addition to Boxes C and D, which are found in many snoRNAs. Therefore, multiple elements are needed for nucleolar localization. In most Box C/D snoRNAs, Boxes C and D abut either side of a terminal stem; this structure is not present in U8 snoRNA and instead two stems separate these Boxes. It is shown here that U8 snoRNA can still localize to nucleoli and function in rRNA processing when the intervening stems are partially or fully deleted, indicating that the spatial relationship of Boxes C and D in U8 snoRNA is not important for these processes.

RESULTS

Localization of *Xenopus* U8 wild-type snoRNA to nucleoli

Using fluorescence microscopy, we have analyzed the sequences within U8 snoRNA necessary for its nucleolar localization. In vitro transcripts of wild-type U8 snoRNA labeled with fluorescein-UTP were injected into

Xenopus oocyte nuclei. After 2 h, the nuclei were manually isolated, the nuclear envelope was removed, and the nuclear contents, including the multiple nucleoli, were centrifuged onto a microscope slide for direct analysis with a fluorescence microscope. In *Xenopus* oocytes, there are multiple nucleoli due to rDNA amplification (Painter & Taylor, 1942; Brown & David, 1968; Gall, 1968; Perkowska et al., 1968). Individual nucleoli can vary in size (Wu & Gall, 1997) and also can fuse into a multinucleolar cluster that sometimes has a vacuolated appearance (Shah et al., 1996). As seen in Figure 1, nucleoli are brightly stained by fluorescein-labeled U8 in foci that encompass the ribosomal DNA stained by DAPI. This localization is specific, because nucleoli are unstained after injection of fluorescein-labeled U2 snRNA (Fig. 1), a small nuclear RNA that is part of the splicing machinery and is not present in nucleoli. In addition, the lampbrush chromosome seen in Figure 2 does not stain with fluorescein, supporting the specificity of nucleolar localization of U8 snoRNA.

The trimethylguanosine cap is used as a signal for nuclear import of snRNAs from the cytoplasm (Fisher & Lührmann, 1990; Hamm et al., 1990; Marshallsay & Lührmann, 1994). Is a cap also needed for snoRNA localization to nucleoli? Nonintronic snoRNAs, such as U8, which are transcribed from their own genes, are made with a monomethylguanosine cap that can be converted in the nucleus to a trimethylguanosine cap (Peculis & Steitz, 1994; Terns & Dahlberg, 1994; Cheng et al., 1995; Terns et al., 1995). Fluorescein-labeled U8 snoRNA that lacks a cap when injected is still localized in nucleoli, as can be seen in Figure 2. As another control, U8 was synthesized with an A-cap rather than its usual G-cap (see Materials and Methods); U8 snoRNA with an A-cap cannot be trimethylated, although it is stable and can function in rRNA processing (Peculis & Steitz, 1994) and, as anticipated, localizes to nucleoli after 2 h (Fig. 2). Therefore, the nucleolar localization in *Xenopus* oocytes does not depend on the presence or nature of the 5' cap on the injected U8 snoRNA. U8 snoRNA synthesized with a monomethylguanosine cap was injected into oocytes for the studies described below, to emulate the *in vivo* situation as closely as possible.

Sequences in U8 snoRNA needed for rRNA processing are not required for nucleolar localization

U8 snoRNA has been dissected by mutagenesis to define the sequences needed for rRNA processing (Peculis & Steitz, 1994; Peculis, 1997). Endogenous U8 snoRNA was depleted by antisense oligonucleotide injection into *Xenopus* oocytes, and mutated U8 snoRNA constructs were subsequently injected to assay if they could rescue the rRNA processing defect. Those ex-

FIGURE 1. U8 wild-type snoRNA localizes specifically to nucleoli, unlike U2 snRNA. Fluorescein-labeled U8 snoRNA or U2 snRNA were injected into nuclei of *Xenopus* oocytes. After 2 h, slides of nucleoli were prepared and DNA was stained with DAPI. Nucleoli are visualized by phase contrast (PC). The rDNA in nucleolar fibrillar centers stains blue with DAPI (DAPI). Fluorescein-labeled U8 snoRNA localizes to the nucleoli (FL, green), but U2 snRNA does not. Some of the nucleoli are clustered into an aggregate, and each nucleolus comprising the cluster has a bright spot of DAPI-stained rDNA. Bar is 10 μ m.

periments indicated that the 5' region of U8 snoRNA was important for function in rRNA processing. We have analyzed the mutated U8 snoRNAs used in those studies that were defective in rRNA processing to see if they can localize to nucleoli. The failure to rescue rRNA processing could possibly reflect a secondary effect caused by inability of the mutant snoRNA to localize to nucleoli where rRNA processing occurs. Alternatively, the sequences that were changed in the mutant snoRNA may be important only for rRNA processing, but not for nucleolar localization. In fact, we will show here that the latter is the case for many sequences in U8.

Mutant U8 molecules examined for their ability to localize to nucleoli are listed in Figure 3. Most of these are substitutions of sequence, but two mutants were constructed in this study that had regions of the molecule deleted (Δ 82–107; Δ St3,4). Table 1 summarizes previous (Peculis & Steitz, 1994) and present results of the ability of mutant U8 snoRNAs to rescue rRNA processing, and compares this to their nucleolar localization, as well as their association with the nucleolar protein, fibrillarin.

U8 snoRNA mutants that had substitutions of sequences found in loops and that lost function in rRNA processing were analyzed for their nucleolar localization. As a positive control, we analyzed U8 loop mutant Lp4M, which is known to retain its function in rRNA processing (Peculis & Steitz, 1994) and therefore must presumably be localized in nucleoli. Indeed, the Lp4M mutant localized to nucleoli (Fig. 4). Similarly, loop mu-

tants Lp1M and Lp2M localized to nucleoli (Fig. 4), even though they cannot function in rRNA processing (Peculis & Steitz, 1994).

Next, a set of U8 molecules carrying substitutions in stems was tested for nucleolar localization. Stem mutants St1Mc (retains the first stem) and St2M (destroys the second stem) localized to nucleoli, although the signal for St2M was somewhat weaker than for wild type (Fig. 4). However, those mutants fail to rescue rRNA processing after U8 snoRNA depletion (Peculis & Steitz, 1994). To assess whether this loss of function in rRNA processing was due to loss of the stem structure per se, a compensatory mutant, St2C, had been prepared that restored the stem structure. Interestingly, compensatory mutant St2C regained some ability to function in rRNA processing (Peculis & Steitz, 1994), suggesting that the stem structure itself was important. Stem mutant St2C was also able to localize to nucleoli (Fig. 4). Thus, in contrast to its role in rRNA processing, changes in sequence or disruption of stem 2 only weakly affected nucleolar localization (Fig. 4), which still occurred whether this stem was present or absent.

Sequences that had been replaced in U8 mutants Lp1M, Lp2M, St1Mc, and St2M were deduced to be important for rRNA processing (Peculis & Steitz, 1994). The data presented here demonstrate that the loss of function of these mutants in rRNA processing cannot be ascribed to failure for proper targeting, because all these mutant U8 snoRNAs were able to localize to nucleoli.

FIGURE 2. U8 wild-type snoRNA localizes to nucleoli regardless of its cap structure. Fluorescein-labeled U8 snoRNA with a G-cap, A-cap, or no cap at its 5' end was injected into *Xenopus* oocyte nuclei. In some cases, the fluorescein label forms ring-like structures surrounding the rDNA core. A lampbrush chromosome is present in the phase contrast view of the G-cap U8-injected material; it stains blue with DAPI, but is not labeled by fluorescein. Other details as in Figure 1. Bar is 10 μ m.

Sequences adjacent to Box C are required for nucleolar localization of U8 snoRNA

In contrast to the U8 mutations above that did not interfere with U8 snoRNA nucleolar localization, other mutations adversely affected U8 nucleolar localization. To guard against the possibility that failure of some snoRNAs to localize to nucleoli was simply due to their degradation, the stability of the mutant U8 snoRNAs was measured. 32 P-labeled mutant U8 snoRNAs were injected into *Xenopus* oocyte nuclei and recovered after injection for analysis by gel electrophoresis. As shown in Figure 5, all mutant U8 snoRNAs examined had sufficient stability 2 h after injection (the same time point used to assay nucleolar localization) to have been detected in nucleoli. Although the $\Delta 82-107$ and $\Delta \text{St}3,4$ deletions (to be discussed later) are only two thirds as stable as wild-type U8 2 h after injection, they are still in the range where a nucleolar signal could be seen if nucleolar localization were to occur: even a fourfold dilution of the standard amount injected of wild-type U8

(0.9 ng/oocyte) can be detected (Fig. 6). All mutant snoRNAs tested were well within this concentration range for detection.

One U8 mutant carrying a substitution of sequences adjacent to evolutionarily conserved Box C (Cup mutant) abolished the ability of U8 snoRNA to localize to nucleoli (Fig. 7). It was previously determined that the Cup mutant, despite its sufficient stability, failed to rescue rRNA processing after U8 snoRNA depletion (Peculis & Steitz, 1996). It is possible that the loss of function in rRNA processing simply reflects the failure of the Cup mutant to localize to nucleoli.

U8 nucleolar localization is independent of the spatial relationship of Boxes C and D

Boxes C and D are evolutionarily conserved sequences found in many different snoRNA species in a variety of eukaryotes (tabulated in Xia et al., 1997). When these conserved sequences are replaced in U8 snoRNA, the

FIGURE 3. Mutations in U8 snoRNA. The predicted secondary structure of *Xenopus laevis* U8 snoRNA is shown in the upper part of the figure, as in Peculis and Steitz (1993, 1994). Evolutionarily conserved Boxes C and D are indicated according to the consensus tabulated by Xia et al. (1997). Regions containing substitutions (Peculis & Steitz, 1994) or deletions (this study) are enclosed by lines; the St2M mutation is a subset of the St2C substitution and is marked by a dotted line. The sequence of wild-type U8 snoRNA and the mutants analyzed here are listed in the lower part of the figure. A dot indicates that the nucleotide is the same as in wild-type U8 snoRNA; a dash indicates that the nucleotide is deleted.

resulting mutants are unstable 6 h or longer after injection, and their ability to function in rRNA processing cannot be determined (Peculis & Steitz, 1994). Nonetheless, they are sufficiently stable 2 h after injection

that their localization can be assayed, and U8 snoRNA carrying substitutions in Box C or Box D failed to localize to nucleoli (Lange et al., 1998). The same was true for U14 snoRNA, where even a single point mu-

TABLE 1. Functions preserved in U8 snoRNA mutants.^a

Mutant	Fibrillarin association ^b	RNA processing ^c	Nucleolar localization ^d
Lp1M	+	—	++
Lp2M	+	—	++
Lp4M	+	+	++
St1Mc	(+)	—	++
St2M	+	—	+
St2C	+	(+)	+
Box C	—	nd	—
Box D	—	nd	—
CupM	—	—	—
Δ82–107		(+), +	++
ΔSt 3, 4		(+)	++

^aData for U8 snoRNA function in rRNA processing and association with fibrillarin shown within the dotted line box are from Peculis and Steitz (1994); nucleolar localization data for Box C and Box D U8 mutants are from Lange et al. (1998). The rest of the data (including Δ82–107 and ΔSt3,4 function in rRNA processing) are from the present study. Note that four mutants lacking function in rRNA processing (Lp1M, Lp2M, St1Mc, St2M) still localize to nucleoli. Also note that the three mutants that fail to localize to nucleoli (Box C, Box D, CupM) also fail to associate with fibrillarin.

^bFibrillarin association: +, >80% WT; (+), like +, but only determined once; —, no association.

^crRNA processing: +, rescues U8 depletion and 28S and 32S reappear; (+), partial rescue; —, no rescue of U8 depletion; nd, not determined.

^dNucleolar localization after 2 h: ++, comparable to WT; +, weaker than wild type; —, no nucleolar localization.

tation in Box C or Box D prevented nucleolar localization (Lange et al., 1998).

Most of the snoRNAs in the Box C/D family have both ends of the molecule base paired together, and Boxes C and D flank either side of this terminal stem (Tycowski et al., 1993). U3 and U8 snoRNAs are exceptions to this structural arrangement. Nonetheless, it could be hypothesized that the two stems between Boxes C and D in U8 snoRNA act to hold the Boxes in the correct topographical arrangement relative to one another. We have tested if alteration of this topography affects nucleolar localization by construction of two mutants where these stems have been altered. In the Δ82–107 mutant, one side of each stem has been deleted, thereby removing both stem structures (Fig. 3). The 49 nt that remain between Boxes C and D in this mutant might fold differently or be unstructured compared with the wild type. The more extensive deletion in the ΔSt3,4 mutant removes both stems, and only 4 nt remain between Boxes C and D. When fluorescein-labeled transcripts of Δ82–107 or ΔSt3,4 are injected into oocyte nuclei, both are localized to nucleoli (Fig. 7). Therefore, sequences and structures of stems 3 and 4 that occur between Boxes C and D are not needed for nucleolar localization of U8 snoRNA. These sequences and structures are not absolutely essential for rRNA processing either, as determined by a depletion and

FIGURE 4. Loop and stem mutants of the 5' portion of U8 snoRNA, needed for rRNA processing, are not nucleolar localization elements. Nucleolar preparations were performed as in Figure 1, and nucleolar localization of mutant U8 snoRNAs were analyzed after injection. Loop mutants: Fluorescein-labeled RNA is seen in nucleoli after injection of Lp1M and of Lp2M U8 mutants that are not functional in rRNA processing, and also of Lp4M U8 that retains function in rRNA processing. Stem mutants: Fluorescein-labeled RNA is seen in nucleoli after injection of St1Mc and St2M U8 mutants that are not functional in rRNA processing, and also St2C U8 that has partial function in rRNA processing. The fluorescein signals for St2M and St2C are somewhat weaker than for wild-type U8 snoRNA. Bar is 10 μm.

rescue experiment. After the depletion of endogenous U8 snoRNA by antisense oligonucleotide injection into oocytes, the rRNA processing defects can be rescued by wild-type U8 snoRNA with or without fluorescein label (Fig. 8). By comparison, the Δ82–107 mutant gives almost complete rescue and the ΔSt3,4 mutant of U8 snoRNA gives partial rescue of rRNA processing. Therefore, the third and fourth stems of U8 snoRNA are not important for nucleolar localization, nor do they play essential roles in rRNA processing.

FIGURE 5. Stability of wild-type and mutated U8 snoRNAs. U2 snRNA was used as an internal control to normalize for any differences in injection or recovery of the samples. ^{32}P -labeled wild-type or mutated U8 molecules were coinjected into *Xenopus* oocytes with ^{32}P -labeled U2 snRNA. Immediately or 2 h after injection, the RNAs were isolated and analyzed by gel electrophoresis. The relative stability was calculated as $[\text{U8}/\text{U2 at 2 h}]/[\text{U8}/\text{U2 at 0 h}]$. The stability was essentially the same for all U8 snoRNA mutants tested except $\Delta 82-107$ and $\Delta \text{St}3-4$, which were two-thirds the value of wild-type U8 snoRNA.

DISCUSSION

Cytological analysis of Nucleolar Localization Elements (NoLEs) in RNA

Just as there are **Nuclear Localization Sequences (NLS)** in some proteins, so too certain *cis*-acting features in RNA are recognized as **Nuclear Localization Elements (NLE)** for import from the cytoplasm to the nucleus (Grimm et al., 1997). Similarly, NoLEs appear to exist to direct RNA traffic from the nucleoplasm to the nucleolus. The method described here can be generally applied to any RNA to decipher the sequences it uses for nucleolar localization. NoLEs were assayed by injection of fluorescein-labeled U8 snoRNA into *Xenopus* oocyte nuclei and subsequent direct visualization of nucleolar labeling by fluorescence microscopy. We deduce that the injected U8 snoRNA localizes to the correct compartment within the nucleolus, because it is functionally active: wild-type U8 carrying this label can rescue rRNA processing after antisense oligonucleotide depletion of the endogenous snoRNA (Fig. 8). Cytological analysis has also been applied to tissue culture cells to analyze the nucleolar localization of fluorescent 7-2/MRP snoRNA (Jacobson et al., 1995) and the RNA of RNase P (Jacobson et al., 1997); these two RNA species comprise a minor family of snoRNAs, distinct from the Box C/D family.

What sequences act as NoLEs in snoRNAs? Because a trimethylguanosine cap is recognized as an

NLE for import of small nuclear RNA (snRNA) from the cytoplasm into the nucleus (Fischer & Lührmann, 1990; Hamm et al., 1990; Marshallsay & Lührmann, 1994), it might be postulated that the 5' cap also could serve as a NoLE for nucleolar localization of snoRNAs. Moreover, Box D is necessary for nucleolar localization of U8 snoRNA (Lange et al., 1998) and is required for cap hypermethylation (Terns et al., 1995). Therefore, it could be argued that the importance of Box D for nucleolar localization is a secondary effect because of cap hypermethylation. Hypermethylation cannot occur on the A-cap (Peculis & Steitz, 1994), yet nucleolar localization of A-cap U8 occurs in *Xenopus* oocytes after 2 h. Thus, the present study demonstrates that the nucleolar localization of U8 snoRNA in *Xenopus* oocytes is independent of the presence or nature of a 5' cap on the injected snoRNA. The opposite conclusion was reached for mammalian cells in culture (Jacobson & Pederson, 1998), and the authors suggested that this may reflect differences in the cell types studied. This possibility has precedence, because the trimethylguanosine cap is needed for nuclear import of U1 and U2 snRNPs in *Xenopus* oocytes (Fischer & Lührmann, 1990; Hamm et al., 1990; Fischer et al., 1991; 1994), but not in mammalian cells (Kleinschmidt & Pederson, 1990; Fischer et al., 1991). Moreover, they point out that there are other RNA species (e.g., snRNAs, pre-mRNAs, spliced mRNAs) that have m⁷G-caps but do not localize in nucleoli. Thus, although the trimethylguanosine cap may assist nucleolar localization of U8

FIGURE 6. Titration of the amount of wild-type U8 snoRNA injected into oocytes. Wild-type U8 snoRNA was injected into oocyte nuclei at an amount of 0.9 ng/oocyte (100%) or at 50% or 25% of this amount. Even the 25% amount yields detectable nucleolar labeling after injection. See Figure 5 for a summary of stability of U8 snoRNA mutants. Some nucleolar clusters are present. Other details as in Figure 1. Bar is 10 μ m.

snoRNA in mammalian cells (Jacobson & Pederson, 1998), it is not needed for this function in *Xenopus* oocytes, and appears unlikely to be a critical NoLE.

Sequences needed for U8 snoRNA function in rRNA processing are not required for nucleolar localization

Nucleolar localization might be the result of diffusion of macromolecules in the nucleus that are trapped in the nucleolus due to molecular interactions there. This is thought to explain the nucleolar localization of several proteins (e.g., nucleolin, NSR1, ribosomal protein S2, and some viral proteins) where the nucleolar localization domains coincide with RNA recognition motifs and glycine/arginine regions that can bind RNA (Bohnelein et al., 1991; Suzuki et al., 1991; McDonald et al., 1992; Créancier et al., 1993; Messmer & Dreyer, 1993; Schmidt-Zachmann & Nigg, 1993; Yan & Melese, 1993; D'Agostino et al., 1995; Hibbard & Sandri-Goldin, 1995; Mears et al., 1995). Presumably, these proteins must bind to RNA in the nucleolus in order to be tethered there. However, in some cases, the glycine/arginine domains are not required or are not sufficient for nucleolar localization (Yan & Mélése, 1993; Girard et al., 1994; Hibbard & Sandri-Goldin, 1995). In the present case, it might be that snoRNAs localize to nucleoli primarily because of their interactions there with pre-rRNA.

Sites of interaction through base pairing with pre-rRNA have been proposed for several of the essential snoRNAs that are required for rRNA processing. Those in U8 snoRNA are among the best explored by mutagenesis (Peculis & Steitz, 1994; Peculis, 1997). Sequences at the 5' end of U8 snoRNA that span stem 1 and loop 1 are thought to base pair with the 5' end of 28S rRNA in pre-rRNA to prevent premature association of that region with 5.8S RNA (Peculis, 1997). Similarly, sequences in stem 2 and loop 2 of U8 snoRNA have the potential to base pair with a region 200 nt downstream of the T1 cleavage site (Peculis & Steitz, 1994). Mutants in each of these regions of U8 snoRNA (stems 1 and 2 or loops 1 and 2) lose their ability to function in rRNA processing (Peculis & Steitz, 1994). However, we show here that these mutant U8 snoRNAs can still localize to nucleoli. This suggests that the potential to base pair with pre-rRNA might not be a crucial step in the nucleolar localization of U8 snoRNA. Most portions of U8 snoRNA needed for rRNA processing are not essential for nucleolar localization. The only possible exception to this found so far is Cup, a single-stranded region that is essential for rRNA processing (Peculis & Steitz, 1994). We show here that it is an important NoLE in U8 snoRNA. Therefore, U8 snoRNA mutated in Cup may fail to rescue rRNA processing simply because it cannot localize properly to nucleoli, rather than due to a direct role of Cup in rRNA processing.

Several sequences in U8 snoRNA are necessary for nucleolar localization

Distinct sequences function as NoLEs in snoRNAs. Evolutionarily conserved Boxes C and D are required for nucleolar localization of U8 and U14 (Lange et al., 1998), and it is probable that this holds true for all snoRNA members of the Box C/D family of snoRNAs. Although Boxes C and D are positioned adjacent to a terminal stem in most snoRNAs, including U14, this is not the case for U8. It is shown here that stems 3 and 4, located between Boxes C and D in U8 snoRNA, are not required for nucleolar localization, and therefore are unlikely to position Boxes C and D in a unique way needed for nucleolar localization.

A truncated version of U20 snoRNA still containing Boxes C and D was found in a nucleolar fraction of a mouse cell line (Cavaillé et al., 1996). Full-length U14 snoRNA carrying just a single point mutation in Boxes C or D fails to localize to nucleoli, demonstrating that Boxes C and D are important for nucleolar localization (Lange et al., 1998). We have addressed the question here whether there are other sequences besides Boxes C and D in U8 snoRNA that are needed for nucleolar localization, and report that the sequence replaced in the Cup mutant of U8 snoRNA is required as a NoLE. Thus, there seem to be multiple NoLEs that act to lo-

FIGURE 7. A flanking sequence of Box C (Cup) is important for U8 nucleolar localization, but the spatial arrangement of Boxes C and D is not. Nucleoli are unlabeled 2 h after injection of fluorescent Cup mutant snoRNA (CupM), but are labeled after injection of $\Delta 82-107$ or $\Delta \text{St} 3,4$ mutant snoRNAs. Other details as in Figure 1. Bar is 10 μm .

calize a snoRNA to the nucleolus. Some of these NoLEs, such as Boxes C and D, are common to many snoRNA species, whereas others (Cup in U8) are unique to a particular snoRNA. Cup-mediated localization of U8 snoRNA could reflect transport to the nucleolus and/or retention there. Cup may localize U8 by binding a protein itself and/or assisting in the binding of protein(s) to Boxes C and D (the other NoLEs).

What protein(s) bind the NoLEs and serve as transporters to the nucleolus or for nucleolar retention? It is interesting to note that Boxes C and D are needed for association of the nucleolar protein fibrillarin to snoRNAs of the Box C/D family (Baserga et al., 1991; Peculis & Steitz, 1994; Watkins et al., 1996). However, in the case of U8 snoRNA, Boxes C and D might not be sufficient for that function, because the Cup mutant fails to bind fibrillarin (Peculis & Steitz, 1994). Therefore, all of the NoLEs in U8 (Box C, Box D, Cup) are also needed for fibrillarin binding. Appealing as the notion may be that fibrillarin localizes snoRNAs to the nucleolus, it might not be the only player, and/or its role may be indirect. Yeast strains that are genetically depleted of fibrillarin contain nucleoli that stain with antibody against the

trimethylguanosine cap found on many snoRNAs (Tollervey et al., 1991). This result, suggesting that snoRNAs containing this cap can still localize to nucleoli (although this was not demonstrated directly by in situ hybridization) argues against fibrillarin playing a major role in snoRNA transport to nucleoli. Therefore, it is possible that some other protein(s) bind Boxes C and D and other NoLEs like Cup to target their cargo to the nucleolus where fibrillarin subsequently binds by protein–protein interactions. Proteins that bind to the Box C/D motif of other snoRNAs of the Box C/D family have been identified recently (Caffarelli et al., 1998; Watkins et al., 1998), and deserve further study to see if they play a role in nucleolar localization of the snoRNP.

We have shown here that U8 snoRNA has a novel NoLE (Cup), in addition to Boxes C and D that are NoLEs common to other Box C/D snoRNAs. Interestingly, the regions needed in U8 for rRNA processing are generally distinct from regions needed for nucleolar localization. Now that NoLEs are defined in U8 snoRNA, further experiments can focus on the molecular mechanism of snoRNA nucleolar targeting mediated by NoLEs.

FIGURE 8. U8 mutants $\Delta 82-107$ and $\Delta St3,4$ retain partial function in rRNA processing. Newly synthesized rRNA was labeled in vivo with ^{32}P -UTP after treatment with (+) or without (-) an antisense oligonucleotide targeted against U8 snoRNA. After depletion of the endogenous U8 snoRNA, wild-type or mutant U8 was injected. 28S rRNA and 32S pre-rRNA disappear after depletion of U8 and novel species of 32S* and 36S* appear as reported previously (Peculis & Steitz, 1993, 1994). 20S pre-rRNA is also decreased in amount even after U8 rescue of 28S and 32S. The same result has been obtained and discussed by Peculis and Steitz (1994). Wild-type U8 snoRNA without or with fluorescein label (FL) can rescue the rRNA processing defects. U8 mutant $\Delta 82-107$ provides almost complete rescue, but U8 mutant $\Delta St3,4$ only gives partial rescue.

MATERIALS AND METHODS

Plasmids, in vitro transcription, and labeling of RNA

U8 snoRNA sense strand run-off transcripts were obtained either from Bst B1-digested pSP64 plasmid carrying the full-length wild-type or mutant sequence (Peculis & Steitz, 1994) or from PCR products generated from pSP64 plasmid containing U8, using a T7 megascript in vitro transcription kit (Ambion, Austin, Texas) according to the manufacturer's instructions. U2 transcripts were generated as described elsewhere (Lange et al., 1998). To label the transcripts, the reactions included either 0.2 mM fluorescein-12-UTP (DuPont New England Nuclear, Boston, Massachusetts) or 1 mM (10 nCi) α -[^{32}P]-UTP (DuPont New England Nuclear, Boston, Massachusetts) in addition to 1 mM ATP, 1 mM CTP, 1 mM GTP, and 0.2 mM UTP. We added 4 mM m⁷G(5')ppp(5')G cap ("G cap") (Ambion, Austin, Texas), 4 mM m⁷G(5')ppp(5')A cap ("A-cap") (Boehringer Mannheim, Indianapolis, Indiana), or no cap analogue to the in vitro transcription reaction. T7 RNA polymerase uses the dinucleotide G-cap analogue for the first position of the synthetic RNA, but it can be inserted

in the forward or reverse orientation (Pasquinelli et al., 1995). The A-cap analogue is only inserted in the reverse orientation, with the A being at the extreme 5' end of the synthetic RNA (Jacobson & Pederson, 1998). Labeling reactions were either incubated for 2 h (fluorescein-UTP) or 1 h (α -[^{32}P]-UTP) at 37 °C. Following the removal of template DNA by a 10-min incubation with 0.2 units/ μ l DNase I (Ambion, Austin, Texas), RNA was recovered by gel filtration with Sephadex G-50 beads (Pharmacia, Uppsala, Sweden), phenol/chloroform (1:1, v/v) extractions, and ethanol precipitation. The integrity of the in vitro transcripts was confirmed by 8% polyacrylamide, 8 M urea gel electrophoresis. The amount of fluorescent transcript was determined as follows. The concentration of in vitro transcripts of unlabeled wild-type snoRNA was calculated by spectrophotometry at 260 nm and formed the basis for a dilution series of known concentrations of snoRNA electrophoresed on a gel stained with methylene blue. Fluorescein-labeled in vitro transcripts of the mutated snoRNAs were fractionated on the same gel and their intensity was compared to that of the wild-type snoRNA. Concentrations were adjusted accordingly so that equivalent amounts of mutated and wild-type snoRNAs were injected.

Construction of U8 snoRNA mutants

Two mutant U8 snoRNAs were constructed ($\Delta 82-107$ and $\Delta St3,4$) by PCR using a pSP64 plasmid carrying the full-length wild-type U8 snoRNA (Peculis & Steitz, 1994) as the template and using the following primers:

WT (5'): 5'- TAA TAC GAC TCA CTA TAG GGA TCG TCA GTG AGG TTA-3'
 $\Delta St3,4$ (3'): 5'- AAT CAG AAT CAT TCA TCA TGT GCT G-3'
 $\Delta 82-107$ (3'): 5'- AAT CAG AAT GAT CCT GCC TGG TAA GTG TTG TCG AAT CAG CGA CTC AGT CTC-3'

PCR products were cloned into pCR3.1 (Invitrogen, Carlsbad, California) and their sequences were confirmed.

Oocyte microinjections

A portion of the ovary was surgically removed from female *Xenopus laevis* (Nasco, Fort Atkinson, Wisconsin) in accordance with IACUC approved guidelines and transferred to OR2 saline (Wallace et al., 1973). Single oocytes were obtained by digesting the connective tissue with collagenase type I and II (3,000 units/mL each) (Sigma, St. Louis, Missouri) in OR2 for 2 h at room temperature. Stage V oocyte nuclei were injected 16–40 h after isolation.

For analysis of U8 snoRNA nucleolar localization by fluorescence microscopy or for stability assays, oocyte nuclei were injected with 9.2 nL of 0.1 μ g/ μ L (0.9 ng/oocyte) of in vitro-transcribed RNA in distilled H₂O. After further incubation for 2 or 18 h at 20 °C, oocytes were transferred to an isolation buffer containing 83 mM KCl, 17 mM NaCl, 1 mM MgCl₂, 6.5 mM Na₂HPO₄, and 3.5 mM KH₂PO₄, pH 7.5, where nuclei were isolated and the nuclear envelopes were removed manually.

For snoRNA depletion and rescue experiments, U8 snoRNA was disrupted by two cytoplasmic injections spaced 4 h apart of 9.2 nL (5 $\mu\text{g}/\mu\text{L}$) each of the antisense oligonucleotide 5'-GCTGTTTCTC-3' complementary to nt 39–48 (as in Peculis & Steitz, 1993). Four hours later, rescue was achieved by injecting 9.2 nL of in vitro-synthesized snoRNA (1 $\mu\text{g}/\mu\text{L}$) with 50 $\mu\text{Ci}/\mu\text{L}$ of α -[^{32}P]-UTP into nuclei. Oocytes were incubated at 20°C for 16 h after the rescue injection. Subsequently, the nuclei were isolated manually and total RNA was extracted using an RNA extraction kit (5',3' Inc.; Boulder, Colorado).

Analysis by fluorescence microscopy

Following the method of Gall et al. (1991) originally designed for lampbrush chromosome preparations, the nuclear contents of one oocyte were dispersed in a chamber on a gelatinized slide containing a solution of 20.75 mM KCl, 4.25 mM NaCl, 0.5 mM MgCl_2 , 10 mM CaCl_2 , 0.1% paraformaldehyde, 6.5 mM Na_2HPO_4 , 3.5 mM KH_2PO_4 , pH 7.2, for 20 min. Subsequently, the slides were centrifuged at 4°C, 4,000 $\times g$ for 40 min, incubated in 2% paraformaldehyde in PBS (137 mM NaCl, 3 mM KCl, 6.4 mM Na_2HPO_4 , 1.5 mM KH_2PO_4 , pH 7.0) for 1 h, washed once in 70% ethanol, 0.3 M ammonium acetate, and then in 100% ethanol, 0.3 M ammonium acetate. Before mounting with FITS-guard (Testog Inc., Chicago, Illinois), DNA in the preparations was stained by adding 20 ng/mL 4'-6-diamidino-2-phenylindole (DAPI) in PBS for 5 min.

All observations were made with a Zeiss Axiophot Epifluorescence microscope equipped with a 100 $\times$ Neofluar Ph3 objective and a 100-watt mercury lamp. For fluorescence analysis, we utilized the following filter sets: Zeiss 48-7709 (fluorescein) and Zeiss 48-7702 (DAPI). Pictures were made with constant exposures for each filter set using Ektachrome 400 $\times$ professional film (Kodak, Rochester, New York).

U8 snoRNA stability assays

Two hours after injection of oocytes with α -[^{32}P]-UTP-labeled U8 and U2 transcripts, the RNA of four oocytes per sample was recovered using an RNA extraction kit (5'-3' Inc., Boulder, Colorado) according to the manufacturer's instructions. The integrity of the U8 wild-type or mutant snoRNA was analyzed by loading two oocyte equivalents of RNA on a 8% polyacrylamide, 8 M urea gel with subsequent autoradiography with Kodak X-OMAT X-ray film and quantitative analysis using a Fuji BAS 1000 phosphorimager and Mac Bas v.1.0 software. U2 snRNA was used as an internal control to normalize for any differences in injection or recovery of the samples. The relative stability of mutant U8 molecules 2 h after injection was compared to its input value at 0 h after injection.

ACKNOWLEDGMENTS

We thank B. Peculis for giving us wild-type and mutant U8 snoRNAs, M. Jacobson and T. Pederson for sharing details of the fluorescein-labeling conditions for in vitro RNA, M. Ezrokhi for technical help, J. Gall for use of his centrifuge rotor, and A. Coleman for generous use of her fluorescence microscope and helpful discussions. We are grateful to A.-K. Bielinsky, M. Ezrokhi, V. Lunyak, and M. North for comments on the manu-

script. We thank C. González and J.C. Boyles for typing this paper. This research was supported by CTR-3983 and by NIH GM 20261 research grants to S.A.G.

Received March 12, 1998; returned for revision April 6, 1998; revised manuscript received April 27, 1998

REFERENCES

- Baserga SJ, Yang XW, Steitz JA. 1991. An intact Box C sequence in the U3 snRNA is required for binding of fibrillarin, the protein common to the major family of nucleolar snRNPs. *EMBO J* 10: 2645–2651.
- Bohnlein E, Berger J, Hauber J. 1991. Functional mapping of the human immunodeficiency virus type 1 Rev RNA binding domain: New insights into the domain structure of Rev and Rex. *J Virol* 65:7051–7055.
- Brown DD, Dawid IB. 1968. Specific gene amplification in oocytes. *Science* 160:272–280.
- Caffarelli E, Losito M, Giorgi C, Fatica A, Bozzoni I. 1998. In vivo identification of nuclear factors interacting with the conserved elements of box C/D small nucleolar RNAs. *Mol Cell Biol* 18:1023–1028.
- Cavaillé J, Nicoloso M, Bachelier JP. 1996. Targeted ribose methylation of RNA in vivo directed by tailored antisense RNA guides. *Nature* 383:732–735.
- Cheng Y, Dahlberg JE, Lund E. 1995. Diverse effects of the guanosine nucleotide exchange factor RCC1 on RNA transport. *Science* 267:1807–1810.
- Créancier L, Prats H, Zanibellato C, Amalric F, Bugler B. 1993. Determination of the functional domains involved in nucleolar targeting of nucleolin. *Mol Biol Cell* 4:1239–1250.
- D'Agostino DM, Ciminale V, Pavlakis GN, Chieco-Bianchi L. 1995. Intracellular trafficking of the human immunodeficiency virus type 1 Rev protein: Involvement of continued rRNA synthesis in nuclear retention. *AIDS Res Hum Retroviruses* 11:1063–1071.
- Dunbar DA, Baserga SJ. 1998. The U14 snoRNA is required for 2'-O-methylation of the pre-18S rRNA in *Xenopus* oocytes. *RNA* 4:1–10.
- Fischer U, Darzynkiewicz E, Tahara SM, Dathan DA, Lührmann R. 1991. Diversity in the signals required for the nuclear accumulation of snRNPs and variety in the pathways of nuclear transport. *J Cell Biol* 113:705–714.
- Fischer U, Heinrich J, VanZee K, Fanning E, Lührmann R. 1994. Nuclear transport of U1 snRNP in somatic cells: Differences in signal requirement compared with *Xenopus laevis* oocytes. *J Cell Biol* 125:971–980.
- Fischer U, Lührmann R. 1990. An essential signaling role for the m³G cap in the transport of U1 snRNP to the nucleus. *Science* 249:786–790.
- Gall JG. 1968. Differential synthesis of the genes for ribosomal RNA during amphibian oogenesis. *Proc Natl Acad Sci USA* 60:553–560.
- Gall JG, Callan HG, Wu Z, Murphy C. 1991. Lampbrush chromosomes. In: Kay BK, Peng HB, eds. *Methods in cell biology*, vol 36. New York: Academic Press. pp 149–166.
- Ganot P, Bortolin ML, Kiss T. 1997. Site-specific pseudouridine formation in preribosomal RNA is guided by small nucleolar RNAs. *Cell* 89:799–809.
- Gerbi SA. 1995. Small nucleolar RNA. *Biochem Cell Biol* 73:845–858.
- Girard JP, Bagni C, Caizergues-Ferrer M, Amalric F, Lapeyre B. 1994. Identification of a segment of the small nucleolar ribonucleoprotein-associated protein GAR1 that is sufficient for nucleolar accumulation. *J Biol Chem* 269:18499–18506.
- Grimm C, Lund E, Dahlberg AE. 1997. In vivo selection of RNAs that localize in the nucleus. *EMBO J* 16:793–806.
- Hamm J, Darzynkiewicz SM, Tahara SM, Mattaj IW. 1990. The trimethylguanosine cap structure of U1 snRNA is a component of a bipartite nuclear targeting signal. *Cell* 62:569–577.
- Hibbard MK, Sandri-Goldin RM. 1995. Arginine-rich regions succeeding the nuclear localization region of the herpes simplex virus type 1 regulatory protein ICP27 are required for efficient nuclear localization and late gene expression. *J Virol* 69:4656–4667.

- Hughes JM. 1996. Functional base-pairing interaction between highly conserved elements of U3 small nucleolar RNA and the small ribosomal subunit RNA. *J Mol Biol* 259:645–654.
- Hughes JM, Ares M Jr. 1991. Depletion of U3 small nucleolar RNA inhibits cleavage in the 5' external transcribed spacer of yeast pre-ribosomal RNA and impairs formation of 18S ribosomal RNA. *EMBO J* 10:4231–4239.
- Jacobson MR, Cao LG, Taneja K, Singer RH, Wang YL, Pederson T. 1997. Nuclear domains of the RNA subunit of RNase P. *J Cell Science* 110:829–837.
- Jacobson MR, Cao LG, Wang YL, Pederson T. 1995. Dynamic localization of RNase MRP in the nucleolus observed by fluorescent RNA cytochemistry in living cells. *J Cell Biol* 131:1649–1658.
- Jacobson MR, Pederson T. 1998. A 7-methylguanosine cap commits U3 and U8 small nucleolar RNAs to the nucleolar localization pathway. *Nucleic Acids Res* 26:756–760.
- Jarmolowski A, Zagorski J, Li HV, Fournier MJ. 1990. Identification of essential elements in U14 RNA of *Saccharomyces cerevisiae*. *EMBO J* 9:4503–4509.
- Kass S, Tyc K, Steitz JA, Sollner-Webb B. 1990. The U3 small nucleolar ribonucleoprotein functions in the first step of pre-ribosomal RNA processing. *Cell* 60:897–908.
- Kiss-László Z, Henry Y, Bachellerie JP, Caizergues-Ferrer M, Kiss T. 1996. Site-specific ribose methylation of pre-ribosomal RNA: A novel function for small nucleolar RNAs. *Cell* 85:1077–1088.
- Kiss-László Z, Henry Y, Kiss T. 1998. Sequence and structural elements of methylation guide snoRNAs essential for site-specific ribose methylation of pre-rRNA. *EMBO J* 17:797–807.
- Kleinschmidt AM, Pederson T. 1990. RNA processing and ribonucleoprotein assembly studied in vivo by RNA transfection. *Proc Natl Acad Sci USA* 87:1283–1287.
- Lange TS, Borovjagin A, Maxwell ES, Gerbi SA. 1998. Conserved boxes C and D are essential nucleolar localization elements of U14 and U8 snoRNAs. *EMBO J*. In press.
- Li HV, Fournier MJ. 1992. U14 function in *Saccharomyces cerevisiae* can be provided by large deletion variants of yeast U14 and hybrid mouse–yeast U14 RNAs. *EMBO J* 11:683–689.
- Li HV, Zagorski J, Fournier MJ. 1990. Depletion of U14 small nucleolar RNA (snR128) disrupts production of 18S rRNA in *Saccharomyces cerevisiae*. *Mol Cell Biol* 10:1145–1152.
- Liang W, Fournier MJ. 1995. U14 base pairs with 18S rRNA: A novel snoRNA interaction required for rRNA processing. *Genes & Dev* 9:2433–2443.
- Maden BEH. 1996. Click here for methylation. *Nature* 383:675–676.
- Maden BEH, Hughes JMX. 1997. Eukaryotic ribosomal RNA: The recent excitement in the nucleotide modification problem. *Chromosoma* 105:391–400.
- Marshallsay C, Lührmann R. 1994. In vitro nuclear import of snRNPs: Cytosolic factors mediate m³G-cap dependence of U1 and U2 snRNP transport. *EMBO J* 13:222–231.
- Matera AG, Tycowski KT, Steitz JA, Ward DC. 1994. Organization of small nucleolar ribonucleoproteins (snoRNPs) by fluorescence in situ hybridization and immunocytochemistry. *Mol Biol Cell* 5:1289–1299.
- Maxwell ES, Fournier MJ. 1995. The small nucleolar RNAs. *Annu Rev Biochem* 35:897–933.
- McDonald D, Hope TJ, Parslow TG. 1992. Posttranscriptional regulation by the human immunodeficiency virus type 1 Rev and human T-cell leukemia virus type 1 Rex proteins through a heterologous RNA binding site. *J Virol* 66:7232–7238.
- Mears WE, Lan V, Rice SA. 1995. Identification of nuclear and nucleolar localization signals in the herpes simplex virus regulatory protein ICP27. *J Virol* 69:935–947.
- Messmer B, Dreyer C. 1993. Requirements for nuclear translocation and nucleolar accumulation of nucleolin of *Xenopus laevis*. *Eur J Cell Biol* 61:369–382.
- Mishra RK, Elicieri GL. 1997. Three small nucleolar RNAs that are involved in ribosomal RNA precursor processing. *Proc Natl Acad Sci USA* 94:4972–4977.
- Ni J, Tien AL, Fournier MJ. 1997. Small nucleolar RNAs direct site-specific synthesis of pseudouridine in ribosomal RNA. *Cell* 89:565–573.
- Nicoloso M, Qu LH, Michot B, Bachellerie JP. 1996. Intron-encoded antisense small nucleolar RNAs: The characterization of nine novel species points to their direct role as guides for the 2'-O-ribose methylation of rRNAs. *J Mol Biol* 260:178–195.
- Painter TS, Taylor AN. 1942. Nucleic acid storage in the toad's egg. *Proc Natl Acad Sci USA* 28:311–317.
- Pasquinelli AE, Dahlberg JE, Lund E. 1995. Reverse 5' caps in RNAs made in vitro by phage polymerases. *RNA* 1:957–967.
- Peculis BA. 1997. The sequence of the 5' end of the U8 small nucleolar RNA is critical for 5.8S and 28S rRNA maturation. *Mol Cell Biol* 17:3702–3713.
- Peculis BA, Steitz JA. 1993. Disruption of U8 nucleolar snRNA inhibits 5.8S and 28S rRNA processing in the *Xenopus* oocyte. *Cell* 73:1233–1245.
- Peculis BA, Steitz JA. 1994. Sequence and structural elements critical for U8 snRNP function in *Xenopus* oocytes are evolutionarily conserved. *Genes & Dev* 8:2241–2255.
- Perkowska E, Macgregor HC, Birnstiel ML. 1968. Gene amplification in the oocyte nucleus of mutant and wild-type *Xenopus laevis*. *Nature* 217:649–650.
- Savino R, Gerbi SA. 1990. In vivo disruption of *Xenopus* U3 snRNA affects ribosomal RNA processing. *EMBO J* 9:2299–2308.
- Schmidt-Zachmann MS, Nigg EA. 1993. Protein localization to the nucleolus: A search for targeting domains in nucleolin. *J Cell Sci* 105:799–806.
- Shah SB, Terry CD, Wells DA, DiMario PJ. 1996. Structural changes in oocyte nucleoli of *Xenopus laevis* during oogenesis and meiotic maturation. *Chromosoma* 105:111–121.
- Smith CM, Steitz JA. 1997. Sno storm in the nucleolus: New roles for myriad small RNPs. *Cell* 89:669–672.
- Sollner-Webb B, Tycowski KT, Steitz JA. 1995. Ribosomal RNA processing in eukaryotes. In: Zimmermann RA, Dahlberg AE, eds. *Ribosomal RNA: Structure, evolution, processing and function in protein synthesis*. Boca Raton, Florida: CRC Press. pp 469–490.
- Suzuki K, Olvera J, Wool IG. 1991. Primary structure of rat ribosomal protein S2. A ribosomal protein with arginine–glycine tandem repeats and RGGF motifs that are associated with nucleolar localization and binding to ribonucleic acids. *J Biol Chem* 266:20007–20010.
- Terns MP, Dahlberg AE. 1994. Retention and 5' cap trimethylation of U3 RNA in the nucleus. *Science* 264:959–961.
- Terns MP, Grimm C, Lund E, Dahlberg JE. 1995. A common maturation pathway for small nucleolar RNAs. *EMBO J* 14:4860–4871.
- Tollervey D. 1996. Small nucleolar RNAs guide ribosomal RNA methylation. *Science* 273:1056–1057.
- Tollervey D, Lehtonen H, Carmo-Fonseca M, Hurt EC. 1991. The small nucleolar RNP protein NOP1 fibrillarin is required for pre-rRNA processing in yeast. *EMBO J* 10:573–583.
- Tycowski K, Shu MD, Steitz JA. 1993. A small nucleolar RNA is processed from an intron of the human gene encoding ribosomal protein S3. *Genes & Dev* 7:1176–1190.
- Tycowski KT, Shu MD, Steitz JA. 1994. Requirement for intron-encoded U22 small nucleolar RNA in 18S ribosomal RNA maturation. *Science* 266:1558–1561.
- Tycowski KT, Smith CM, Shu MD, Steitz JA. 1996. A small nucleolar RNA requirement for site-specific ribose methylation of rRNA in *Xenopus*. *Proc Natl Acad Sci USA* 93:14480–14485.
- Venema J, Tollervey D. 1995. Processing of pre-ribosomal RNA in *Saccharomyces cerevisiae*. *Yeast* 11:1629–1650.
- Wallace RA, Jared DW, Dumont JN, Sega NW. 1973. Protein incorporation by isolated amphibian oocytes. Optimum incubation conditions. *J Exp Zool* 184:321–333.
- Watkins NJ, Leverette RD, Ling X, Andrews MT, Maxwell ES. 1996. Elements essential for processing intronic U14 snoRNA are located at the termini of the mature snoRNA sequence and include conserved nucleotide Boxes C and D. *RNA* 2:118–133.
- Watkins NJ, Newman DR, Kuhn JF, Maxwell ES. 1998. In vitro assembly of the mouse U14 snoRNP core complex and identification of a 65-kDa box C/D-binding protein. *RNA* 4:582–593.
- Wu Z, Gall JG. 1997. "Micronucleoli" in the *Xenopus* germinal vesicle. *Chromosoma* 105:438–443.
- Xia L, Watkins NJ, Maxwell ES. 1997. Identification of specific nucleotide sequences and structural elements required for intronic U14 snoRNA processing. *RNA* 3:17–26.
- Yan C, Mélése T. 1993. Multiple regions of NSR1 are sufficient for accumulation of fusion protein within the nucleolus. *J Cell Biol* 123:1081–1091.