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An H/ACA guide RNA directs U2 pseudouridylation at two different sites in the branchpoint recognition region in *Xenopus* oocytes

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ABSTRACT

U2 is the most extensively modified of all spliceosomal snRNAs. We previously showed that at least some of the internally modified nucleotides in U2 snRNA are required for snRNP biogenesis and pre-mRNA splicing. Recent work from several laboratories suggests that nuclear guide RNAs facilitate U2 snRNA internal modification, including pseudouridylation and 2′-*O*-methylation. Here, we present a novel approach to identifying guide RNAs for U2 pseudouridylation. Several *Xenopus* oocyte nuclear RNAs were affinity selected with U2 snRNA substituted with 5-fluorouridine, a pseudouridylation inhibitor that sequesters pseudouridylases. One of these RNAs was sequenced and found to be a novel RNA of 134 nt. This small RNA contains an H/ACA motif and folds into a typical H/ACA RNA structure, and its authenticity as an H/ACA RNA was confirmed by immunoprecipitation analysis. The RNA contains two guide sequences for pseudouridylation (Ψ) of U2 snRNA at positions 34 and 44 in the branch-site recognition region, and we demonstrate that this RNA indeed guides the formation of Ψ_{34} and Ψ_{44} in U2 using a *Xenopus* oocyte reconstitution system. Therefore, this novel RNA was designated pugU2-34/44, for pseudouridylation guide for U2 snRNA U_{34} and U_{44}. Intranuclear localization analyses indicate that pugU2-34/44 resides within the nucleoplasm rather than nucleoli or Cajal bodies where other guide RNAs have been localized. Our results clarify the mechanism of U2 snRNA pseudouridylation in *Xenopus* oocytes, and have interesting implications with regard to the intranuclear localization of U2 snRNA pseudouridylation.

Keywords: 5-fluorouridine; H/ACA guide RNA; nucleoplasm; pseudouridylation; U2 snRNA; *Xenopus* oocytes

INTRODUCTION

The small nuclear RNAs (snRNAs) U1, U2, U4, U5, U6, U11, U12, U4atac, and U6atac are spliceosomal components essential for catalyzing the removal of introns from pre-mRNAs (reviewed in Burge et al., 1999; Yu et al., 1999). All these snRNAs contain posttranscriptional modifications (reviewed in Reddy & Busch, 1988; Massenet et al., 1998), and in addition to 5′ cap hypermethylation, a number of internal nucleotides are modified by pseudouridylation and 2′-*O*-methylation. U2 contains the greatest number of modifications among all the spliceosomal snRNAs. In fact, more than 10% of the nucleotides of U2 snRNA are modified, including 10 2′-*O*-methylated residues and 13 pseudouridines (Ψ).

All of the modified nucleotides of U2 are concentrated in the 5′ half of the molecule, which plays essential roles in pre-mRNA splicing (reviewed in Reddy & Busch, 1988; Massenet et al., 1998; Yu et al., 1999). We previously demonstrated that at least some of these modified nucleotides are required for snRNP assembly and pre-mRNA splicing (Yu et al., 1998). Recent NMR studies from Greenbaum's group indicate that base pairing between Ψ_{35} in yeast U2 (equivalent to Ψ_{34} in vertebrate U2) and the nucleotide next to the branchpoint adenosine in a pre-mRNA induces a change in conformation of the U2-pre-mRNA branch site helix, favoring the configuration in which the branchpoint adenosine is bulged out (Newby & Greenbaum, 2001; N.L. Greenbaum, pers. comm.).

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We have recently shown that internal modifications of U2 snRNA (and perhaps other spliceosomal snRNAs) occur exclusively in the nucleus (Yu et al., 2001), and several nuclear subcompartments have been implicated as sites where modification may occur. Based on the correlation between its modification and nucleolar localization, we suggested that U2 snRNA internal modification likely occurs within the nucleolus (Yu et al., 2001), as do the modification of U6 snRNA (Tycowski et al., 1998; Ganot et al., 1999) and ribosomal RNA (rRNA; reviewed in Peculis, 1997; Smith & Steitz, 1997; Tollervey & Kiss, 1997; Weinstein & Steitz, 1999; Kiss, 2001; Filipowicz & Pogacic, 2002; Terns & Terns, 2002). However, our previous work does not exclude the possibility that the modifications also occur in Cajal bodies (Yu et al., 2001). Very recent work from the Kiss group demonstrates that several small RNAs containing potential guide sequences for spliceosomal snRNA modifications are localized to Cajal bodies, suggesting that the internal modifications of spliceosomal snRNAs may indeed occur in these nuclear structures (Darzacq et al., 2002). In another case, a small trypanosome RNA containing a putative guide sequence for the pseudouridylation of spliced leader RNA (SL RNA) is localized primarily to the nucleoplasm (Liang et al., 2002), suggesting a possible nucleoplasmic modification process.

Recently, several groups identified a large number of potential guide RNAs for modification of spliceosomal snRNA (Tycowski et al., 1998; Ganot et al., 1999; Huttenhofer et al., 2001; Jady & Kiss, 2001; Darzacq et al., 2002), and in fact, the guide function of a few of these RNAs has been experimentally confirmed (Tycowski et al., 1998; Jady & Kiss, 2001). However, the number of guide RNAs identified to date is still far smaller than the number of modified nucleotides identified within spliceosomal snRNAs. In addition, most of these recently identified guide RNAs are from mammalian cells and their guide function has yet to be experimentally proven (Huttenhofer et al., 2001; Darzacq et al., 2002). Here, we identify and characterize a new H/ACA motif RNA (pugU2-34/44) from *Xenopus* oocytes that functions to guide U2 pseudouridylation at positions 34 and 44. As expected, pugU2-34/44 specifically associates with GAR1, an H/ACA motif snoRNP protein. However, unlike other H/ACA RNPs that are localized to the nucleolus or Cajal bodies, pugU2-34/44 resides within the nucleoplasm.

RESULTS

A *Xenopus* H/ACA snoRNA copurifies with U2 snRNA substituted with 5-fluorouridine

To identify possible small guide RNAs for U2 pseudouridylation, we took advantage of the fact that U2 snRNA that is fully substituted with 5-fluorouridine constitutes a potent pseudouridylation inhibitor that may tightly bind the enzymes responsible for U2 pseudouridylation (Patton et al., 1994; Yu et al., 1998). A uniformly radiolabeled 5-fluorouridine-substituted U2 was incubated with stage VI *Xenopus* oocyte nuclear extracts, which exhibit potent pseudouridylation activity (data not shown). As shown in Figure 1A, a large complex was observed on a native polyacrylamide gel (lane 2). In contrast, incubation of uniformly radiolabeled nonsubstituted U2 with the extracts resulted in only a poorly defined complex (Fig. 1, lane 1). These results suggested that the 5-fluorouridine-containing U2 may indeed form a tight complex with the U2 pseudouridylation machinery, thus permitting the use of a biotin-streptavidin affinity pull-down assay to purify the U2 pseudouridylases to which the putative pseudouridylation guide RNAs are bound.

A fully substituted 5-fluorouridine U2 with biotinylated cytidine incorporated at random positions was incubated for 30 min with stage VI oocyte nuclear extracts. Streptavidin agarose beads were then used to pull down the pseudouridylases and guide RNAs associated with the 5-fluorouridine-containing U2 snRNA. The beads were treated with proteinase K, and the associated RNAs were subsequently recovered, radiolabeled at the 3′ end with ^{32}pCp, and resolved on a denaturing polyacrylamide gel (Fig. 1B). No RNA was pulled down by the streptavidin beads in the absence of biotinylated U2 (Fig. 1B, lane 1). In contrast, in the presence of the biotinylated 5-fluorouridine-containing U2, a number of RNAs were affinity selected (Fig. 1B, lane 3). Many of these RNAs (e.g., those between 100 and 160 nt) appeared to be selected more effectively by 5-fluorouridine-containing U2 (Fig. 1B, lane 3) than by nonsubstituted U2 (Fig. 1B, lane 2). Several of the selected RNAs were eluted from the gel and sequenced using a combination of chemical and primer-extension sequencing. The strongest radioactive bands of equivalent intensity in Figure 1B, lanes 2 and 3, represent U2 and its breakdown products (most likely the biotinylated U2 RNA used for affinity selection). However, at least one moderately labeled RNA of 134 nt was identified as a novel RNA. This RNA contains an H/ACA motif and has the potential to fold into a typical H/ACA snoRNA structure (Fig. 1C). Inspection of the guide sequences suggested a role in guiding U2 pseudouridylation at positions 34 and 44 (Fig. 1C). We named this H/ACA RNA pugU2-34/44 (pseudouridylation guide for U2 snRNA U_{34} and U_{44}). Database searches identified a homologous sequence within a *Xenopus* cDNA clone (GenBank accession number BE507485), a mouse RNA fragment predicted to guide U2 snRNA pseudouridylation at positions 34 and 44 in mouse (Huttenhofer et al., 2001), and U92 predicted to guide U2 pseudouridylation at position 44 in human (Darzacq et al., 2002; see Fig. 2 for sequence alignments).

To confirm that pugU2-34/44 is specifically associated with the 5-fluorouridine-containing U2, RNAs iso-

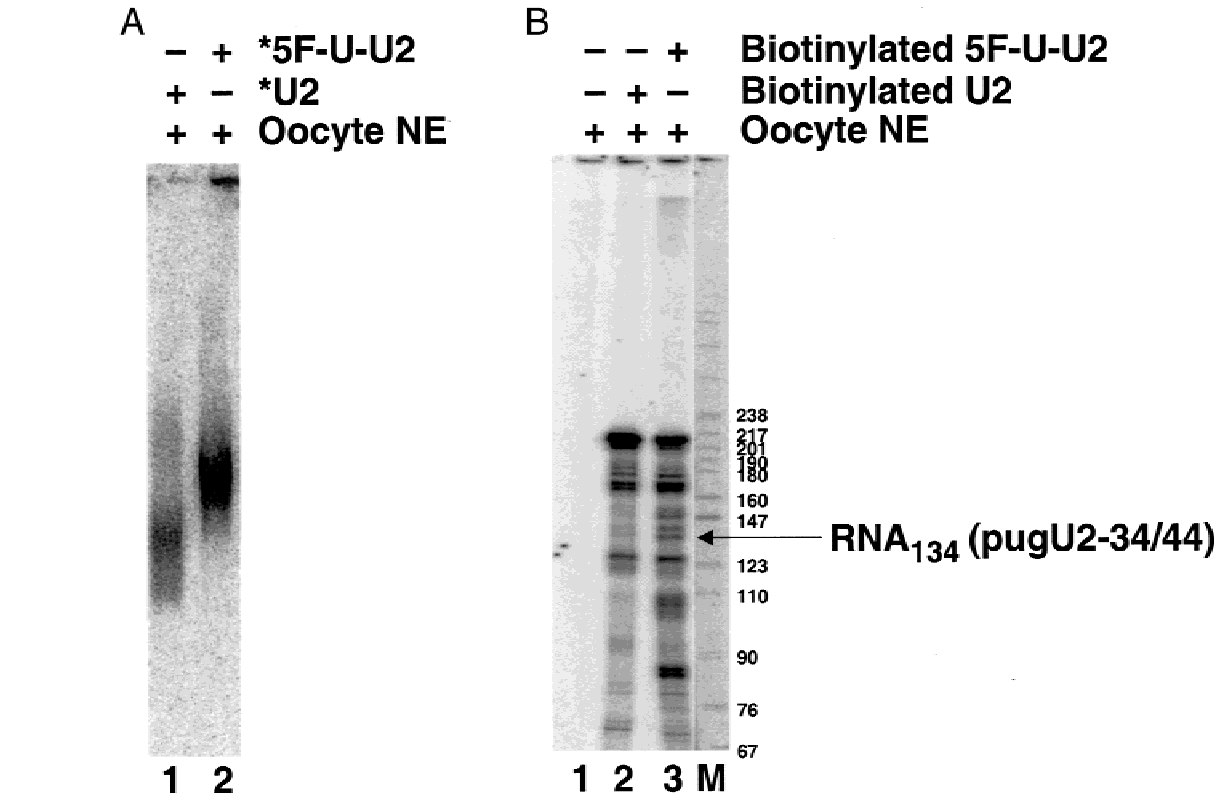


FIGURE 1. Affinity selection of guide RNA using a biotinylated fully substituted 5-fluorouridine U2. **A:** Formation of a complex between a 5-fluorouridine-substituted U2 and the nuclear components of *Xenopus* oocytes. Uniformly [α-^{32}P]GTP-labeled nonsubstituted U2 (lane 1) or fully substituted 5-fluorouridine U2 (lane 2) was incubated with *Xenopus* oocyte nuclear extract for 30 min, after which the samples were loaded on a 4% native polyacrylamide gel. **B:** Selection of guide RNAs. Biotinylated U2 (lane 2) or biotinylated fully substituted 5-fluorouridine U2 (lane 3) was incubated with oocyte nuclear extracts for 30 min, after which streptavidin beads were used to pull down the complex assembled on the U2 RNA. Following proteinase K treatment, RNAs were eluted, 3′-end radiolabeled with ^{32}pCp, and resolved on a 6% denaturing gel (see Materials and Methods). Lane 1 is a parallel experiment in the absence of biotinylated U2 or biotinylated 5-fluorouridine-substituted U2. The numbers on the right are size markers (in nucleotides) of *Msp*I-digested pBR322 DNA. The band with the slowest mobility is the biotinylated U2 of 189 nt (U2 containing biotin-14-CTP migrates slower than unmodified U2 does). The band of 134 nt is indicated [RNA_{134} (pugU2-34/44)]. **C:** RNA_{134} (pugU2-34/44) was sequenced using a combination of chemical and primer-extension sequencing, and its primary sequence and putative secondary structure are shown. The H/ACA motif is indicated by shaded boxes. The guide sequences that direct U2 pseudouridylation are shown base paired with the appropriate sequences in U2 snRNA. Pseudouridines (Ψ) 34 and 44 are indicated. *Xenopus* U2 snRNA is schematized at the bottom of the figure. The sequence of the pre-mRNA branch site recognition region, which includes Ψ_{34} and Ψ_{44}, is shown. The line under the sequence indicates the 6 nt involved in branch site recognition.

```
                1                                                    38
pugU2-34/44    (1)  ----------------------------GTGTGGGAGA
be507485-re    (1)  CATAATGGGATCAGTCAAGTGTTTCCCAGTGTGGGAGA
   AF357402    (1)  -----------------------------------
AY077742-U92   (1)  ----------------------------TGGGAGG
                39                                                   76
pugU2-34/44   (11)  TTGATACTCGGACAGAGA-GAAACTCTGCTGTGTCTGA
be507485-re   (39)  TTGATACTCGGACAGAGA-GAAACTCTGCTGTGTCTGA
   AF357402    (1)  ------CGCAACTTGGGCAGAAGTACTGCTCCAGTTGT
AY077742-U92   (8)  CTGATACACAAATTGGGCTGAAATACTGCTCTACTTGT
                77                                                  114
pugU2-34/44   (48)  CACTAGATCTCCCTATAGTGACCCTGCCTTTTGATGAC
be507485-re   (76)  CACTAGATCTCCCTATAGCGACCCTGCCTTTTGATGAC
   AF357402   (33)  CACTGGACCTCCCCAGAGTAAAC-TGCCTTTTGATGAC
AY077742-U92  (46)  CACCATGCCTCCCTAGAATAAAC-TGCCTTTTGATGAC
               115                                                  152
pugU2-34/44   (86)  CGGGACGAAATGAGAGAAATCGTAACGGACAGATACGA
be507485-re  (114)  -GGGACGAAATGAGAGAAATCGTAACGGACAGATACGA
   AF357402   (70)  CAGGGCGAATTGAGTGAAATCGTCATGGACAGATACGG
AY077742-U92  (83)  CGGGACGAATTGAGTGAAATCGTAACGGACAGATACGG
               153                                                  190
pugU2-34/44  (124)  GGCAGACAGGT----------------------------
be507485-re  (151)  GGCAGACAGGTTTTATAGTCTCTTATCTGCTCTGATTT
   AF357402  (108)  GGCAGACAATT----------------------------
AY077742-U92 (121)  GGCAGACAGAT----------------------------
```

FIGURE 2. Sequence alignment of pugU2-34/44 and its homologs. BLAST searches identified homologous sequences: BE507485-re is the minus strand of a *Xenopus* cDNA clone (GenBank accession number BE507485); AY077742-92 is a potential H/ACA guide RNA for U2 pseudouridylation at position 44 in human (GenBank accession number AY077742; Darzacq et al., 2002); AF357404 is the partial sequence of a potential H/ACA guide RNA for U2 pseudouridylation at positions 34 and 44 in mouse (GenBank accession number AF357404; Huttenhofer et al., 2001). The lines above pugU2-34/44 indicate the guide sequences for U2 pseudouridylation at positions 34 and 44 in *Xenopus* oocytes (this study). The entire sequence of the *Xenopus* cDNA clone (GenBank accession number BE507485) is 415 nt in length, of which nt 228–415 (or 1–188 of the minus strand) are shown.

lated using biotinylated U2 or biotinylated 5-fluorouridine-substituted U2 were analyzed on northern blots using DNA oligonucleotide probes for pugU2-34/44. Figure 3 shows that only background levels of pugU2-34/44 copurified with biotinylated U2 (compare lane 3 with lane 2), whereas biotinylated 5′-fluorouridine-substituted U2 specifically selected pugU2-34/44 (lane 4). Collectively, our results indicate that pugU2-34/44 copurifies with 5-fluorouridine-substituted U2, and therefore represents a potential guide for U2 pseudouridylation.

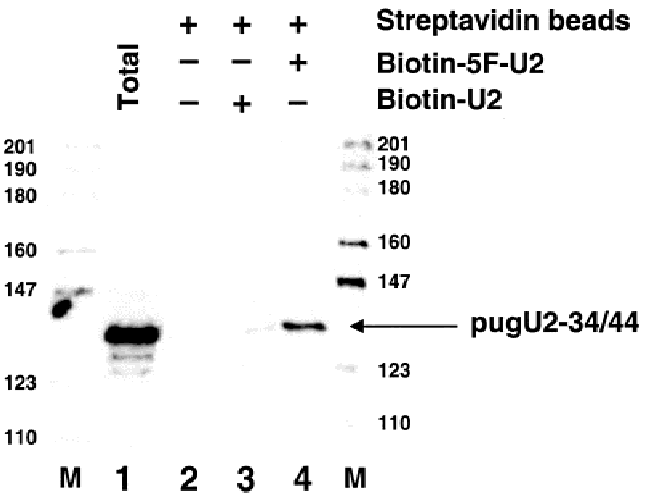


FIGURE 3. Northern analysis showed that pugU2-34/44 specifically copurified with biotinylated 5-fluorouridine-substituted U2 snRNA. Biotinylated U2 (nonsubstituted or 5-fluorouridine substituted) was incubated in the *Xenopus* oocyte nuclear extracts for 30 min. Streptavidin agarose beads were added to pull down the complex(es) associated with U2. RNA components of the complex(es) were recovered and probed with radiolabeled oligonucleotides complementary to pugU2-34/44 (see Materials and Methods). Lane 1 contains total RNA isolated from *Xenopus* oocyte nuclei. Lane 2 is a mock experiment where U2 was omitted. In lane 3, biotinylated nonsubstituted U2 was used. In lane 4, biotinylated 5-fluorouridine-substituted U2 was used. The arrow on the right indicates the signal of PugU2-34/44. Lanes M on both sides are the size markers (in nucleotides) of *Msp*I-digested pBR322 DNA.

pugU2-34/44 is an authentic H/ACA snoRNA

To verify that pugU2-34/44 is a genuine H/ACA snoRNA, we carried out immunoprecipitation analysis. Proteins were immunoprecipitated from nuclear extracts prepared from stage VI oocytes using antibodies specific for GAR1, an H/ACA snoRNP protein (reviewed in Venema & Tollervey, 1999; Weinstein & Steitz, 1999; Filipowicz & Pogacic, 2002; Terns & Terns, 2002) or for fibrillarin, a C/D box snoRNP protein (reviewed in Venema & Tollervey, 1999; Weinstein & Steitz, 1999; Filipowicz & Pogacic, 2002; Terns & Terns, 2002). Coprecipitated RNAs were eluted from protein A-Sepharose beads, resolved on a denaturing gel, and analyzed by northern blotting. Antifibrillarin antibodies nearly quantitatively precipitated mgU6-77, a C/D box snoRNA that guides U6 2′-*O*-methylation (Tycowski et al., 1998; see Fig. 4A,C, lanes 2 and 6 for pellet and supernatant, respectively), but no pugU2-34/44 was detected (Fig. 4B,C, lanes 2 and 6 for pellet and supernatant, respectively). In contrast, anti-GAR1 antibodies precipitated pugU2-34/44 (Fig. 4B,C, lanes 3 and 7), but not mgU6-77 (Fig. 4A,C, lanes 3 and 7). No RNA was precipitated using a control preimmune serum (Fig. 4A,B,C, lanes 1 and 5), and both RNAs were present in the total nuclear RNA population (Fig. 4A,B,C, lane 4). These results indicate that pugU2-34/44 specifically associates with the H/ACA snoRNP protein GAR1, and therefore is an authentic H/ACA motif RNA.

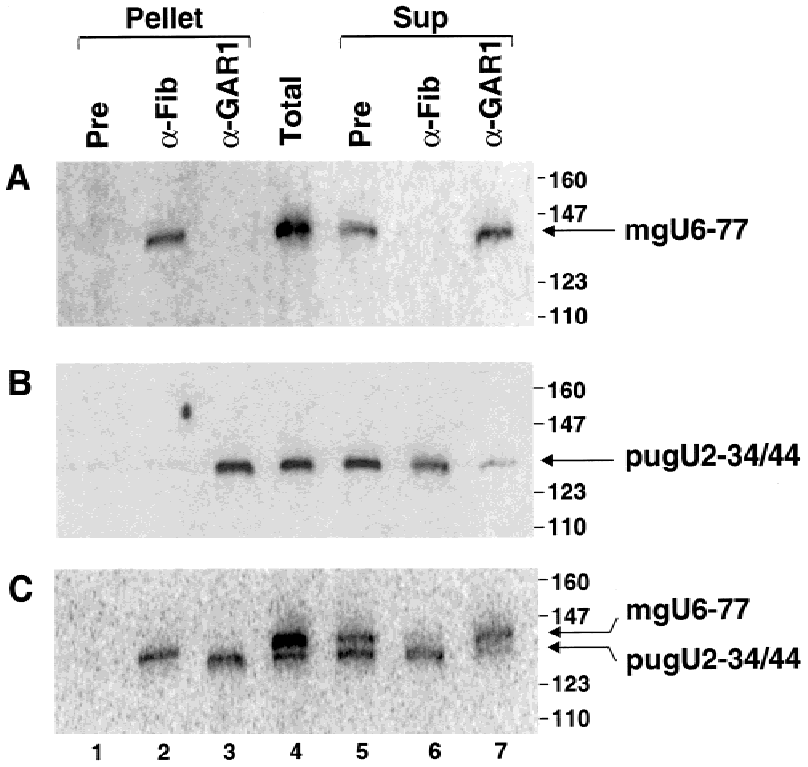


FIGURE 4. pugU2-34/44 associated tightly with Gar1, an H/ACA RNA specific protein. Antibodies prebound to protein A sepharose beads that are specific for Gar1 (lanes 3 and 7), fibrillarin (a C/D box snoRNP protein; lanes 2 and 6), or preimmune antibodies (lanes 1 and 5) were incubated with the oocyte nuclear extracts at 4 °C for 30 min. The beads (pellet, lanes 1–3) were then washed and the coprecipitated RNAs eluted (Material and Methods). The RNAs were also recovered from the unbound fraction (sup, lanes 5–7). The RNAs recovered from both bound (pellet) and unbound (sup) fractions were resolved on a 6% polyacrylamide–8 M urea gel, and probed with radiolabeled DNA oligonucleotides complementary to pugU2-34/44 (**B** and **C**) or mgU6-77 (**A** and **C**), a C/D box snoRNA guiding U6 2′-*O*-methylation at position 77 (Tycowski et al., 1998). Lane 4 is a control that contained total oocyte nuclear RNA. The signals of mgU6-77 and pugU2-34/44 are indicated by arrows.

pugU2-34/44 serves as a guide for U2 pseudouridylation at U_{34} and U_{44} in *Xenopus* oocytes

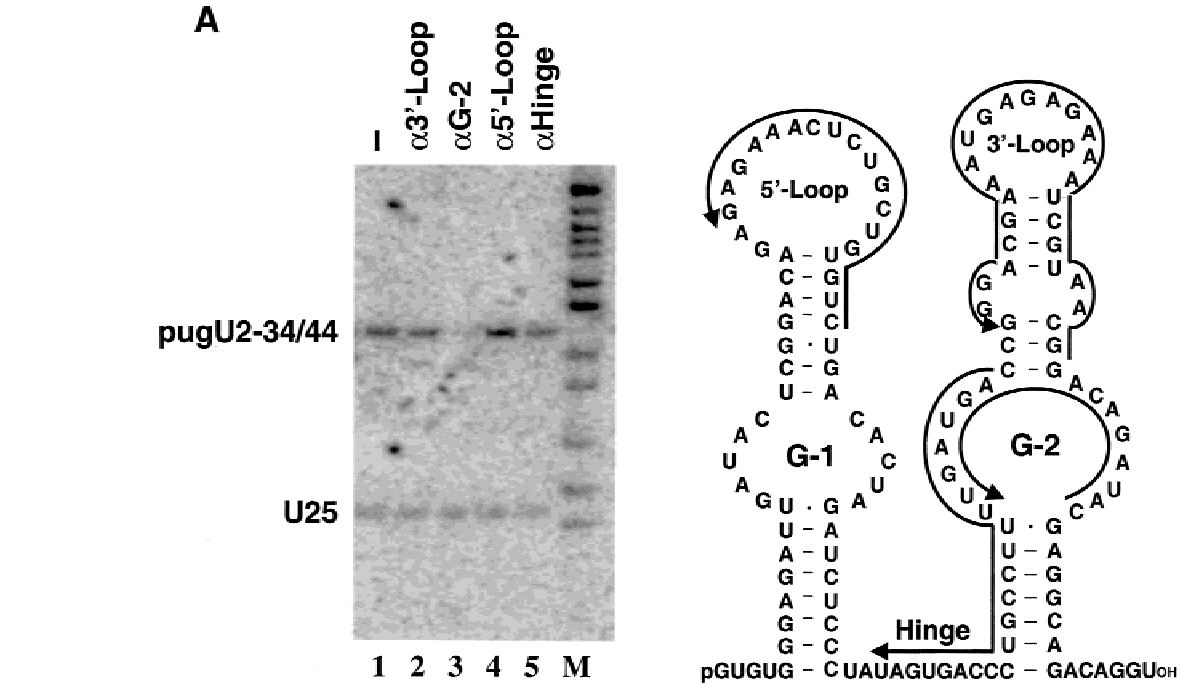
To directly address whether pugU2-34/44 plays a role in U2 pseudouridylation, DNA oligonucleotide-directed RNase H digestion was used to deplete endogenous pugU2-34/44 in *Xenopus* oocytes. A number of DNA oligonucleotides covering almost the entire pugU2-34/44 sequence were injected. Surprisingly, none of these oligonucleotides elicited RNase H degradation of endogenous pugU2-34/44 based on northern analysis (Fig. 5A, lanes 1, 2, 4, and 5; data not shown). This observation suggests that the RNA might be covered by proteins or that its structure masks the binding sites for the chosen oligonucleotides. To overcome this problem, we designed additional DNA oligonucleotides complementary to the predicted guide sequences G-1 (guide 1) and G-2 (see Figs. 5A and 1C). Injection of antisense G-1 facilitated partial degradation of pugU2-34/44 (data not shown), and injection of antisense G-2 completely depleted endogenous pugU2-34/44 (Fig. 5A, lane 3).

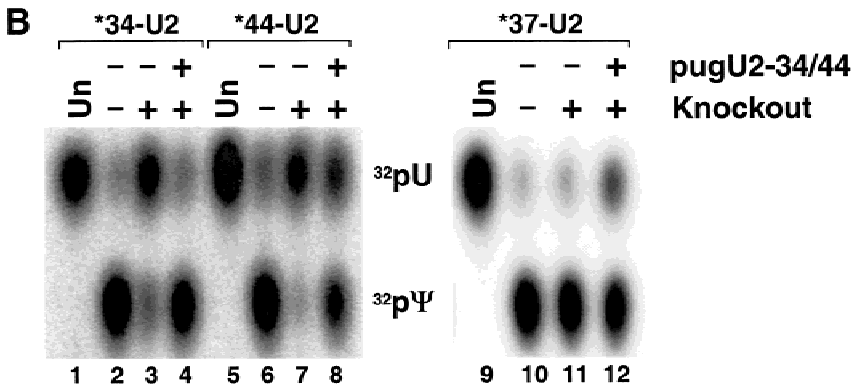
Using the antisense G-2 oligonucleotide, the effect of depleting endogenous pugU2-34/44 on U2 pseudouridylation was examined. Sixteen hours after injection of antisense G-2, oocytes were injected with U2 RNA in which a single phosphate 5′ to U_{34}, U_{37} or U_{44} was substituted with ^{32}P. Following an additional 1- or 4-h incubation, total nuclear RNA was recovered, digested with nuclease P1, and assayed for pseudouridylation by thin layer chromatography. Figure 5B shows the results of the 1-h incubation. Control oocytes received no antisense G-2 and exhibited robust activity in converting U_{34} (Fig. 5B, lane 2), U_{44} (Fig. 5B, lane 6) and U_{37} (Fig. 5B, lane 10) to pseudouridines under these conditions. However, injection of antisense G-2 severely inhibited pseudouridylation at U_{34} (Fig. 5B, lane 3) and U_{44} (Fig. 5B, lane 7) whereas pseudouridylation at U_{37} was not affected (Fig. 5B, lane 11). Importantly, pseudouridylation at U_{34} and U_{44} in pugU2-34/44-depleted oocytes was restored by preinjecting in vitro-transcribed pugU2-34/44 (Fig. 5B, lanes 4 and 8), but not by injecting other irrelevant RNAs (e.g., yeast tRNA; data not shown). We conclude from these experiments that pugU2-34/44 is necessary and specific for U2 pseudouridylation at U_{34} and U_{44} in *Xenopus* oocytes.

Interestingly, when single-radiolabeled U2 was incubated for 4 h or more (instead of 1 h) in oocytes depleted of pugU2-34/44, recovery of pseudouridylation at position 34 was observed, even without supplementing with in vitro transcribed pugU2-34/44 (Fig. 5C, lane 3). Such longer incubations had no effect on pseudouridylation at position 44 (Fig. 5C, lane 7). Thus, there may be redundant activities that catalyze the formation of Ψ_{34} in *Xenopus* oocytes (see Discussion).

pugU2-34/44 resides in the nucleoplasm

Most C/D box and H/ACA motif guide RNAs localize to the nucleolus, where they function as guides for rRNA





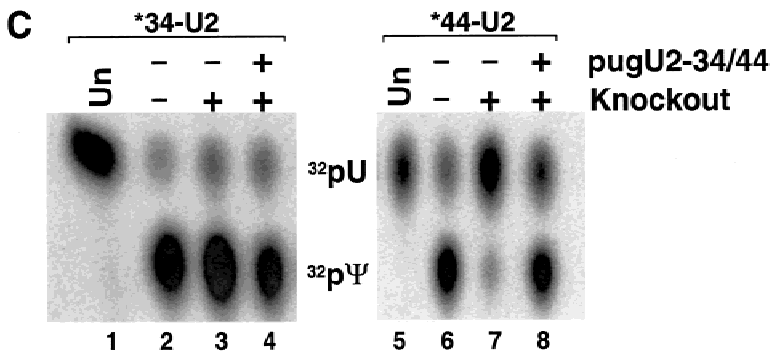


FIGURE 5. pugU2-34/44 functioned as a guide that directs U2 pseudouridylation at positions 34 and 44 in *Xenopus* oocytes. **A:** Targeted depletion of pugU2-34/44. Various antisense DNA oligonucleotides (indicated by arrowed lines in the diagram on the right and by names above each lane on the left) were injected into the cytoplasm of *Xenopus* oocytes. After a 16-h incubation, total nuclear RNA was isolated and probed for pugU2-34/44 and U25 (as a control) with radiolabeled DNA oligonucleotides complementary to pugU2-34/44 and U25, respectively (see Material and Methods). The signals of pugU2-34/44 and U25 are indicated. Note that in lane 3 where anti-G2 was injected, pugU2-34/44 was completely depleted. **B:** Reconstitution of U2 pseudouridylation at positions 34 and 44 in *Xenopus* oocytes. Oocytes depleted of pugU2-34/44 (as in **A**) were supplemented either with in vitro transcribed pugU2-34/44 (lanes 4, 8, and 12) or with water (lanes 3, 7, and 11). Sixteen hours later, U2 RNAs containing a single ^{32}P at the 5′ side of U_{34} (lanes 1–4), U_{44} (lanes 5–8), or U37 (lanes 9–12) were injected separately into the depleted oocytes and incubated for ~1 h. Total nuclear RNA was isolated, treated with nuclease P1, and analyzed on TLC plates. Lanes 2, 6, and 10 are positive controls where single-labeled U2 RNAs were injected into undepleted oocytes. Lanes 1, 5, and 9 are pure single-labeled U2 prior to oocyte injection. Radiolabeled uridylate and pseudouridylate are indicated. **C:** Recovery of U2 pseudouridylation at position 34 upon longer incubation in oocytes depleted of pugU2-34/44. The experiments were carried out exactly as in **B**, except that after injection of single-labeled U2, the oocytes were incubated for 4 h (instead of 1 h). The other experimental conditions are as in **B**.

and spliceosomal snRNA modification (reviewed in Peculis, 1997; Tollervey & Kiss, 1997; Smith & Steitz, 1997; Weinstein & Steitz, 1999; Kiss, 2001; Terns & Terns, 2002; Filipowicz & Pogacic, 2002). Recently, Kiss and colleagues reported that some guide RNAs, including the H/ACA and C/D-H/ACA hybrid guides for spliceosomal snRNA modification, are localized to Cajal bodies (Darzacq et al., 2002), a nuclear structure whose function remains largely unclear. These findings prompted us to investigate the intranuclear localization of pugU2-34/44.

Following injection of fluorescein- and ^{32}P-labeled pugU2-34/44 into *Xenopus* oocyte nuclei, nuclear spreads were prepared and analyzed by fluorescence microscopy. Surprisingly, no signal was observed in either nucleoli or Cajal bodies (Fig. 6A) even up to 48 h after injection (data not shown). As expected, U65 Box H/ACA snoRNA and U7 snRNA were observed primarily in nucleoli and Cajal bodies, respectively, in these experiments (Wu et al., 1996; Narayanan et al., 1999a). When total nuclear and cytoplasmic RNAs were recovered from the oocytes and analyzed on a denaturing

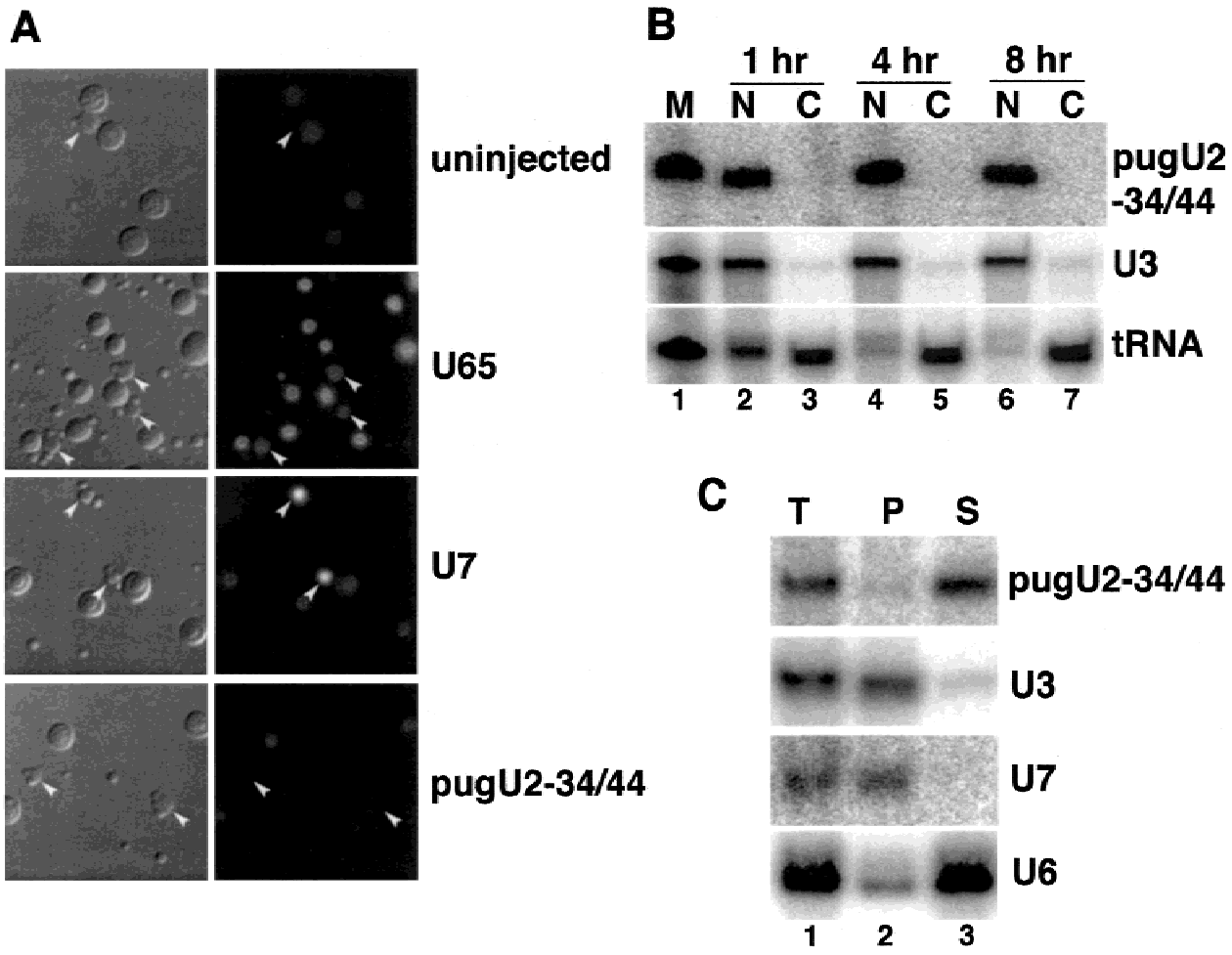


FIGURE 6. pugU2-34/44 resides in the nucleoplasm of *Xenopus* oocytes. **A:** ^{32}P- and fluorescently labeled pugU2-34/44, U65 snoRNA, or U7 snRNA (1 fmol each) were injected separately into *Xenopus* oocyte nuclei and the intranuclear localization of the RNAs were analyzed in nuclear spreads prepared 1 h after injection. As expected, U65 and U7 localize to nucleoli and Cajal bodies, respectively (Wu et al., 1996; Narayanan et al., 1999a). In contrast, pugU2-34/44 does not localize to nuclear structures. A nuclear spread prepared from an uninjected oocyte was used as control for the background fluorescence of the preparation. The nuclear spreads were analyzed by differential interference (DIC) and fluorescence (FL) microscopy. Cajal bodies are indicated by arrowheads. **B:** Nucleocytoplasmic distribution of pugU2-34/44. Oocytes injected with ^{32}P-labeled pugU2-34/44 (from the same batch analyzed above) were manually dissected into nuclear (N; lanes 2, 4, and 6) and cytoplasmic (C; lanes 3, 5, and 7) fractions at indicated time points. U3 snoRNA and tRNA served as controls for nuclear retention and export, respectively. The labeled RNAs were detected by gel electrophoresis and autoradiography. Marker lane (M, lane 1) shows the RNAs before injection. **C:** Endogenous pugU2-34/44 is present in the nucleoplasm. The subnuclear distribution of pugU2-34/44, U3, U6, U7 RNAs was determined by northern blot analysis of RNA present in isolated, unfractionated nuclei (total, T, lane 1) as well as nuclear fractions containing structures (pellet, P, lane 2) and nucleoplasm (supernatant, S, lane 3). U3 and U7 (found predominately in nucleoli and Cajal bodies, respectively) were observed mainly in the pellet (lane 2), whereas the majority of the nucleoplasmic U6 was detected in the supernatant fraction (lane 3). pugU2-34/44 RNA was present mainly in the nucleoplasmic (supernatant) fraction (lane 3).

gel, stable, mature pugU2-34/44 was observed in the nuclear fraction (Fig. 6B, lanes 2, 4, and 6 in subpanel pugU2-34/44) but not in the cytoplasmic fraction (Fig. 6B, lanes 3, 5, and 7 in the pugU2-34/44 subpanel). Coinjected radiolabeled U3 snoRNA (a nucleolar RNA) remained in the nucleus throughout the course of experiment (Fig. 6B, lanes 2, 4, and 6 in the U3 subpanel), whereas a radiolabeled mature tRNA was localized to the cytoplasm following nuclear injection (Fig. 6B, lanes 3, 5, and 7 in the tRNA subpanel). Together, these results suggested that pugU2-34/44 resides within the nucleoplasm. To test this hypothesis, we carried out nuclear fractionation analysis of endogenous RNAs. Using a previously established centrifugation procedure (Peculis & Gall, 1992; Terns et al., 1995), we separated the dense nuclear structures such as the nucleoli and Cajal bodies from the nucleoplasm. RNAs recovered from the pellet (containing nuclear structures) and supernatant (nucleoplasm) fractions were then analyzed by northern blotting with specific antisense probes (Fig. 6C). pugU2-34/44 was detected in the nucleoplasmic fraction (Fig. 6C, lane 3 in subpanel pugU2-34/44) but not in the structural fraction (Fig. 6C, lane 2 in the pugU2-34/44 subpanel). Controls included U6 snRNA, a nucleoplasmic RNA, which was observed in the nucleoplasmic fraction (Fig. 6C, lane 3 in the U6 subpanel), and U3 and U7, which are present in the nucleoli and Cajal bodies, respectively (Terns et al., 1995; Wu et al., 1996), and were detected mainly in the structural fraction (Fig. 6C, lane 2 in the U3 and U7 subpanels). We conclude that pugU2-34/44 resides predominantly in the nucleoplasm.

DISCUSSION

We have identified a novel small nuclear RNA termed pugU2-34/44 in *Xenopus* oocytes from a complex formed on biotinylated 5-fluorouridine-substituted U2. pugU2-34/44 is predicted to adopt a typical H/ACA structure with two guide sequences for U2 pseudouridylation at U_{34} and U_{44}. Immunoprecipitation analysis indicates that pugU2-34/44 is associated with GAR1, a protein specific to H/ACA snoRNPs, demonstrating that this RNA is an authentic H/ACA snoRNA. Intranuclear fractionation suggests that pugU2-34/44 is found in the nucleoplasm. Using the *Xenopus* oocyte depletion/reconstitution system, we demonstrate that pugU2-34/44 is indeed required for guiding the formation of Ψ_{34} and Ψ_{44} in the *Xenopus* U2 snRNA. Interestingly, however, pseudouridylation at position 34 appears to be only partially dependent on pugU2-34/44, as Ψ_{34} formation occurred, albeit at a relatively slow rate, in oocytes depleted of endogenous pugU2-34/44. Together with previous findings, our results raise some interesting questions about the mechanism and intranuclear localization of U2 snRNA pseudouridylation.

The RNA-guided mechanism for the internal modification of spliceosomal snRNAs

It has been suggested that the internal modifications (pseudouridylation and 2′-*O*-methylation) of spliceosomal snRNAs are generated by snoRNPs/scaRNPs in which the RNA components serve as guides via a mechanism identical to that for eukaryotic rRNA modifications (reviewed in Kiss, 2001). Support for this hypothesis comes from recent discoveries of multiple Box C/D or H/ACA RNAs in various organisms with sequence features that suggest roles as guides for spliceosomal snRNA modifications (Tycowski et al., 1998; Ganot et al., 1999; Huttenhofer et al., 2001; Jady & Kiss, 2001; Darzacq et al., 2002). Direct evidence for the function of a few of these putative guide RNAs has been reported, including two 2′-*O*-methylation guides for U6 in *Xenopus* oocytes (Tycowski et al., 1998), and one 2′-*O*-methylation/pseudouridylation hybrid guide for U5 in HeLa cell extracts and COS7 cells (Jady & Kiss, 2001). Here, we identified and experimentally confirmed a new pseudouridylation guide for both positions 34 and 44 of U2 snRNA in *Xenopus* oocytes.

At least three independent lines of evidence suggest that guide RNAs constitute a general mechanism for the internal modification of spliceosomal snRNAs as well as eukaryotic rRNAs. First, extensive searches have identified similar spliceosomal snRNA modification guide RNAs in a wide variety of organisms including human (Tycowski et al., 1998; Ganot et al., 1999; Jady & Kiss, 2001; Darzacq et al., 2002), mouse (Tycowski et al., 1998; Huttenhofer et al., 2001), *Caenorhabditis elegans* (Tycowski et al., 1998), fruit fly (Jady & Kiss, 2001), trypanosome (Liang et al., 2002), and *Xenopus* oocytes (Tycowski et al., 1998; this work). Second, modification guide RNAs have been identified for almost all the spliceosomal snRNAs (U2, U5, U6, U4, and U1; Tycowski et al., 1998; Ganot et al., 1999; Huttenhofer et al., 2001; Jady & Kiss, 2001; Darzacq et al., 2002; this work). Third, some guide RNAs are predicted to direct modifications at different sites within a single spliceosomal snRNA (Tycowski et al., 1998; Ganot et al., 1999; Huttenhofer et al., 2001; Jady & Kiss, 2001; Darzacq et al., 2002; this work). However, to demonstrate that RNA-guided modification is a general mechanism for all spliceosomal snRNA modifications in different organisms, an extensive search for additional guide RNAs and experimental verification of their function is necessary.

Functional redundancy for spliceosomal snRNA modification

Our results in *Xenopus* oocytes indicate that pseudouridylation at position 34 of U2 RNA is only partially dependent on pugU2-34/44, as pseudouridylation activity slowly recovered in oocytes depleted of endogenous

pugU2-34/44 (Fig. 5C). Although this phenomenon could be due to incomplete depletion or regeneration of pugU2-34/44, we consider this possibility unlikely because no pugU2-34/44 was detected by northern blotting of depleted oocytes throughout the course of our experiments (Fig. 5A; data not shown). Moreover, we observed that Ψ_{44} formation, which is also guided by pugU2-34/44, was blocked in the depleted oocytes even when the incubation of U2 RNA in oocytes was extended beyond 4 h (Fig. 5C). This suggests that pugU2-34/44 depletion was very effective. Alternatively, the slow recovery of pseudouridylation activity for Ψ_{34} in depleted oocytes could be due to the generation of a 5′ half fragment of pugU2-34/44 during targeted RNase H digestion (see Fig. 1C). Conceivably, such a 5′ half fragment could function to guide the formation of Ψ_{34}. However, we also consider this possibility unlikely because no 5′ half fragment was detected in northern blots using a probe complementary to the 5′ half of pugU2-34/44 (data not shown). One likely explanation for the partial dependence that we observed is that *Xenopus* oocytes contain a redundant pseudouridylation activity. This assertion is supported by recent work from the Kiss group (Darzacq et al., 2002) in which many scaRNAs were identified. Although the sequences of several of these scaRNAs are quite different, the putative guide sequences they contain are identical, suggesting that modification of any particular site within a spliceosomal snRNA may be guided by not just one, but several different RNAs. If true, then *Xenopus* oocytes should contain functionally redundant small RNAs that guide pseudouridylation of U2 at position 34. Identification of such redundant RNAs is necessary to address this hypothesis.

Is U2 snRNA modified at multiple sites within the nucleus?

The observation that pugU2-34/44 resides in the nucleoplasm raises an interesting question as to the pathway by which U2 snRNA modification occurs. It is clear that although the U2 snRNP cycles through the cytoplasm during its biogenesis, U2 snRNA internal modifications (including pseudouridylation and 2′-*O*-methylation) occur exclusively in the nucleus of *Xenopus* oocytes (Yu et al., 2001). Furthermore, based on an excellent correlation between overall U2 modification and its nucleolar targeting, we suggested that U2 snRNA modifications occur in the nucleolus (Yu et al., 2001). This view is supported by the fact that a vast majority of the RNP modifying enzymes are localized to the nucleolus, including the C/D snoRNPs for 2′-*O*-methylation and the H/ACA snoRNPs for pseudouridylation (for review, see Peculis, 1997; Smith & Steitz, 1997; Weinstein & Steitz, 1999; Kiss, 2001; Filipowicz & Pogacic, 2002; Terns & Terns, 2002). At the same time, we could not rule out the possibility that U2 modifications may also occur in Cajal bodies (Yu et al., 2001). Indeed, recent work from the Kiss group demonstrates that a number of human small RNAs containing guide sequences predicted to target spliceosomal snRNA modification are found in Cajal bodies (Darzacq et al., 2002). It is possible that while *Xenopus* pugU2-34/44 resides in the nucleoplasm (Fig. 6), it transits nucleoli or Cajal bodies where it functions in U2 modification. Alternatively, although it is likely that the majority of spliceosomal snRNA nucleotide modifications occur in the nucleolus and/or Cajal bodies, our results require that we consider that some modifications may occur in the nucleoplasm.

It should be noted that although *Xenopus* pugU2-34/44 is localized to the nucleoplasm (this work), its human homolog (U92) is among those found in Cajal bodies (Darzacq et al., 2002). The apparent discrepancy may simply be due to the different cell systems used in these studies (*Xenopus* oocytes versus HeLa cells). However, further study is necessary to clarify this issue.

MATERIALS AND METHODS

Preparation of various U2 snRNAs

T7 in vitro transcription was used to generate biotinylated U2 snRNA. The transcription reaction mixture contained 1.2 mM each of ATP, CTP, UTP, and GpppG (New England Biolabs), 0.6 mM GTP (New England Biolabs), 0.12 mM biotin-14-CTP (Invitrogen), 40 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$, 2 mM spermidine, 5 mM DTT, 0.1 mg/mL *Sma*I-linearized T7-U2 plasmid, and 4 U/μL T7 RNA polymerase. To create biotinylated fully substituted 5-fluorouridine U2, 1.2 mM UTP was replaced by 1.2 mM 5-fluorouridine-5′-triphosphate. To produce radiolabeled, nonbiotinylated U2 (unmodified or fully substituted with 5-fluorouridine), biotin-14-CTP was left out and [α-^{32}P]GTP (2 μCi/μL) was included.

Native gel analysis

Forty nuclei were manually isolated from stage VI *Xenopus* oocytes, as previously described (Yu et al., 1998). Nuclear extracts were prepared by pipetting the nuclei up and down 20 times using P10 tips. Extracts were then incubated with 50 ng of radiolabeled U2 RNA (~1,000,000 cpm) for 15 min, after which the mixture was loaded on a 4% polyacrylamide gel without urea (acrylamide:bis = 80:1). After electrophoresis for 4 h at 250 V, the gel was exposed to a phosphor screen and the complexes were visualized using a PhosphorImager (Molecular Dynamics).

Biotin-streptavidin affinity purification

Oocyte nuclear extracts prepared from 200 isolated nuclei were incubated with 2 μg of biotinylated U2 snRNA for 30 min. The mixture was then added to 10 μL (bed volume) of streptavidin agarose beads (Pierce) in 250 μL of Buffer A (100 mM Tris-HCl, pH 8.0, 100 mM ammonium acetate, 5 mM $MgCl_2$,

2 mM DTT, 0.1 mM EDTA, and 0.05% NP-40). The tube containing the beads was then gently rotated on a nutator at 4 °C for 2 h. The beads were subsequently washed with 1 mL Buffer A four times. The washed beads then received 250 μL G50 buffer (20 mM Tris-HCl, pH 7.5, 300 mM sodium acetate, 2 mM EDTA, and 0.3% SDS) and 200 μg of proteinase K, and the reaction was carried out at 42 °C for 15 min. The proteinase K-digested sample was then transferred to 85 °C for 10 min. Following a brief centrifugation, the supernatant was transferred to a new tube and extracted twice with 400 μL PCA (phenol-chloroform-isoamyl alcohol, 50:49:1). RNA was recovered by ethanol precipitation, radiolabeled at the 3′ end with ^{32}pCp using T4 RNA ligase, and resolved on a 6% denaturing polyacrylamide gel. RNA bands were visualized by autoradiography and excised for sequencing according to published procedures (Peattie, 1979).

Xenopus oocyte microinjection

Microinjection procedures were performed essentially as described previously (Yu et al., 1998). The antisense pugU2-34/44 oligodeoxynucleotides were: α5′-Loop, complementary to nt 27–44 of pugU2-34/44; α3′-Loop, complementary to 88–112 of pugU2-34/44; αHinge, complementary to nt 63–84 of pugU2-34/44; αG2, complementary to the guide sequence for position 44 (see Figs. 1C and 5A). Briefly, 32 nL of the antisense pugU2-34/44 oligodeoxynucleotides (3 μg/μL) were injected individually into the cytoplasm of *Xenopus* oocytes that were then incubated at 18 °C for 16 h. To check the effectiveness of oligonucleotide-targeted depletion of endogenous pugU2-34/44, oocyte nuclei were manually isolated, and total nuclear RNA was recovered and analyzed by northern blotting (Yu et al., 1996). In Figures 3 and 5A, α3′-Loop was used as a probe for pugU2-34/44, and the control probe was an oligodeoxynucleotide complementary to nt 1–51 of the snRNA U25.

For functional reconstitution experiments, the oocytes depleted of pugU2-34/44 were injected cytoplasmically with 41 nL of synthetic pugU2-34/44 at a concentration of 300 ng/μL, or with an equal volume of water as a control. After an overnight incubation, 18 nL of synthetic U2 snRNA containing a single ^{32}P-label at the 5′ of either U_{34}, U_{44}, or U_{37} (~25 ng/μL; ~10^6 cpm/25 ng) were injected. Following a 1-h or 4-h incubation, total nuclear RNA was recovered, digested with nuclease P1, and assayed for pseudouridylation by thin layer chromatography (Yu et al., 1998).

Immunoprecipitation of pugU2-34/44

Immunoprecipitations with anti-GAR1 and antifibrillarin were carried out essentially as described (Dragon et al., 2000). Briefly, 40 nuclei were isolated from stage VI *Xenopus* oocytes. A nuclear suspension was prepared by pipetting the nuclei up and down 20 times using P10 tips followed by vigorous mixing in Net-2 buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% NP-40). Antibodies (anti-GAR1 or antifibrillarin) prebound to protein A-Sepharose were added, and the mixture was incubated with gentle mixing at 4 °C for 2 h. After centrifugation, the beads were washed four times with Net-2 buffer and digested with proteinase K as above in G50 buffer. RNAs were recovered by PCA extraction and ethanol precipitation, and analyzed by northern blotting.

RNA localization assays

The method for *Xenopus* oocyte nuclear injections was described (Yu et al., 2001). Briefly, oocyte nuclei were injected with 10 nL of solution containing 1 fmol of ^{32}P-labeled and/or fluorescently labeled mature pugU2-34/44 transcribed in vitro from template DNA generated by PCR. Templates for generation of U3, U7, U65, and tRNA control RNAs have been described (Narayanan et al., 1999a; Yu et al., 2001). To assay the intranuclear localization of fluorescently labeled RNAs, nuclear spreads were prepared from dissected nuclei and observed under a Zeiss (Thornwood, New York) Axiovert S 100 inverted fluorescence microscope (Narayanan et al., 1999b). The nucleocytoplasmic distribution of the injected RNAs were performed by manual dissection of the injected oocytes into nuclear and cytoplasmic fractions followed by RNA extraction and gel electrophoresis (Speckmann et al., 1999). The subnuclear distribution of endogenous RNAs was determined by fractionating oocyte nuclei into pellet (containing nuclear structures including nucleoli and Cajal bodies) and supernatant (nucleoplasmic) fractions followed by northern blot analysis of the fractionated RNA (Terns et al., 1995). The blots were probed with ^{32}P-labeled, full-length antisense RNA probes transcribed in vitro against pugU2-34/44, U6, U3, and U7. The results were visualized using a PhosphorImager (Molecular Dynamics).

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