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Role of the Box C/D Motif in Localization of Small Nucleolar RNAs to Coiled Bodies and Nucleoli

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Small nucleolar RNAs (snoRNAs) are a large family of eukaryotic RNAs that function within the nucleolus in the biogenesis of ribosomes. One major class of snoRNAs is the box C/D snoRNAs named for their conserved box C and box D sequence elements. We have investigated the involvement of *cis*-acting sequences and intranuclear structures in the localization of box C/D snoRNAs to the nucleolus by assaying the intranuclear distribution of fluorescently labeled U3, U8, and U14 snoRNAs injected into *Xenopus* oocyte nuclei. Analysis of an extensive panel of U3 RNA variants showed that the box C/D motif, comprised of box C′, box D, and the 3′ terminal stem of U3, is necessary and sufficient for the nucleolar localization of U3 snoRNA. Disruption of the elements of the box C/D motif of U8 and U14 snoRNAs also prevented nucleolar localization, indicating that all box C/D snoRNAs use a common nucleolar-targeting mechanism. Finally, we found that wild-type box C/D snoRNAs transiently associate with coiled bodies before they localize to nucleoli and that variant RNAs that lack an intact box C/D motif are detained within coiled bodies. These results suggest that coiled bodies play a role in the biogenesis and/or intranuclear transport of box C/D snoRNAs.

INTRODUCTION

The generation of eukaryotic ribosomes takes place predominantly inside the nucleus within nucleoli. Nucleoli are composed of a complex mixture of macromolecules, and considerable intracellular trafficking of macromolecules is required to assemble functional nucleoli and to produce ribosomal subunits. Scores of ribosomal and nonribosomal proteins synthesized in the cytoplasm must move to nucleoli. Indeed, several nucleolar proteins have been demonstrated to shuttle continuously between the cytoplasm and nucleus (Borer *et al.*, 1989; Meier and Blobel, 1992; Shaw and Jordan, 1995). In addition, >150 distinct small nucleolar RNAs (snoRNAs) are each targeted to nucleoli where they function in the modification and processing of rRNA (Bachellerie *et al.*, 1995; Maxwell and Fournier, 1995; Smith and Steitz, 1997; Tollervey and Kiss, 1997). snoRNAs are produced in the nucleus and remain within the nucleus where they are matured at unidentified intranuclear sites (Terns and Dahlberg, 1994; Terns *et al.*, 1995). Thus, production of functional snoRNAs involves transport from the nucleoplasm to nucleoli and may involve other nuclear structures.

The box C/D snoRNAs are one of two major classes of snoRNAs distinguished by the presence of conserved sequence elements and common secondary structures (Balakin *et al.*, 1996). Box C/D snoRNAs each associate with common proteins including fibrillarin (Schimmang *et al.*, 1989; Lapeyre *et al.*, 1990; Baserga *et al.*, 1991), Nop 56/58 (Wu *et al.*, 1998; Lafontaine and Tollervey, 1999), and recently identified proteins (Caffarelli *et al.*, 1998; Watkins *et al.*, 1998). Only a few box C/D snoRNAs, namely, U3, U8, U14, and U22, are required for rRNA processing (Li *et al.*, 1990; Savino and Gerbi, 1990; Hughes and Ares, 1991; Peculis and Steitz, 1993; Tycowski *et al.*, 1994; Enright *et al.*, 1996). The majority of box C/D snoRNAs function as guide RNAs to direct methylation of ribose 2′-

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hydroxyl groups at conserved positions in rRNA (Cavaille *et al.*, 1996; Kiss-Laszlo *et al.*, 1996; Tollervey, 1996; Tycowski *et al.*, 1996).

The defining structural element of the box C/D snoRNAs is the box C/D motif. The box D element (core consensus sequence CUGA [Xia *et al.*, 1997]) is generally found in a single-stranded region near the 3′ terminus of the box C/D RNAs. The box C element (core consensus sequence GANG [Xia *et al.*, 1997]) exists in a single-stranded region opposite box D within the predicted secondary structure of most box C/D snoRNAs (Tycowski *et al.*, 1993; Kiss-Laszlo *et al.*, 1996; Watkins *et al.*, 1996; Samarsky and Fournier, 1998). Thus the two common sequence elements, box C and box D, are generally distant from each other in the primary sequence of the snoRNAs but are brought into proximity in the folded RNAs as a result of the base pairing of complementary sequences flanking the box elements. The resultant structure, consisting of box C and box D and one or two adjacent helices, has been referred to as the stem-box structure (Qu *et al.*, 1995), the terminal core motif (Xia *et al.*, 1997), or, simply, the box C/D motif (Samarsky *et al.*, 1998).

In this study, we have focused our analysis on the mechanisms governing the localization of the box C/D class of snoRNAs to the nucleolus. Extensive studies were performed using U3 snoRNA, which is the most abundant and well-characterized box C/D snoRNA. Comparison of U3 RNAs from diverse organisms reveals that U3 snoRNA contains a box D and two box C (referred to as boxes C and C′) sequence elements as well as U3-specific elements known as boxes A, A′, and B. A two-domain secondary structure is predicted for U3 RNA from all organisms examined. The 5′ domain contains sequences (boxes A and A′) that participate in base-paired interactions with 18S rRNA (Hughes, 1996; Mereau *et al.*, 1997). The 3′ domain of U3 RNA is structurally diverse (Fournier *et al.*, 1998) but invariably contains boxes B, C, C′, and D, which are protein-binding sites (Parker and Steitz, 1987; Jeppesen *et al.*, 1988; Hartshorne and Agabian, 1994; Mereau *et al.*, 1997), as well as a 3′ terminal stem. The "hinge," a short, single-stranded sequence that links the 5′ and 3′ domains, recognizes complementary sequences in the 5′ external transcribed sequences of pre-rRNA (Beltrame and Tollervey, 1995; Mereau *et al.*, 1997).

We have introduced substitution mutations throughout the U3 molecule including all conserved box elements, the 3′ terminal stem, and the hinge sequence, and have analyzed the intranuclear localization of these variant U3 RNAs after their injection into *Xenopus* oocyte nuclei. In addition, we have also examined the intranuclear localization of additional box C/D snoRNAs (U8 and U14) to test the generality of our observations. We have found that the targeting of box C/D snoRNAs to nucleoli depends on their common sequence elements (the box C/D motif) and is temperature dependent. Furthermore, we have characterized the association of the box C/D snoRNAs with an additional intranuclear organelle, the coiled body. Our results suggest that box C/D snoRNAs associate with coiled bodies transiently before localization to nucleoli. Important differences between the results obtained in this study and those of similar recent studies (Lange *et al.*, 1998a–c; Samarsky *et al.*, 1998) will be discussed.

MATERIALS AND METHODS

Generation of snoRNA Mutant Constructs

U3 Constructs. A plasmid containing a genomic clone encoding *Xenopus* U3A snoRNA (Savino *et al.*, 1992) was used as the source of wild-type U3 RNA-coding sequence. Templates used to transcribe wild-type or mutant U3 RNAs were DNA fragments generated by PCR amplification. DNA fragments encoding U3 RNAs with specific mutations were generated by PCR-based strategies described in detail below. In general, block substitution mutations were introduced in which each nucleotide of a conserved box element was replaced with a complementary nucleotide. The precise changes are noted in the specific description of the generation of each mutant below. The deoxyoligonucleotide primers used in PCR reactions to prepare wild-type and mutated *Xenopus* U3 templates are listed. SP6 promoter sequences are underlined, and sites of mutation are in bold. All PCR reactions were performed using Pfu DNA polymerase (Stratagene, La Jolla, CA) and an annealing temperature of 52°C.

5′ primers were as follows: 1) GATTTAGGTGACACTATAGAAGACTATACTTTCAGGGATCA; 2) GATTTAGGTGACACTATAGAAGACTA**ATGAA**TCAGGGATCA; 3) CAGTAAGACTATACTTTCAG**CCTAGTAAAGAT**TAGGTTGTACCTGGTGA; 4) GTGCTCGAAAGTGTGT**GACTTGAGTGTT**ACCACGAGGAAGAGC; 5) CTGAACTCACAAACCAC**CTCCTTCT**GCGTCAGTGTTCTCTC ; 6) CGTCAGTGTTCTCTC**CTCTCGCA**CTTGTGAGCTCACAGTGCTG; 7) GGCTGCTGTTTGCTAT**ACTACTTGC**TTCTGCTCCCCTTTA; 8) GATTTAGGTGACACTATAGACCACGAGGAAGAGCG; and 9) AAAAAGAATTCCCAAATTCAGAAGTGACTGCG. 3′ primers were as follows: 10) GGGTGTCAGCCTGTGTTCTCTCCCTCC; 11) ACCACTCAGCCTGTGTTCTCTCCCTCC; 12) TCACCAGGTACAACCTA**ATCTTTACTAGG**CTGAAAGTATAGTCTTACTG; 13) GCTCTTCCTCGTGGT**AACACTCAAGTC**ACACTTTCGAGCACAT; 14) GAGAGAACACTGACGC**AGAAGGAG**GTGGTTTGTGAGTTCAG; 15) CAGCACTGTGAGCTCAC**AAGTGCGAG**AGGAGAGAACACTGACG; 16) TAAAGGGGAGCAGAA**GCAAGTAGT**ATAGCAAACAGCAGC; 17) ACCAC**AGTCGG**TGTGTTC; 18) ACCACTCAT**C**CTGTGTTCTCTCCCTCC; 19) ACCACTC**C**GCCTGTGTTCTCTCCCTCC; 20) ACCACT**G**AGCCTGTGTTCTCTCCCTCC; 21) ACCAC**A**CAGCCTGTGTTCTCTCCCTCC; 22) ACCACTCAGCCTGTGTTCTCTCCCGAAGG; and 23) AAAAAAAGCTTCAGCCCCACTTTTCCATTC.

Two different PCR strategies were used: one to introduce mutations near the termini of U3 and to generate subfragments of U3 and another to introduce mutations at internal positions within the U3-coding region.

Generation of Terminal U3 Mutations and U3 Subfragments. Wild-type U3 transcription template DNA was generated by PCR amplification from wild-type U3 plasmid using oligonucleotides 1 + 11. Block substitutions of box A′ (nucleotides [nt] 8–12; UACUU to AUGAA), box D (nt 210–215; GGCUGA to CCGACU), and box D point mutants (see below) were generated by direct PCR amplification from wild-type U3 plasmid using the following primer pairs: box A′, 2 + 11; box D, 1 + 17; box D C212B, 1 + 18; box D U213G, 1 + 19; box D G214B, 1 + 20; and box D A215U, 1 + 21. The

subfragment of U3 comprised of the 3′ domain (nucleotides 75–220) was generated using primers 8 + 11. The U3 subfragment containing box C′ and box D (nucleotides 75–104/GCUU tetraloop/198–220) was generated using primers 8 + 22 and the following oligonucleotide template: TAATACGACTCACTATAGGGAAGACTACCACGAGGAAGAGCGTCAGTGTTCTCTCCTTCGGGAGAGAACACAGGCTGAGTGGT. In all other cases, the wild-type U3 gene was used as the PCR template. The point mutation U213G in the subfragment containing box C′ and box D was produced using primers 8 + 19 and the unmutated subfragment as the template in a PCR reaction. All U3 mutant DNA fragments were subcloned into the *Sma*I site of pUC19 and confirmed by sequencing.

Generation of Internal U3 Mutations. Mutations of the internal elements, box A (nt 17–28; GGAUCAUUUCUA to CCUAGUAAAGAU), hinge (nt 63–74; CUGAACUCACAA to GACUUGAGUGUU), box C′ (nt 80–87; GAGGAAGA to CUCCUUCU), box B (nt 106–114; GAGCGUGAA to CUCGCACUU), and box C (nt 157–165; UGAUGAACG to ACUACUUGC) of the U3 gene were produced by recombinant PCR (Higuchi, 1990). Block substitution of box A was accomplished by combining the products of two independent PCR reactions (from the wild-type U3 gene by the use of primer pairs 9 + 12 and 3 + 23) and PCR amplification using the outermost primers (9 + 23). The first series of PCR reactions to produce block substitutions in each of the following elements used the indicated primer pairs: the hinge region, primers 9 + 13 and 4 + 23; box C′, primers 9 + 14 and 5 + 23; box B, primers 9 + 15 and 6 + 23; and box C, primers 9 + 16 and 7 + 23. For each of these PCR reactions, the wild-type U3 plasmid was used as the template. Primers 9 + 23 were used to amplify from the combined products in the second step. All U3 mutant DNA fragments were digested with *Eco*RI and *Hin*dIII (sites incorporated into primers 9 + 23), subcloned into the *Eco*RI/*Hin*dIII site of pGEM3Zf−, and sequenced.

U14 Constructs. pBT20, a plasmid containing the mouse U14.5 gene (Shanab and Maxwell, 1992), was used as a template to generate wild-type and variant U14 RNAs. Mutagenesis of the box C, box D, and 3′ terminal stem sequences of U14 genes was performed by PCR amplification using mutagenic primers. Base substitutions introduced were ACTACT (wild-type is TGATGA) in the position of box C and TCTAGA (wild-type is GTCTGA) for box D. To disrupt terminal stem formation in the U14 RNA, we substituted the 3′ terminal nucleotides of the U14 gene (GCGAAT) with CGCTTA.

In Vitro RNA Synthesis

PCR products (100 ng) or linearized plasmids (1 μg) were used as templates for in vitro transcription. Wild-type and mutant U3 and U14 RNAs were transcribed from PCR-derived DNA fragments. The following additional RNAs were prepared by in vitro transcription from plasmids as described previously: *Xenopus* U8 wild type and a box C mutant (Peculis and Steitz, 1994); *Xenopus* U8 box D mutant and *Xenopus* U3 terminal stem mutant (Terns *et al.*, 1995); *Xenopus* U1, U1Sm−, and U6 (Terns *et al.*, 1993); and DHFR mRNA, 5S rRNA, and $tRNA_i^{Met}$ (Jarmolowski *et al.*, 1994).

The reaction conditions used to generate capped, ^{32}P-labeled RNAs by SP6 or T7 RNA polymerase were essentially as described previously (Terns *et al.*, 1993). To generate fluorescein-labeled RNAs, we used an equal mixture (250 μM each) of UTP and fluorescein-12-UTP (Boehringer Mannheim, Indianapolis, IN). The RNAs were quantitated by measuring incorporation of trace amounts of [^{32}P]GTP (ICN Pharmaceuticals, Costa Mesa, CA). RNA transcripts were either gel purified or purified by two successive isopropanol precipitations, suspended in distilled water, and stored at −20°C wrapped in aluminum foil to avoid exposure to light. The integrity and size of the RNA were assessed by electrophoresis on 8% denaturing polyacrylamide gels and ethidium bromide staining.

Injection of RNAs into Xenopus *Oocytes*

The method by which we microinject and micromanipulate oocytes has been described recently in detail (Terns and Goldfarb, 1998). In brief, stage V and VI *Xenopus laevis* oocytes were separated from each other and from the surrounding follicle cells by treatment with 2 mg/ml collagenase for 60–90 min. The collagenase-treated cells were washed thoroughly in MBSH buffer before microinjection. Injections were performed using the model PL1–100 picoinjector microinjector (Medical Systems Corporation, Greenvale, NY) and a glass needle with a tip of 10 μm inner diameter. The RNA sample was dried using a Savant (Farmingdale, NY) speed vacuum unit and resuspended in a solution of filter-sterilized blue dextran in water (20 mg/ml; 2×10^6 molecular weight; Sigma, St. Louis, MO). Oocyte nuclei were injected with 10 nl of solution containing 5 fmol of fluorescein-labeled RNA. In control experiments (e.g., see Figure 2B), 132 fmol of fluorescein-12-UTP was injected. The oocytes were maintained in MBSH buffer at 18°C. Nuclear injections were monitored by determining whether nuclei gained a blue appearance as the result of the injected blue dextrans (Jarmolowski *et al.*, 1994).

For the analysis of RNA stability, nuclear retention, fibrillarin binding, and 5′ cap hypermethylation, oocytes were each injected with 10 nl of solution containing ~1 fmol each of [^{32}P]GTP-labeled U1, U8, and U6 RNAs and either [^{32}P]GTP-labeled or fluorescein-12-UTP + [^{32}P]GTP–labeled U3 RNA. After injection, the oocytes were incubated at 18°C for 8 h before they were manually dissected in J-buffer (Birkenmeier *et al.*, 1978) into nuclear and cytoplasmic fractions. The nucleocytoplasmic distribution was determined by isolating the RNAs from two to four dissected nuclear and cytoplasmic fractions by proteinase K digestion, phenol extraction, and ethanol precipitation. The RNA equivalent of one nucleus or one cytoplasm was resolved on an 8% denaturing polyacrylamide gel and detected by autoradiography with an intensifying screen.

Immunoprecipitations

Antibodies used in these experiments included polyclonal antibodies directed against either the m^7G (Munns *et al.*, 1982) or $m^{2,2,7}G$ cap (Bringmann *et al.*, 1983) and a monoclonal antibody (72B9) against fibrillarin (Reimer *et al.*, 1987). Antibodies were coupled to preswollen protein A–Sepharose CL-4C beads (Sigma) by end-over-end rotation in 0.5 ml of Ipp-500 buffer (500 mM NaCl, 10 mM Tris-HCl, pH 8.0, 0.1% [vol/vol] NP-40, and 0.1% [wt/vol] sodium azide) at 4°C for 12–16 h. Immunoprecipitations with cap-specific antibodies were performed with purified nuclear RNAs (1 nuclear equivalent/reaction) in Net-2 buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, and 0.05% NP-40). Immunoprecipitations with fibrillarin antibodies were performed with nuclear extracts (5 nuclear equivalents/reaction) in Net-100 buffer (100 mM NaCl, 50 mM Tris-HCl, pH 7.5, and 0.05% NP-40). In both cases, immunoprecipitations were performed at 4°C for 12–16 h, supernatants were collected, and the beads were washed extensively. RNA from both supernatants and pellets were obtained by digestion with proteinase K, phenol extraction, and ethanol precipitation, and the purified RNAs were analyzed on 8% denaturing polyacrylamide gel by autoradiography.

Nuclear Spreads, Immunofluorescence, and Microscopy

After injection of fluorescently labeled RNAs into *Xenopus* oocytes, nuclear spreads were prepared from dissected nuclei as described (Gall *et al.*, 1991; Wu *et al.*, 1996). After centrifugation, the spreads were fixed in 2% paraformaldehyde in 1× PBS, pH 7.2, for 1 h. After washing with 1× PBS, mounts were made using 50% glycerol (in 1× PBS) containing 1 mg/ml phenylenediamine, pH 9, and were stored at −20°C.

Indirect immunofluorescence was performed on the nuclear spreads after fixation essentially as described (Wu and Gall, 1997). Briefly, slides were blocked using 10% horse serum (Hyclone, Logan, UT) in 1× PBS for 15 min at 37°C. After thorough washing with

1× PBS, the sample area was incubated with antibody (mAb 17C12) directed against fibrillarin at 1:1000 dilution in 1× PBS (Hultman *et al.*, 1994) or with antibody (mAb H1) directed against the *Xenopus* p80 coilin homologue (Tuma *et al.*, 1993) at 1:10 dilution in 1× PBS for 30 min at 37°C. The excess primary antibodies were washed away with 1× PBS, and the secondary antibody (Texas Red–conjugated anti-mouse antibody at 1:150 dilution in 1× PBS) was added and incubated at 37°C for 30 min. Excess secondary antibody was washed away with 1× PBS, and mounts were made as described above.

A Zeiss (Thornwood, NY) Axiovert S 100 inverted fluorescence microscope equipped with differential interference contrast optics was used for all observations. All images were acquired using a cooled charge-coupled device camera (Quantix-Photometrics) and IP Lab Spectrum software.

RESULTS

U3 RNA Synthesized with Fluorescein-12-UTP Retains Its Biological Properties

U3 RNA intranuclear transport was studied by examining the localization of fluorescein-labeled RNAs in nuclear spread preparations by fluorescence microscopy. As a first step, we compared the nuclear retention, 5′ cap hypermethylation, and fibrillarin binding of fluorescein-labeled U3 snoRNA with that of control RNA to verify that the fluorescein label did not affect known biological properties of the RNA.

U3 RNA injected into oocyte nuclei is stable and retained in the nucleus (Terns and Dahlberg, 1994; Terns *et al.*, 1995). To ensure that incorporation of fluorescein-12-UTP into U3 RNA would not affect the stability or nuclear retention of the RNA, we injected RNA transcribed in vitro with fluorescein-12-UTP (see MATERIALS AND METHODS) into the nuclei of *Xenopus* oocytes. Eight hours after injection, RNAs present in the nuclear and cytoplasmic fractions were analyzed by PAGE. Coinjected control RNAs included a U1 small nuclear RNA (snRNA) mutant (U1sm$^-$), U6 snRNA, and U8 snoRNA (Figure 1A). As expected, U1sm$^-$ that lacks the Sm protein-binding site was exported to the cytoplasm (and not reimported to the nucleus [Mattaj and De Robertis, 1985; Terns *et al.*, 1993]). U6, an snRNA that is not exported to the cytoplasm (Hamm and Mattaj, 1989; Terns *et al.*, 1993), and U8 snoRNA were retained within the nucleus (Figure 1A) (Terns *et al.*, 1995). These controls ensure that, in each oocyte, RNAs were being exported (U1) and retained (U6 and U8) appropriately and that the nuclear injections and dissections were precise. We found that U3 snoRNA labeled with fluorescein-12-UTP (Figure 1A, lanes 2 and 3) is stable and retained within the nucleus similarly to nonfluorescein-labeled U3 RNA (lanes 5 and 6).

We also tested for effects of fluorescein labeling on the ability of U3 RNA to undergo cap hypermethylation. U3 snoRNA is normally synthesized with a 5′ m^{7}G cap that is hypermethylated to an m^{2,2,7}G cap within the nucleus (Terns *et al.*, 1995). Cap hypermethylation was assayed by immunoprecipitation of nuclear RNAs with antibodies that specifically recognize either the m^{7}G (Munns *et al.*, 1982) or m^{2,2,7}G cap structures (Bringmann *et al.*, 1983). The antibodies immunoprecipitated the fluorescein-12-UTP–labeled U3 and the nonfluorescein-labeled U3 to the same extent (Figure 1B, compare lanes 2 and 3 with lanes 7 and 8), indicating that fluorescein labeling does not interfere with cap hypermethylation.

Fibrillarin is a nucleolar protein that binds box C/D family snoRNAs including U3 (Baserga *et al.*, 1991). We tested whether fluorescein-labeled U3 RNA associated with fibrillarin by assaying the ability of anti-fibrillarin antibodies to coimmunoprecipitate the RNA after injection of the RNA into oocyte nuclei. Monoclonal antibody 72B9 against fibrillarin (Reimer *et al.*, 1987) immunoprecipitates U3 RNA labeled with fluorescein as well as nonfluorescein-labeled U3 (Figure 1C, compare lanes 2 and 3 with lanes 5 and 6). Thus, fluorescein-labeled U3 RNA interacts with fibrillarin.

Our results clearly show that labeling U3 RNA with fluorescein by transcription with fluorescein-12-UTP does not significantly alter any of the biological properties of the RNA that we examined, which included stability, nuclear retention, hypermethylation, and fibrillarin binding.

snoRNAs but Not Other Classes of RNA Localize to Nucleoli

U3 snoRNA has been shown to be involved in 18S rRNA processing within the nucleolus (Kass *et al.*, 1990; Savino and Gerbi, 1990; Hughes and Ares, 1991; Beltrame and Tollervey, 1995). Amplified nucleoli and other nuclear structures including chromosomes, coiled bodies, and snurposomes can be observed and distinguished by morphology and molecular markers in oocyte nuclear spread preparations (Gall *et al.*, 1991; Wu *et al.*, 1993). For example, nucleoli can be identified by immunostaining using antibodies against nucleolar proteins such as fibrillarin (Shah *et al.*, 1996). Fibrillarin is enriched in the dense fibrillar regions of nucleoli that can be distinguished as internal substructures by differential interference contrast microscopy (Figure 2A). Coiled bodies, which are highly conserved intranuclear organelles of unclear function (Gall *et al.*, 1995; Matera, 1998), are distinguished by their size and morphology (Gall and Callan, 1989) and the presence of p80 coilin (Andrade *et al.*, 1991). Fluorescein-labeled U3 RNA colocalized with endogenous fibrillarin within the dense fibrillar region of nucleoli and was not detectable in coiled bodies 1 h after injection into *Xenopus* oocytes (Figure 2A).

Other box C/D snoRNAs, namely, U8 and U14, also localized to the multiple amplified nucleoli after injection into oocyte nuclei (Figure 2B). As expected, a spliceosomal snRNA (U1 snRNA), an mRNA (dihydrofolate reductase

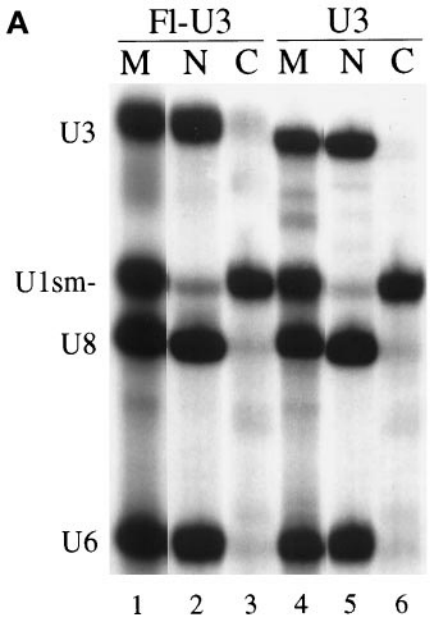
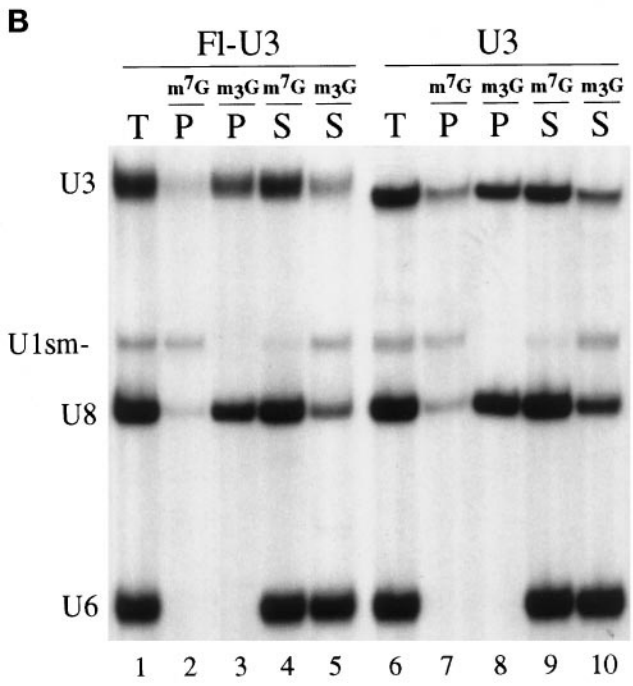
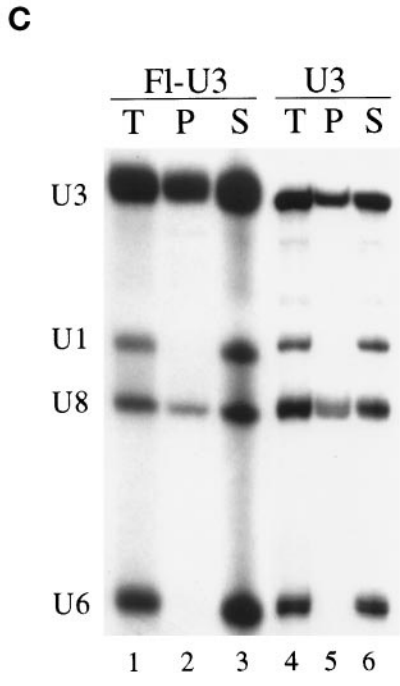


Figure 1. Nuclear retention, cap hypermethylation, and fibrillarin binding of fluorescein-labeled U3 RNA in *Xenopus* oocytes. (A) Nucleocytoplasmic distribution of fluorescein-labeled U3 snoRNA (Fl-U3) or nonfluorescein-labeled U3 snoRNA (U3) and U1sm− snRNA, U8 snoRNA, and U6 snRNA after their injection into oocyte nuclei. ^{32}P-labeled, m^{7}G-capped Fl-U3, U3, U1sm−, U8, and U6 were synthesized in vitro, and mixtures of the RNAs were injected into nuclei of *Xenopus* oocytes. After 8 h of incubation at 18°C, the labeled RNAs present in the nuclear (N) and cytoplasmic (C) fractions of the oocytes were isolated and analyzed by electrophoresis in a denaturing polyacrylamide gel. Lanes 1 and 4 (M) show the RNAs before injection. Lanes 2 and 3 show the distribution of Fl-U3, and lanes 5 and 6 show that of nonfluorescein-labeled U3. (B) Identification of the 5′ cap structure (m^{7}G or m^{2,2,7}G) by immunoprecipitation. The RNAs present in the nucleus 4 h after injection were precipitated using anti-m^{7}G (m^{7}G) (Munns *et al.*, 1982) or anti-m^{2,2,7}G (m$_3$G) (Bringmann *et al.*, 1983) cap antibodies as indicated. The RNAs present in the total sample (T), precipitate (P), and supernatant (S) fractions were separated by gel electrophoresis as described in A. Lanes 1–5 show the precipitation profile of Fl-U3, and lanes 6–10 show that of nonfluorescein-labeled U3. (C) Determination of fibrillarin binding to fluorescein-labeled U3. Immunoprecipitations were performed on nuclear extracts prepared 4 h after injection of RNAs using anti-fibrillarin antibodies (72B9) (Reimer *et al.*, 1987). RNAs present in the precipitate (P), 20% of the total sample (T), and 20% of the supernatant (S) were separated by gel electrophoresis as described in A. Lanes 1–3 show the precipitation profile of Fl-U3, and lanes 4–6 show that of nonfluorescein-labeled U3. Note that the gel electrophoretic mobility of the fluorescein-labeled U3 RNA is reduced relative to that of the unlabeled U3 in A–C.





mRNA), and a tRNA (tRNA$_i^{Met}$) did not localize to nucleoli after injection (Figure 2B). Furthermore, injection of fluorescein-12-UTP alone (at molar amounts that exceed those incorporated into the injected RNAs, see MATERIALS AND METHODS) did not result in significant fluorescence in nucleoli (Figure 2B). Thus, fluorescein-labeled box C/D snoRNAs (including U3, U8, and U14 RNA) specifically localize to the dense fibrillar region of nucleoli within the nucleus of *Xenopus* oocytes.

Nucleolar Localization of U3 snoRNA Is Temperature Dependent

Energy-dependent, active transport processes in the cell are generally inhibited at low temperatures. To

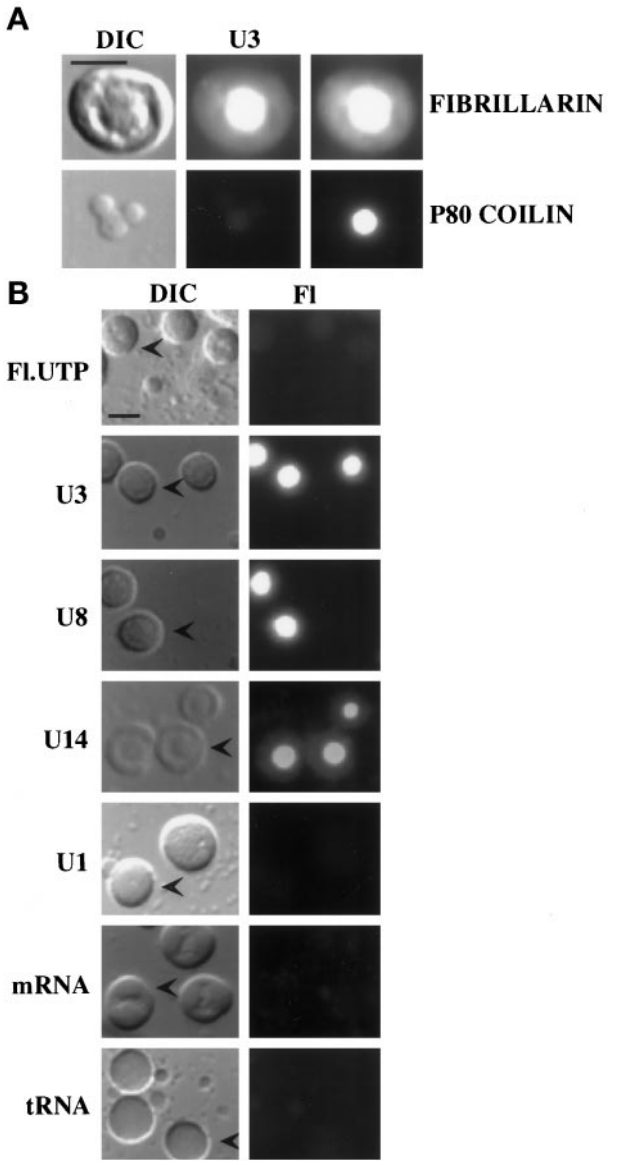


Figure 2. Microinjected snoRNAs localize to the fibrillar regions of nucleoli. (A) U3 colocalizes with endogenous fibrillarin. Fluorescein-labeled U3 was injected into oocytes, and nuclear spreads were analyzed 1 h after injection. Top, a single, representative nucleolus is shown. Bottom, a single, coiled body with two snurposomes attached to its surface is shown. Left, differential interference contrast (DIC) images of the structures are shown. Right, indirect immunofluorescence using the anti-fibrillarin antibody 17C12 (Hultman *et al.*, 1994) or antibody H1 (Tuma *et al.*, 1993) directed against the coiled body marker protein p80 coilin confirm the identity of the structures as nucleoli and coiled bodies, respectively. Middle, the fluorescein signal from U3 RNA (U3) colocalized with fibrillarin in nucleoli but not p80 coilin in the coiled body. Bar, 10 μm. (B) Selective nucleolar localization of U3, U8, and U14 snoRNAs. RNAs (U3, U8, and U14 snoRNAs; U1 spliceosomal snRNA; DHFR mRNA; and $tRNA_i^{Met}$) were fluorescently labeled by in vitro transcription in the presence of fluorescein-12-UTP (see MATERIALS AND METHODS), and each RNA was injected into *Xenopus* oocytes. Fl.UTP indicates injection of fluorescein-12-UTP alone. Nuclear spreads were prepared 1 h after injection (except

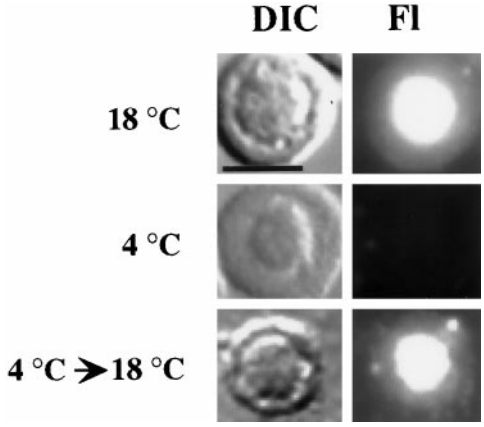


Figure 3. Effect of temperature reduction on the nucleolar localization of U3 snoRNA. Fluorescently labeled, in vitro–synthesized U3 RNA was injected into nuclei of oocytes that were maintained either at 18°C (top) or at 4°C (middle) for 1 h before nuclear spread preparations were analyzed. In the temperature-shift experiment (bottom), the oocytes were shifted to 18°C for 1 h after being maintained at 4°C for 1 h. Each DIC image shows a single nucleolus, and the RNA signal is shown in the corresponding Fl image. Bar, 10 μm.

determine whether the nucleolar localization of U3 is a temperature-dependent process, we examined localization at reduced temperatures. Fluorescein-labeled U3 RNA was injected into the nuclei of oocytes that had been preincubated at 4°C for 1 h. The injections were performed at 4°C, and after injection the oocytes were maintained at 4°C for 1 h. U3 RNA was not localized to the nucleolus in oocytes that were maintained at 4°C (Figure 3). The inhibitory effect of temperature reduction on nucleolar localization was reversible because U3 RNA localized to the nucleolus in injected oocytes maintained at 4°C for 1 h and subsequently incubated at 18°C for 1 h (Figure 3) but not in oocytes maintained at 4°C for 2 h (our unpublished results). Thus, localization of U3 RNA to nucleoli is a temperature-dependent process.

Box C′ and Box D Are Required for Localization of U3 RNA to the Nucleolus

To identify *cis*-acting signals essential for nucleolar targeting of U3 snoRNA, we analyzed the localization of multiple sequence variants of U3. We began our analysis with block substitutions of the phylogenetically conserved sequence elements in U3: boxes A, A′,

Figure 2 (cont). that of tRNA that was analyzed after 15 min because this RNA is rapidly exported [Jarmolowski *et al.*, 1994] and that of U14 that was analyzed after 4 h), and the RNAs were observed by fluorescence microscopy. Both DIC and fluorescence (Fl) images are shown for each sample. The arrowheads in the DIC images point to representative nucleoli. Bar, 10 μm.

B, C, C′, and D (Figure 4A). A series of U3 mutants was generated in which each nucleotide of a given box element was replaced by its complement (see MATERIALS AND METHODS). In addition, similar substitutions were introduced into the single-stranded hinge region of the RNA. Although the primary sequence of the hinge is not conserved, there is evidence that it is a functionally important region of the RNA (Beltrame and Tollervey, 1995; Mereau *et al.*, 1997). In addition, to determine whether the m^7G cap (or its hypermethylated form) is required for nucleolar localization, we replaced the m^7G cap of U3 RNA with a nonphysiological, nontrimethylatable ApppG cap.

Among the eight variants analyzed, six were found in nucleoli in patterns similar to that in wild-type U3; substitution of boxes A, A′, B, and C and the hinge region did not prevent the nucleolar localization of U3 (Figure 4B). Likewise, ApppG-capped U3 RNA was also localized to nucleoli, indicating that the m^7G or trimethylated cap is not essential for this process. On the other hand, disruption of box C′ and box D abolished the nucleolar localization of the RNA (Figure 4B) even at times up to 24 h after injection (our unpublished results). All experiments were repeated at least nine times, and nucleolar fluorescence intensity relative to that of wild-type was qualitatively consistent for a given RNA in various experiments. Importantly, the lack of nucleolar fluorescence does not simply reflect the lack of RNA in the nucleus because all of the variant U3 RNAs, including those that did not localize to nucleoli, were found to be stable and retained within the nucleus 1 h after injection (Figure 4C) (Terns *et al.*, 1995). Our results clearly indicate that box C′ and box D are necessary for the nucleolar localization of U3 RNA.

We performed a more detailed mutational analysis on box D, one of the elements found to be essential for nucleolar localization. The core box D element conserved among C/D family snoRNAs consists of CUGA (Xia *et al.*, 1997). We tested the contribution that each of the four conserved nucleotides makes on nucleolar localization by analyzing U3 molecules with point mutations at each position. Substitution of C212 did not disrupt localization of U3 RNA to the nucleolus (Figure 5). However, mutations at U213, G214, and A215 each resulted in a loss of localization similar to that observed when the entire box D sequence was substituted (Figure 5). Thus, the conserved nucleotides UGA (213–215) of box D are each important for localization of U3 to the nucleolus.

A Fragment of U3 Containing Box C′ and Box D Localizes to the Nucleolus

Box C′ and box D are the only conserved sequence elements that we found to be essential for nucleolar localization of U3 (Figure 4B). These sequence elements are present within the 3′ domain of the U3 molecule (nt 75–220; see Figure 4A and MATERIALS AND METHODS). We found that a fragment comprised of the 3′ domain of U3 that is stable and retained within the nucleus localized to the nucleolus (Figure 6 and our unpublished results). Stable subfragments of the 3′ domain were also analyzed. A subfragment comprised of box C′, box D, and flanking stems localized to the nucleolus (Figure 6). A single point mutation within the box D element of this subfragment abolished nucleolar localization (Figure 6). In addition, a subfragment containing box B, box C, and flanking stems does not localize to the nucleolus (our unpublished results). These results suggest that box C′ and box D are sufficient to direct nucleolar localization of U3 RNA.

An Intact 3′ Terminal Stem Is Essential for Nucleolar Localization of U3 and U14

For many box C/D snoRNAs, the 3′ terminal sequences are predicted to be involved in the formation of a short-stem structure that results in positioning the box C and box D sequences adjacent to one another in the predicted secondary structures of the RNAs (Maxwell and Fournier, 1995; Tollervey and Kiss, 1997) (Figure 4A). Phylogenetically conserved 3′ terminal stems have been experimentally demonstrated for both U3 and U14 snoRNAs (Parker and Steitz, 1987; Jeppesen *et al.*, 1988; Shanab and Maxwell, 1991; Baserga *et al.*, 1992; Hartshorne and Agabian, 1994; Mereau *et al.*, 1997). To determine whether the integrity of the 3′ terminal stem is important for nucleolar localization of box C/D snoRNAs, we examined the effect of disrupting the terminal stems of both U3 and U14 snoRNAs on nucleolar localization. Variant U3 and U14 snoRNAs predicted to be unable to form 3′ terminal stems were generated by substituting the nucleotides of one side of the stem with complementary sequences to abolish Watson–Crick base-pairing potential (see MATERIALS AND METHODS). In contrast to their wild-type U3 and U14 counterparts, both variant RNAs failed to localize to nucleoli (Figure 7). The lack of nucleolar signal did not simply reflect a lack of RNA in the nucleus at the time of analysis (Figure 4C). Thus, an intact 3′ terminal stem is essential for the nucleolar localization of both U3 and U14 snoRNAs.

Box C/D snoRNAs Transiently Localize to Coiled Bodies before Nucleoli

At very early time points (15 min after injection of RNA), before significant localization of U3 to nucleoli, the RNA was clearly observed within coiled bodies (Figure 8A). Over a time period of 1 h after injection, we observed an increase in U3 RNA present in nucleoli and a coincident decrease in the RNA observed in

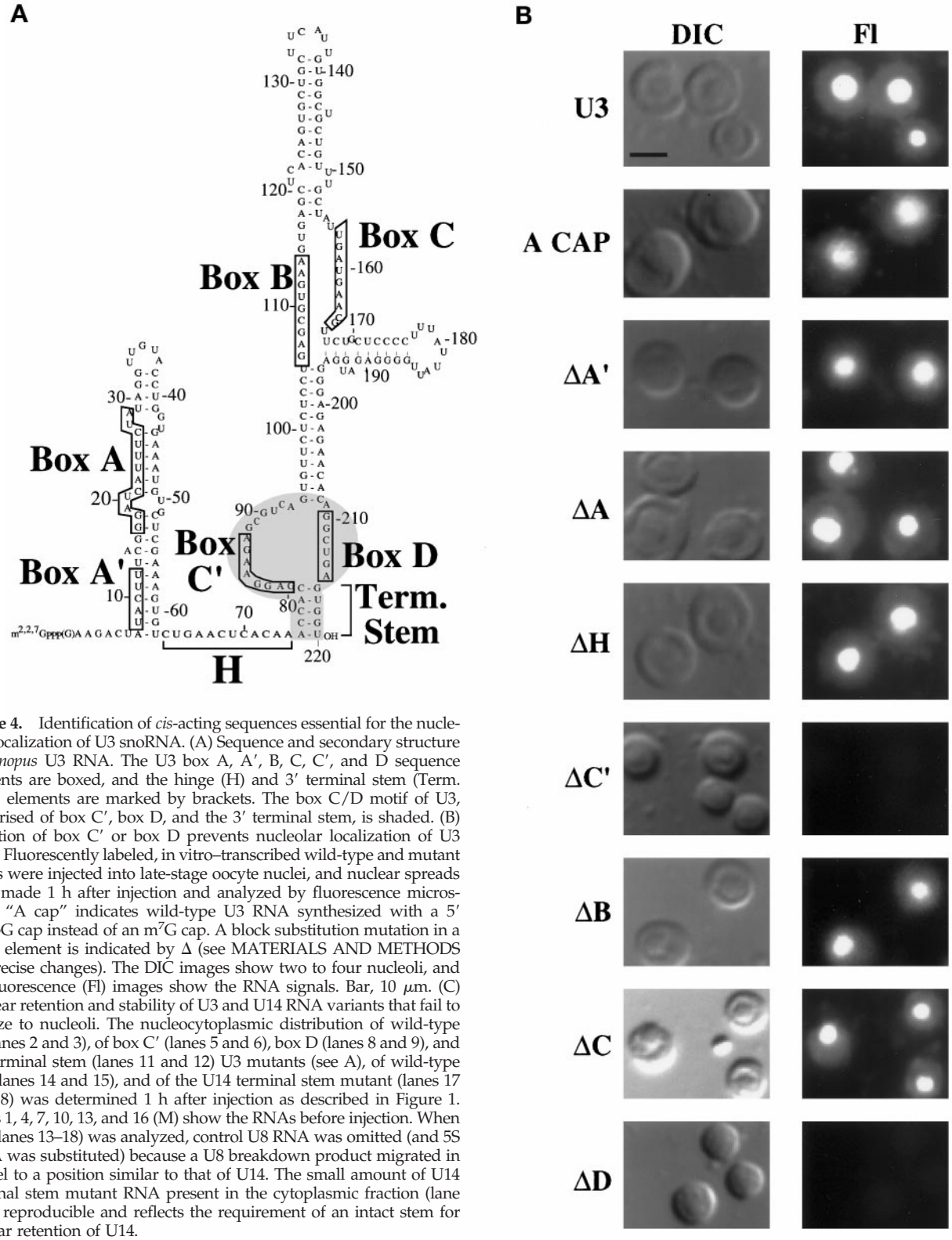


Figure 4. Identification of *cis*-acting sequences essential for the nucleolar localization of U3 snoRNA. (A) Sequence and secondary structure of *Xenopus* U3 RNA. The U3 box A, A′, B, C, C′, and D sequence elements are boxed, and the hinge (H) and 3′ terminal stem (Term. Stem) elements are marked by brackets. The box C/D motif of U3, comprised of box C′, box D, and the 3′ terminal stem, is shaded. (B) Mutation of box C′ or box D prevents nucleolar localization of U3 RNA. Fluorescently labeled, in vitro–transcribed wild-type and mutant RNAs were injected into late-stage oocyte nuclei, and nuclear spreads were made 1 h after injection and analyzed by fluorescence microscopy. "A cap" indicates wild-type U3 RNA synthesized with a 5′ ApppG cap instead of an m^7G cap. A block substitution mutation in a given element is indicated by Δ (see MATERIALS AND METHODS for precise changes). The DIC images show two to four nucleoli, and the fluorescence (Fl) images show the RNA signals. Bar, 10 μm. (C) Nuclear retention and stability of U3 and U14 RNA variants that fail to localize to nucleoli. The nucleocytoplasmic distribution of wild-type U3 (lanes 2 and 3), of box C′ (lanes 5 and 6), box D (lanes 8 and 9), and the terminal stem (lanes 11 and 12) U3 mutants (see A), of wild-type U14 (lanes 14 and 15), and of the U14 terminal stem mutant (lanes 17 and 18) was determined 1 h after injection as described in Figure 1. Lanes 1, 4, 7, 10, 13, and 16 (M) show the RNAs before injection. When U14 (lanes 13–18) was analyzed, control U8 RNA was omitted (and 5S rRNA was substituted) because a U8 breakdown product migrated in the gel to a position similar to that of U14. The small amount of U14 terminal stem mutant RNA present in the cytoplasmic fraction (lane 18) is reproducible and reflects the requirement of an intact stem for nuclear retention of U14.

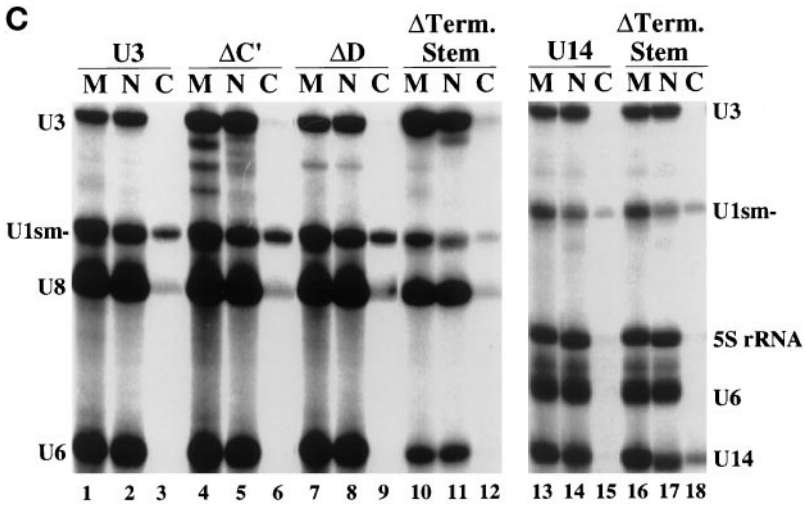


Figure 4 (facing page).

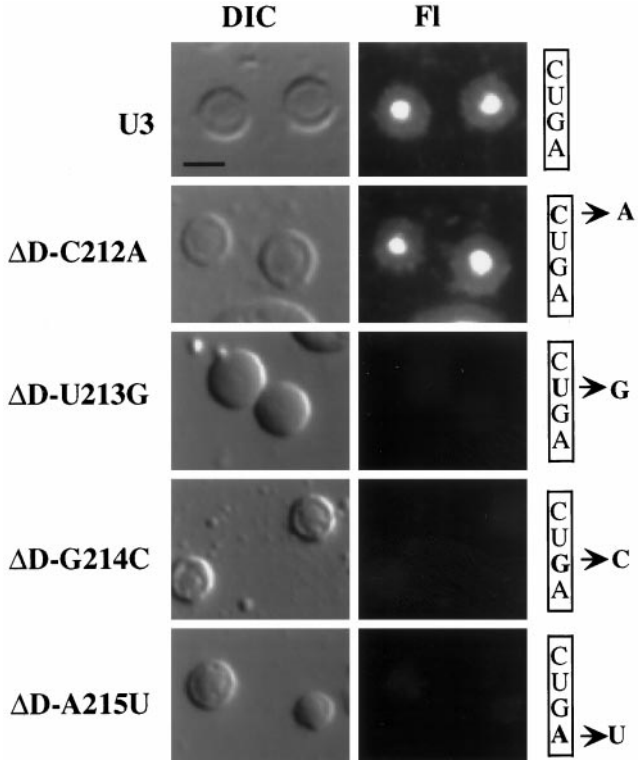


Figure 5. Effect of box D point mutations on the nucleolar localization of U3 snoRNA. Fluorescently labeled wild-type (U3) and variant U3 RNAs with single-nucleotide changes in the box D element (ΔD-C212B, -U213G, -G214B, and -A215U) were injected into oocytes, and nuclear spreads were made 1 h after injection. The particular nucleotides in box D (CUGA) that were altered are indicated on the right. The DIC images show two to three nucleoli, and the RNA signal is shown in the Fl images. Bar, 10 μm.

coiled bodies (Figure 8A). By 1 h after injection, U3 was observed predominantly in nucleoli (Figure 8A). Although U3 RNA remained in nucleoli for at least 24 h (our unpublished results), we only observed U3 in coiled bodies at early times after injection. Injection of fluorescein-12-UTP (as in Figure 2B) did not result in significant fluorescence in coiled bodies (our unpublished results). Our results suggest that box C/D snoRNAs transiently localize to coiled bodies before their localization to nucleoli.

Strikingly, U3 mutants that did not localize to nucleoli accumulated in coiled bodies (Figure 8B). U3 box C′, box D, and 3′ terminal stem mutants were not observed in nucleoli at any time tested. On the other hand, we observed these mutants within coiled bodies for at least 4 h after injection (Figure 8B), well beyond the 1 h time point when very little wild-type RNA remains in coiled bodies. The signal observed in coiled bodies 1 h after injection of other U3 mutants (boxes A, A′, B, and C and hinge) was similar to that of wild-type U3 RNA (our unpublished results). Furthermore, the subfragment of U3 RNA containing box B and box C, which does not localize to nucleoli, was found in coiled bodies for up to 4 h (our unpublished results). Thus, the snoRNA variants that lack intact box C/D motifs remained in coiled bodies longer than did wild-type RNAs. The box C/D motif (i.e., U3 box C′, box D, and the 3′ terminal stem; Figure 4A, shaded area) is essential for the localization of snoRNAs to nucleoli but is not required for localization to coiled bodies.

The specificity of the association of the RNAs with coiled bodies was supported by the localization observed with additional RNAs including a box H/ACA family snoRNA, U65 (Ganot *et al.*, 1997), tRNA, and a spliceosomal snRNA, U1. Although U65 readily localized to nucleoli as expected, we did not detect U65 or

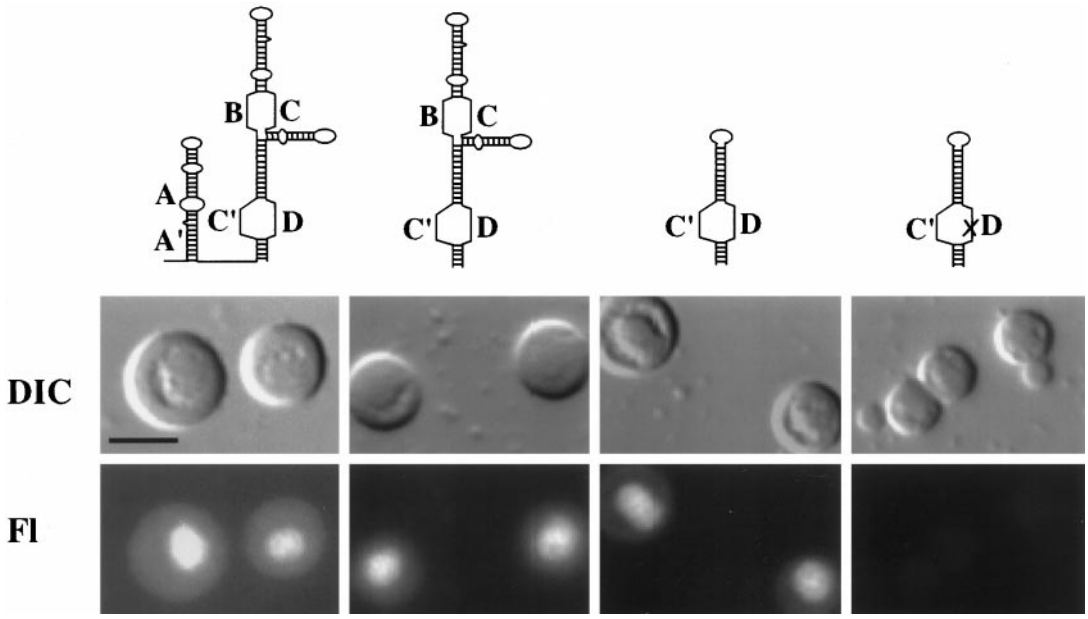


Figure 6. Analysis of the ability of stable subfragments of U3 snoRNA to localize to nucleoli. Fluorescently labeled, m^7G-capped RNAs were injected into oocyte nuclei, and spreads were made 1 h after injection and examined by microscopy. Top, the diagrams represent the RNAs injected (for sequences, see MATERIALS AND METHODS). Left, wild-type U3 RNA is shown. Middle, a U3 fragment lacking the 5′ domain and hinge (second from left) localizes to nucleoli, as does a U3 subfragment containing box C′ and box D sequences and adjacent stems (second from right). Right, Nucleolar localization of this subfragment was abolished by a single point mutation (U213G; represented as an X) in the box D element of the RNA. Bar, 10 μm.

tRNA in coiled bodies at any time point examined (Figure 8B and our unpublished results). The lack of association of U65 with coiled bodies is intriguing because U65 snoRNA-associated proteins including homologues of Cbf5p (Meier and Blobel, 1994) and GAR-1 (our unpublished results) have been observed in coiled bodies. In contrast, U1 RNA localized to coiled bodies for several hours but did not localize to nucleoli (weak localization of U1 to snurposomes [Wu *et al.*, 1993] was observed at late time points [our unpublished results]). These controls clearly indicate that coiled bodies are not a simple default destination for injected RNAs.

To examine the association of other box C/D snoRNAs with coiled bodies, we analyzed the localization of U8 and U14. As was the case for U3, a transient localization to coiled bodies was observed with U8 and U14 snoRNAs (Figure 9). Moreover, replacement of box C or box D sequences prevented localization of U8 to nucleoli, and the mutants were detained in coiled bodies longer than were wild-type U8 (Figure 9). The severe instability of box C and box D mutants of U14 RNA (even when artificially capped with m^7GpppG or ApppG dinucleotides), coupled with the failure of these mutants to be retained in the nucleus, prevented meaningful analysis of these RNAs (our unpublished results). However, significant levels of the U14 terminal stem mutant are present in the nucleus 1 h after injection (Figure 4C), and although the RNA does not localize to nucleoli, it does localize to coiled bodies and remains in coiled bodies longer than does wild-type U14 RNA (Figure 9). Taken together, our results indicate that box C/D snoRNAs transiently localize to coiled bodies before nucleoli and that the box C/D motif is not necessary for the localization of RNAs to coiled bodies but is required for subsequent localization to nucleoli.

DISCUSSION

In eukaryotic cells, mechanisms exist to ensure that RNAs are properly transported from the sites of their synthesis to the sites of their function. In this study, we have identified determinants governing the localization of members of the box C/D class of snoRNAs to the nucleolus. We have found that the nucleolar localization of box C/D snoRNAs requires *cis*-acting sequences and structural features that are common to this class of RNAs. Moreover, the RNAs apparently travel through coiled bodies en route to nucleoli.

The Box C/D Motif Targets Box C/D snoRNAs to the Nucleolus

The members of the box C/D snoRNA family contain two common sequence elements (box C and box D)

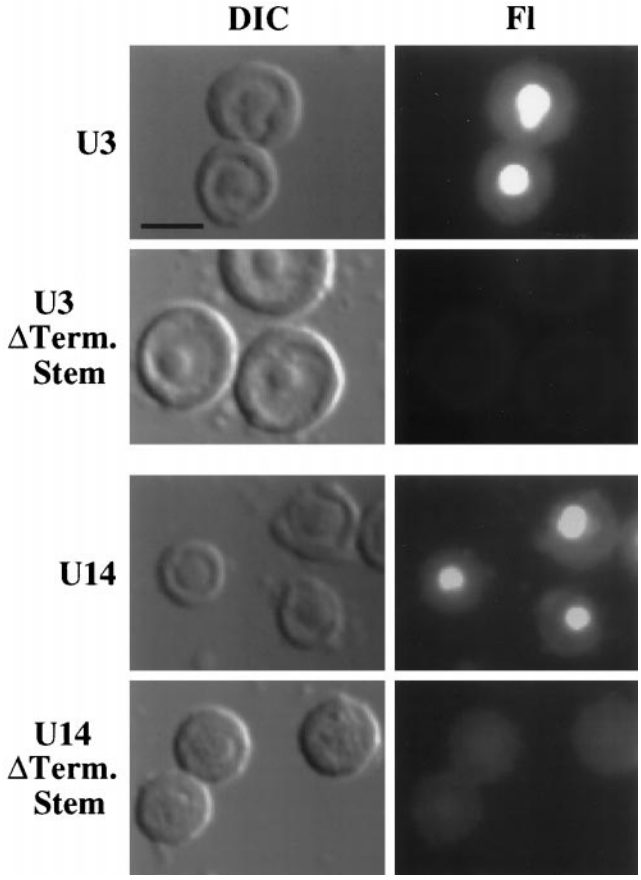


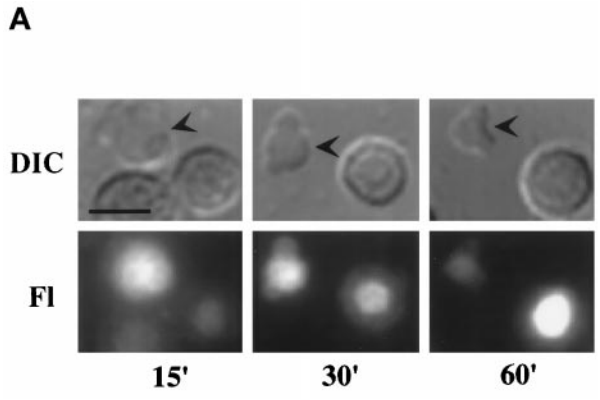
Figure 7. Disruption of the 3′ terminal stem structure prevents nucleolar localization of U3 and U14 RNA. Mutation of the 3′ terminal stem (ΔTerm. Stem) of U3 or U14 is predicted to disrupt stem formation completely (for sequences, see Figure 4A, MATERIALS AND METHODS, and Xia *et al.* [1997]). Fluorescently labeled, m^7G-capped wild type (rows labeled U3 and U14) and stem mutants (rows labeled ΔTerm. Stem) were injected into oocyte nuclei, and spreads were made 1 h after injection and examined by microscopy. The DIC images show two to four nucleoli, and the RNA signal is shown in the Fl images. Bar, 10 μm.

that are brought together in the folded RNAs as a result of the base pairing of complementary sequences flanking the box elements. Our results indicate that the key elements of the box C/D motif (i.e., box C, box D, and a nearby stem; Figure 4A, shaded area) are each essential for localization of box C/D snoRNAs.

U3 snoRNA, the primary focus of this work, contains two conserved elements with homology to box C, termed box C and box C′, and a single box D. In U3 RNA, box C′ exists opposite box D in a conserved stem-loop motif (Figure 4A) (Parker and Steitz, 1987; Jeppesen *et al.*, 1988; Tycowski *et al.*, 1993; Hartshorne and Agabian, 1994; Mereau *et al.*, 1997; Samarsky and Fournier, 1998). Interestingly, box C of U3 is found opposite box B in the predicted secondary structure (Mereau *et al.*, 1997; Samarsky and Fournier, 1998), and this conserved box B/C motif is unique to U3 RNAs. In addition, box C′ (and not box C) of U3 functions in accumulation of the RNA in yeast similar to box C of other C/D snoRNAs (Samarsky and Fournier, 1998). Thus, the box C/D motif of U3 RNA is comprised of U3 box C′, box D, and the flanking stems including the 3′ terminal stem.

Our mutational analysis of U3 showed that substitution of sequences in box C′, box D, or the 3′ terminal stem but not of other elements (boxes A, A′, B, and C or hinge) disrupted localization of the RNA to the nucleolus (Figures 4B and 7). These variant RNAs were not observed in nucleoli even at times up to 24 h after injection, indicating that these mutations did not simply reduce the rate of localization to nucleoli but rather rendered the RNAs defective in their ability to localize to nucleoli (our unpublished results). A recent study of the localization of fluorescently labeled U3 RNA in *Xenopus* oocyte nuclear spreads concludes that box C (not box C′) and box D are the elements critical for nucleolar localization of U3 (Lange *et al.*, 1998c). Furthermore, box C′ and the 3′ terminal stem were found not to be necessary for nucleolar localization (although it was noted that variable results were obtained with box C′ mutants in that study [Lange *et al.*, 1998c]). It is difficult to reconcile the striking differences between our results and those of Lange *et al.* (1998c) given the similarity of the studies. In yeast, mutation of U3 box C′ results in the loss of RNA stability (Mereau *et al.*, 1997; Samarsky and Fournier, 1998), and Lange *et al.* (1998c) postulated that the reduced localization that they occasionally observed with *Xenopus* box C′ mutants may reflect the lack of stability. However, we have consistently observed that essentially all of the *Xenopus* U3 box C′ mutant RNA injected into *Xenopus* oocytes is present at the time of analysis (Figure 4C) and that significant amounts of the RNA remain for several hours (with a half-life of ~6 h) but that the RNA is not observed in nucleoli at times up to 24 h after injection (Figure 4B and our unpublished results). The lack of nucleolar localization of the variant U3 RNAs in our experiments clearly is not simply a reflection of degradation of the RNAs.

Importantly, our results indicate that box C (U3 box C′) and box D function together as parts of the box C/D motif to direct nucleolar localization of this class of RNAs. Neither element appears to be capable of supporting nucleolar localization in the absence of the other, because substitution of either element (block substitution of box C′ or block substitution or point mutation of box D) resulted in a loss of localization (Figures 4B and 5). Similarly we found that disruption of either box C or box D prevents localization of U8 to nucleoli (Figure 9). Furthermore, disruption of the 3′ terminal stem of U3 RNA, which likely maintains the proximity of box C′ and box D, abolished the nucleolar localization of the RNA (Figure 7). Our finding that



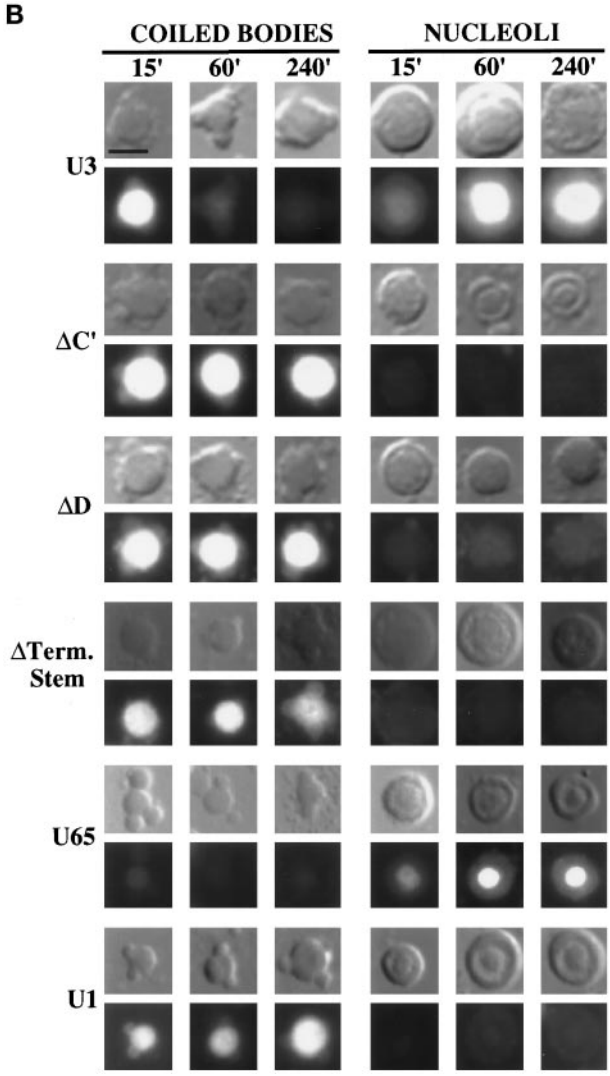


Figure 8. Dynamic association of U3 RNAs with coiled bodies. (A) The localization of U3 RNA to the coiled bodies is transient. Fluorescently labeled wild-type U3 RNA was injected into oocyte nuclei, and nuclear spreads were made at the times indicated in minutes. The arrowheads in the DIC images indicate coiled bodies. The other large structure(s) found in each image is a nucleolus. (B) U3 mutants that fail to localize to nucleoli (right) are retained for longer periods of time in coiled bodies (left). Wild-type U3, as well as box C′ (ΔC′), box D (ΔD), and the terminal stem (ΔTerm. Stem) mutants, was injected into oocyte nuclei, and nuclear spreads were made at the times indicated. The localization of U65 and U1 control RNAs revealed that U65 (a snoRNA of the box H/ACA class) localizes to nucleoli but not coiled bodies and that U1 (a spliceosomal snRNA) localizes to coiled bodies but not nucleoli over the time course analyzed. Bar, 10 μm.

an intact 3′ terminal stem was also essential for the nucleolar localization of U14 indicates that the presence of a stem that brings together the box C and box D elements in the folded RNA molecules is likely of general importance for the nucleoloar localization of box C/D snoRNAs. Lange and colleagues report the fundamentally different observation that neither box C′ nor the terminal stem of the box C/D motif of U3 (Lange *et al.*, 1998c) nor the terminal stem of U14 (Lange *et al.*, 1998b) are essential for nucleolar localization. In addition, although they also found that box C and box D are essential for nucleolar localization of U8 (Figure 9) (Lange *et al.*, 1998b), Lange *et al.* (1998b,c) have suggested that the spatial relationship of U8 box C and box D sequence elements is not important. The secondary structure of U8 has not been experimentally determined, and it is possible and indeed might be predicted based on an alternate proposed structure model (Watkins *et al.*, 1996) that the spatial relationship of box C and box D has not been significantly altered in the variants examined. The box C/D motif and specifically the terminal stem that is thought to maintain the spatial relationship of box C and box D have been demonstrated to be important for the stability of snoRNAs (Terns *et al.*, 1995), snoRNA biogenesis (Baserga *et al.*, 1992; Xia *et al.*, 1997), small nucleolar ribonucleoprotein (snoRNP) assembly (Watkins *et al.*, 1998), and the binding of box C/D–specific proteins (Caffarelli *et al.*, 1998). Our results indicate that the box C/D motif is also essential for the transport of the box C/D family snoRNAs to the nucleolus.

Our findings further indicate that sequences common to box C/D snoRNAs, rather than sequences specific to particular snoRNAs, are responsible for localizing these RNAs to nucleoli. It is conceivable that mutations in sequences other than the box C/D motif have subtle, nonessential effects on the rate or extent of nucleolar localization that were not detected in our assay. However, none of the other variant RNAs tested exhibited obvious diminished nucleolar localization relative to that of wild-type U3 RNA. It is noteworthy that U3 mutations that are predicted to disrupt base pairing of U3 with pre-rRNA (Beltrame and Tollervey, 1995; Hughes, 1996; Mereau *et al.*, 1997) did not prevent the nucleolar localization of the RNA (box A, box A′, and hinge mutants; Figure 4B). Fur-

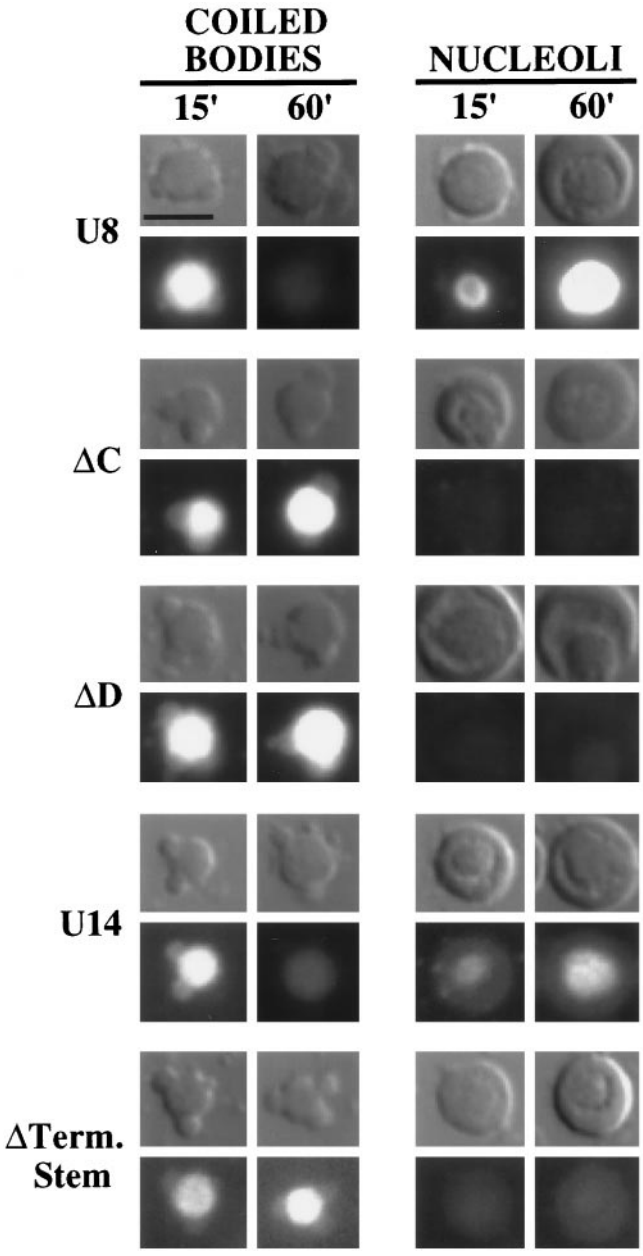


Figure 9. Dynamic association of U8 and U14 RNAs with coiled bodies. Fluorescein-labeled, wild-type, or nucleolar localization-defective variant U8 and U14 RNAs (including U8 box C [ΔC] and box D [ΔD] mutants and the U14 terminal stem mutant [ΔTerm. Stem]) were injected into oocyte nuclei. Nuclear spreads were made at the times indicated in minutes, and the RNA signals were analyzed by fluorescence microscopy. Variant snoRNAs that fail to localize to nucleoli (right) are retained for longer periods of time in coiled bodies (left) than are wild-type RNAs. Bar, 10 μm.

thermore, stable fragments of U3 RNA that lack all three of these known pre-rRNA–binding sequence elements were also targeted to nucleoli (Figure 6). These results indicate that base pairing with rRNA, which is essential for U3 function (Beltrame and Tollervey, 1995; Hughes, 1996; Mereau *et al.*, 1997), is not essential for its localization to nucleoli. Likewise, sequences within two other box C/D snoRNAs (U14 and U8 snoRNA) that exhibit complementarity to pre-rRNA were shown recently to be dispensable for their nucleolar localization (Lange *et al.*, 1998a–c; Samarsky *et al.*, 1998). Finally, although box B and box C are important for U3 function in rRNA processing (Samarsky and Fournier, 1998), we have shown that box B and box C are not essential for localization to nucleoli.

In addition, our results indicate that the box C/D motif is sufficient to direct localization of RNA to the nucleolus (Figure 6). Recent work with a snoRNA in different experimental systems supports this conclusion (Samarsky *et al.*, 1998). Samarsky *et al.* (1998) demonstrated that minimal RNAs derived from U14 that maintain the box C/D motif are localized to nucleoli in both yeast and mammalian cells. It was not possible for Samarsky *et al.* (1998) to assess the necessity of the box C/D motif for U14 localization in those systems because of the lack of stability of the relevant mutant RNAs. Together the results indicate that the highly conserved box C/D motif mediates the nucleolar targeting of snoRNAs in diverse cell types including *Xenopus* oocytes (U3, U8, and U14 [this study]), yeast, and mammalian somatic cells (U14 [Samarsky *et al.*, 1998]).

5′ Cap Hypermethylation Is Not Essential for Nucleolar Localization of Box C/D snoRNAs

Certain box C/D snoRNAs, including U3 and U8 RNA, are synthesized with m^7G caps that become hypermethylated in the nucleus (Tyc and Steitz, 1989; Terns and Dahlberg, 1994; Terns *et al.*, 1995). There is evidence that cap hypermethylation of snoRNAs occurs in the nucleoplasm before their localization to nucleoli (Terns *et al.*, 1995). We found that U3, U8, and U14 RNAs transcribed with either their natural m^7GpppG cap structure or with a cap structure that cannot be trimethylated (ApppG) were comparably localized to nucleoli (Figure 4B and our unpublished results). Thus, our results and those of others (Lange *et al.*, 1998a) directly demonstrate that 5′ cap hypermethylation is not essential for the nucleolar localization of the capped box C/D snoRNAs. This conclusion is perhaps not surprising given that the majority of vertebrate snoRNAs are processed out of introns and do not contain 5′ cap structures but are nevertheless targeted to nucleoli (Maxwell and Fournier, 1995; Tollervey and Kiss, 1997). Previously, it was suggested that cap hypermethylation is not essential for nucleolar localization of U8 RNA because ApppG-capped U8 RNA is functional in *Xenopus* oocytes (Peculis and Steitz, 1994). On the other hand, cap hypermethylation was reported to be essential for the localization of U3

and U8 RNAs to nucleoli of injected mammalian cells (Jacobson and Pederson, 1998). This latter study raises the possibility that the nucleolar localization of box C/D snoRNAs may depend on cap hypermethylation in some but not all cell types.

The Function of the Box C/D Nucleolar-Targeting Motif Is Likely Mediated by Specific RNA-Binding Proteins

It is clear from a number of studies that box C/D sequences play key roles in important aspects of snoRNA metabolism (Baserga *et al.*, 1991; Huang *et al.*, 1992; Peculis and Steitz, 1994; Caffarelli *et al.*, 1996; Cavaille and Bachellerie, 1996; Kiss-Laszlo *et al.*, 1996, 1998; Nicoloso *et al.*, 1996; Watkins *et al.*, 1996; Xia *et al.*, 1997). In vivo and in vitro structure probing of U3 RNA from a diverse range of organisms demonstrates that both the box C′ and box D sequences of U3 RNA are phylogenetically conserved protein-binding sites (Parker and Steitz, 1987; Jeppesen *et al.*, 1988; Hartshorne and Agabian, 1994; Mereau *et al.*, 1997). Moreover, we have found via competition experiments that newly synthesized snoRNAs are actively retained in the nucleus by a mechanism that is both saturable and sequence specific (Terns *et al.*, 1995), indicating that specific nuclear factors associate with snoRNAs to prevent them from exiting the nucleus. Recently, excellent candidates for protein mediators of box C/D function have been identified (Caffarelli *et al.*, 1998; Watkins *et al.*, 1998; Wu *et al.*, 1998; Lafontaine and Tollervey, 1999). Further research will be required to understand how such RNA–protein interactions contribute to the maturation, transport, and function of box C/D snoRNAs.

Potential Role of Coiled Bodies in the Maturation and Transport of Box C/D snoRNAs

A major gap in our knowledge of RNA trafficking is how specific subcellular structures participate in RNA transport and localization. We have found that box C/D family snoRNAs associate with nucleoli and coiled bodies. snoRNAs have been observed both in nucleoli (Fischer *et al.*, 1991; Puvion-Dutilleul *et al.*, 1991; Matera *et al.*, 1994; Samarsky *et al.*, 1998) and in coiled bodies (Bauer *et al.*, 1994; Jimenez-Garcia *et al.*, 1994; Samarsky *et al.*, 1998; Shaw *et al.*, 1998) in diverse cell types by in situ hybridization and light and electron microscopy. However, because analysis of the steady-state distribution of endogenous RNAs yields little information about the mechanism of ongoing RNA transport, we have performed a temporal analysis of the intranuclear distribution of injected snoRNAs. We show that box C/D snoRNAs interact dynamically with coiled bodies (Figures 8 and 9). Our finding that the injected box C/D snoRNAs are readily detected first in coiled bodies and only later in nucleoli suggests that movement of the precursor U3, U8, and U14 snoRNAs through coiled bodies may be a step in the pathway toward nucleolar localization. Our observation that snoRNA mutants that fail to localize to nucleoli are detained in coiled bodies suggests that these mutant RNAs are stalled at an intermediate step in the nucleolar localization pathway.

In addition to box C/D snoRNAs, other nuclear RNAs may also transiently localize to coiled bodies. Indeed, we have found that the U1 spliceosomal snRNA is targeted to coiled bodies rapidly after injection into *Xenopus* oocytes before localization to snurposomes (Figure 8 and our unpublished results). Previous examination of the steady-state localization of U1 snRNA by in situ hybridization in *Xenopus* oocytes did not indicate significant amounts of the RNA in coiled bodies (Wu *et al.*, 1991). However, U1 snRNA has been readily observed in coiled bodies of somatic cells where snRNAs are more actively synthesized (Carmo-Fonseca *et al.*, 1992; Huang and Spector, 1992; Matera and Ward, 1993). In addition, studies with U7 snRNA indicate a dynamic localization with coiled bodies after injection into oocytes (Wu *et al.*, 1996).

It is unclear why snoRNAs traverse coiled bodies. Numerous models for coiled body function have been proposed to account for the fact that these structures contain a multitude of factors including both snRNAs and snoRNAs and their associated proteins (Lamond and Carmo-Fonseca, 1993; Gall *et al.*, 1995; Matera, 1998). Coiled bodies might be involved in the early metabolism of snoRNAs and may be sites of snoRNA modification or assembly into RNP complexes. Conceivably, the reason why mutant snoRNAs that do not localize to nucleoli are detained in coiled bodies is because they lack the ability (e.g., modifications or box C/D–binding factors) to exit these structures efficiently or to be transferred to nucleoli.

Coiled bodies could serve as intranuclear transport vehicles that shuttle components (including snoRNPs) to the sites of their function. We detected snoRNAs in coiled bodies at early but not late time points after injection, suggesting that the flow of snoRNAs between coiled bodies and nucleoli is unidirectional. The box C/D motif is not essential for localization of snoRNAs to coiled bodies, but our results suggest that the motif is required to move snoRNAs from coiled bodies to nucleoli. Coiled bodies have been detected in close association with snoRNA (and snRNA) gene loci (reviewed in Matera, 1998) and are often found physically associated with nucleoli (Raska *et al.*, 1990; Lafarga *et al.*, 1991; Malatesta *et al.*, 1994; Ochs *et al.*, 1994; Bohmann *et al.*, 1995; Lyon *et al.*, 1997). Thus, coiled bodies could conceivably be important in the synthesis, modification, and transport of snoRNAs and other nuclear RNAs.

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