Sequence and structural elements critical for U8 snRNP function in *Xenopus* oocytes are evolutionarily conserved

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We have generated mutants in *Xenopus* U8 RNA, a nucleolar snRNA required for the maturation of 5.8S and 28S rRNAs, to identify sequences and structural domains essential for RNA stability, particle assembly, and function of the U8 RNP. Activity of the mutants was assayed by microinjection of in vitro-synthesized U8 RNAs into the cytoplasm of *Xenopus* oocytes. Most of the mutant RNAs were stable, bound fibrillarin, a protein common to several of the nucleolar-specific snRNPs, and became hypermethylated. Although hypermethylation of the 5' cap of U8 RNA and fibrillarin binding can occur in either the cytoplasmic or nuclear compartment of *Xenopus* oocytes, neither is required for nuclear import. We find that the trimethylguanosine cap, although present on the endogenous U8 RNA, is not essential for stability, particle assembly, or functioning of U8 in the coordinate processing of pre-rRNA at sites 3' of 28S and 5' of 5.8S RNA. Several conserved single- and double-stranded sequences within the 5' domain of U8 RNA are essential for function.

[**Key Words:** nucleolar snRNP function; *Xenopus* oocyte; RNA stability; particle assembly; rRNA processing; ribosome biogenesis]

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The nucleolus is the site of rRNA transcription and ribosome biogenesis. Pre-rRNA is synthesized as a long precursor that binds ribosomal proteins and undergoes processing at several sites to yield mature 18S, 5.8S, and 28S rRNAs, which exit the nucleus in the form of ribosomal subunits. Recently, several nucleolar small nuclear ribonucleoprotein particles (snRNPs) have been shown to play critical roles in the processing of pre-rRNA. In yeast, genetic depletion of U3 and U14 snRNPs resulted in the absence of mature 18S rRNA (Li et al. 1990; Hughes and Ares 1991). Similar results were obtained when U3 RNA was depleted from *Xenopus* oocytes by microinjecting U3 antisense deoxyoligonucleotides (Savino and Gerbi 1990); in vitro, the U3 snRNP has been shown to act in early cleavage near the 5' end of the rRNA transcript (Kass et al. 1990). Genetic depletion of snR30, an essential snRNA in yeast, also affects 18S maturation (Morrissey and Tollervey 1993). Previously, we demonstrated that U8 snRNA is involved in the processing of 5.8S and 28S rRNAs (Peculis and Steitz 1993). Depletion of the endogenous pool of U8 RNA from *Xenopus* oocytes caused an inhibition of pre-rRNA processing at specific sites leading to an absence of mature 28S rRNA and of a 12S precursor to 5.8S rRNA, resulting in an accumulation of abnormal precursors of

28S rRNA. Subsequent microinjection of in vitro-synthesized U8 RNAs into the cytoplasmic compartment of U8-depleted oocytes could restore, or "rescue," pre-rRNA processing (Peculis and Steitz 1993).

Although snRNAs are transcribed and function within the nucleus, several snRNAs are known to undergo bidirectional transport across the nuclear envelope. The abundant splicing RNAs (U1, U2, U4, and U5) are transcribed by RNA polymerase II in the nucleus (Dahlberg and Lund 1988) and exported to the cytoplasm where they assemble with the common Sm proteins and the 7-methyl G cap is hypermethylated to 2,2,7-trimethylguanosine (TMG) (Mattaj 1986, 1988; Hamm et al. 1990a). After particle assembly the snRNPs accumulate in the nucleus where they function in the splicing of pre-mRNAs (DeRobertis et al. 1982; Fischer et al. 1991; for review, see Moore et al. 1993). The requirements for nuclear import vary. U1 and U2 snRNAs require binding of the Sm proteins for hypermethylation to occur (Mattaj 1986, 1988; Hamm et al. 1990a). In *Xenopus* oocytes, a bipartite signal of Sm proteins plus TMG cap is essential for nuclear import (Fischer and Lührmann 1990; Hamm et al. 1990a). In somatic cells the requirements for import of U1 and U2 are less stringent and resemble more closely those of U4 and U5 snRNAs, which bind the Sm

proteins in the cytoplasm but do not require hypermethylation for nuclear import (Fischer et al. 1991, 1993; Marshallsay and Lührmann 1994).

Like the splicing RNPs, some of the nucleolar small nuclear RNAs (snRNAs) associate with a common protein, fibrillarin, and are synthesized by RNA polymerase II. The genes for these RNAs (U3, U8, and U13) have their own promoters, and their transcripts become hypermethylated (Reddy et al. 1979, 1985; Tyc and Steitz 1989; Savino and Gerbi 1990; Peculis and Steitz 1993). Other fibrillarin-associated nucleolar snRNAs (U14, U15, U16, U18, and U21) are intron-encoded (Liu and Maxwell 1990; Fragapane et al. 1993; Prislei et al. 1993; Tycowski et al. 1993; K. Tycowski, pers. comm.). Fibrillarin is a 34-kD protein that has been conserved through evolution from yeast to mammals (Lischwe et al. 1985; Schimmang et al. 1989; Jansen et al. 1991), and a possible homolog has been identified recently in the Archaea (Amiri 1994). Previous studies have demonstrated that fibrillarin binding requires two conserved sequences in the RNA, boxes C and D (Jarmolowski et al. 1990; Baserga et al. 1991). Studies with in vitro-synthesized U3 RNA showed that fibrillarin binding could occur in the cytoplasmic compartment of *Xenopus* oocytes (Baserga et al. 1992). However, oocytes do not stockpile fibrillarin in the cytoplasm; most of the fibrillarin is located in the nucleus (Caizergues-Ferrer et al. 1991). It is not known whether fibrillarin binding is essential for the localization of snRNAs to the nucleolus; there are several nucleolar snRNAs that do not bind fibrillarin (U17, U19, E2, and E3) (Kiss and Filipowicz 1993; Ruff et al. 1993; Cecconi et al. 1994). Likewise, fibrillarin is not required to retain the nucleolar snRNAs within the nucleolus; genetic depletion of NOP1, the yeast homolog of fibrillarin, does not alter the nucleolar localization of U3 RNA (Tollervey et al. 1991).

Mutations in snRNP RNAs can affect function in several ways. They may disrupt sequences or structures in the RNA essential for substrate recognition, for protein binding, or for interaction with other components of the processing apparatus. Alternatively, mutations may interfere with the cellular localization of the snRNP particles, precluding access to the substrate. We have generated mutants to examine the particle assembly, cellular localization, and function of U8 RNA. We have focused on evolutionarily conserved nucleotides or secondary structures and have utilized injection of in vitro-synthesized U8 RNA into the cytoplasm of *Xenopus* oocytes. Although the endogenous U8 RNA is hypermethylated, to our surprise, we find that the TMG cap is not critical for stability, assembly, or function of the U8 RNP. We identify certain evolutionarily conserved sequences in single- and double-stranded regions that are important for function.

Results

The hypermethylated cap is not essential for U8 function

We first examined whether the TMG cap is essential for

function of the *Xenopus* U8 RNP. The endogenous pool of U8 RNA was depleted in oocytes by microinjection of a deoxyoligonucleotide complementary to nucleotides 39–48 in U8 RNA (Peculis and Steitz 1993). After 4 hr, sufficient time for this deoxyoligonucleotide to be degraded in vivo (data not shown), the oocytes were microinjected with U8 RNA synthesized in vitro. RNAs transcribed from a U8 cDNA template were primed with either a GpppG (G-cap) or ApppG (A-cap) dinucleotide and contained three extra guanosine residues at the 5' end (to enhance T7 transcription). The 3' end of the transcribed RNA was identical to the endogenous U8 RNA (Peculis and Steitz 1993). A subsequent 4-hr incubation allowed particle assembly and nuclear accumulation, after which oocytes were microinjected with [³²P]UTP to label nascent RNAs.

Figure 1 (lanes 1) shows the in vivo-labeled RNAs isolated from the cytoplasmic (A) or nuclear (B) compartments of untreated oocytes. The normal pre-rRNA processing pattern is seen. Mature 18S and 28S rRNAs accumulate in the cytoplasm (Fig. 1A, lane 1), whereas pre-rRNAs are observed in the nucleus (Fig. 1B). In *Xenopus*, there are two alternative processing pathways that can coexist in a single oocyte (Fig. 2B) (Savino and Gerbi 1990; Peculis and Steitz 1993). The oocytes shown in Figure 1 utilize both pathways, although pathway A is predominant; 20S rRNA accumulates at the expense of

Figure 1. Rescue of rRNA processing with in vitro-synthesized U8 RNA. In vivo-labeled RNAs isolated from the cytoplasmic (A) or nuclear (B) compartments of oocytes. (Lanes 1) RNA from untreated oocytes; (lanes 2), RNA from U8-depleted oocytes. RNA from U8-depleted oocytes subsequently injected with G-cap U8 RNA is shown in lanes 3, or A-cap U8 RNA in lanes 4.

Figure 2. (A) S1 nuclease analysis of in vivo-labeled rRNAs. S1 digestion pattern produced after in vivo-labeled RNA from untreated oocytes (lane 1), U8 RNA-depleted oocytes (lane 2), or U8-depleted oocytes subsequently injected with G-capped U8 RNA (U8 rescue) (lane 3) was annealed with an unlabeled probe extending from within the 3' end of 28S through part of the 3' nontranscribed spacer region of the *Xenopus* rDNA repeat (bottom). Protected fragments corresponding to the correct 3' end of 28S rRNA (T1) and the 3' extension present in U8-depleted oocytes (bracket, T2), to near T2 or beyond, are indicated. The numbers indicate the size of 123-bp DNA markers. The origin of the faint bands at ~550 nucleotides is not known. These highly reproducible bands may be attributable to either the presence of a processing site within the 3' ETS, or, more likely, nibbling by S1 nuclease. (B) Pre-rRNA processing pathways utilized in normal *Xenopus* oocytes, as well as in U8-depleted and U8-rescued oocytes.

36S rRNA. Figure 1 (lanes 2) contains in vivo-labeled RNA from oocytes previously depleted of their endogenous pool of U8 RNA. The typical U8-depleted rRNA processing pattern is seen: No mature 28S rRNA is produced. Instead, "36S" and 32*S rRNAs accumulate. These abnormal precursors to 28S migrate slightly slower than their normal counterparts because of addi-

tional nucleotides at the 3' end of 28S (Peculis and Steitz 1993). In addition, no 12S RNA (a long-lived nuclear precursor to 5.8S RNA) is observed (data not shown).

Figure 1 (lanes 3) contains RNA from oocytes, which like those in lanes 2, were depleted of their endogenous U8 RNA, but were subsequently microinjected with in vitro-synthesized G-capped U8 RNA. Oocytes will hy-

permethylate snRNAs carrying the G-cap (Fischer and Lührmann 1990; Hamm et al. 1990a), whereas A-cap is not a substrate for hypermethylation (Baserga et al. 1992). The “rescued” rRNA processing pattern appears; mature 28S rRNA is seen, and the normal form of 32S accumulates. The “36S” rRNA is also present, although after rescue this precursor fails to produce the tight, well-defined band seen in untreated oocytes. In addition, the 12S precursor to 5.8S rRNA reappears (data not shown).

Figure 1 (lanes 4) contains RNA from oocytes depleted of their endogenous U8 RNA and later injected with U8 RNA capped with the A-cap dinucleotide. The rescued processing pattern shows mature 28S rRNA (Fig. 1A,B, lanes 4), the normal form of 32S (Fig. 1B, lane 4), as well as the normal 12S rRNA intermediate (data not shown). These results, plus titration studies showing that the A-cap RNA functions as efficiently as the G-cap RNA (data not shown), indicate that the TMG cap normally present on U8 RNA is not essential for nuclear accumulation, particle assembly, nucleolar localization, or function of U8 RNP.

5'-End processing of 5.8S and 3'-end processing of 28S RNAs are linked

When U8 RNA is injected into the cytoplasm of U8-depleted oocytes, pre-rRNA processing is rescued; mature 28S and the normal 32S precursor to 5.8S and 28S appear. However, the gel mobility of 36S, the alternate precursor to 5.8S and 28S, indicated that the rescue with respect to this species may not be complete. S1 nuclease analysis was performed on total RNA isolated from the nuclei of untreated (Fig. 2A, lane 1), U8-depleted (lane 2), or U8 G-cap RNA-rescued (lane 3) oocytes. As was seen previously (Peculis and Steitz 1993), in oocytes depleted of U8 RNA, processing fails to occur at site T1, leaving a sizable 3' extension on precursors to 28S rRNA (Fig. 2A, lane 2; see Fig. 2B). In *Xenopus* oocytes, rRNA transcribed past T2 is highly unstable (Labhart and Reeder 1986), so we presume that the new 3' end maps at or very near to site T2, which is 235 nucleotides downstream of T1.

Subsequent injection of U8 RNA restores 3' termini at site T1, as evidenced by the normal migration of the 28S and 32S pre-rRNAs (Fig. 1B, lanes 3,4; see also Fig. 5A, below) and the reappearance of a normal band in the S1 nuclease protection assay (Fig. 2A, lane 3). However, the S1 assay also reveals that the longer RNAs seen in depleted oocytes do not disappear completely, even when the amount of U8 RNA injected is in fourfold excess of that needed for rescue of 32S rRNA. Because the “36S” pre-rRNA appears as a thicker, more ill-defined band in rescued samples relative to untreated samples (Fig. 1B, lanes 3,4), we propose that U8 RNA facilitates coordinate processing at sites 3 and T1 (Fig. 2B). If processing occurs at site 3, cuts at site T1 will produce a normal 32S species (Fig. 1B, lanes 3,4) with the correct 3' terminus (Fig. 2A, lane 3). However, if processing at site 3 is debilitated (or if processing occurs at site 2 first), then there

is inhibition of processing at T1. Alternatively, it is possible that the initial inhibition of processing is at site T1, such that in the absence of processing at T1, no processing can occur at site 3, causing site 2 to be utilized (see Fig. 2B).

Site-directed mutagenesis produces U8 RNA mutants

In generating mutants in U8 RNA (Fig. 3), we focused on conserved structures or nucleotides. The complete sequence of U8 RNA is shown at the top of Figure 3; uppercase letters indicate those nucleotides conserved between mammalian (Kato and Harada 1984; Reddy et al. 1985; Tyc and Steitz 1989) and *Xenopus* U8 (Peculis and Steitz 1993); lowercase letters represent nucleotides that are not identical. The 5' end of the U8 RNA is highly conserved between *Xenopus* and mammals with only 4 nucleotide changes out of 38. Likewise, the lengths of stems 1 and 2 are conserved. The conserved elements in the 3' 100 nucleotides include boxes C and D, but neither the sequences nor the lengths of stem-loops 3 or 4 are conserved. The shaded nucleotides in Figure 3 indicate the nucleotides altered by site-directed mutagenesis (see Materials and methods) in each mutant; only the relevant portion of the U8 RNA is shown, the rest being the wild-type sequence.

The A-cap mutant, used above, is identical to the wild-type RNA with the exception of the cap itself. Another mutant (5' end) changes the first 4 nucleotides in U8 to the complementary nucleotides. Several loop mutants (Lp1M, Lp1-23, Lp1-45, Lp2M, Lp2-13, Lp2-24, Lp3M, and Lp4M) changed selected nucleotides within the four predicted hairpin loops of U8 to nucleotides complementary to those in the wild type. For the first and second stems of U8 RNA, pairs of mutants (St1M5' and St1M3', St2M and St2M3') change the nucleotides on either side of the conserved stem (5' and 3', left and right side, respectively) to their complementary nucleotides; the compensatory mutants (St1Mc and St2C) change the sequence on both sides of the stem to restore base-pairing. Two mutants (C boxM and D boxM) alter the conserved sequences in box C and box D, respectively. The mutant C-upM changes the entire single-stranded region upstream of box C, including several nucleotides conserved between mammals and *Xenopus*. Two additional mutants in this region change only those nucleotides that are conserved between *Xenopus* and human (C-upconM) or only those not conserved between these two species (C-upNC).

Assaying hypermethylation and nuclear accumulation of U8 mutants

Mutant U8 RNAs transcribed in vitro in the presence of [³²P]UTP and the G-cap dinucleotide (except for the A-cap mutant) were microinjected into the cytoplasmic compartment of oocytes. After incubation, the ability of each mutant RNA to accumulate within the nucleus, become hypermethylated, assemble into fibrillarin-pre-

Figure 3. Mutant U8 RNAs generated by site-directed mutagenesis. (Top) The complete sequence of *Xenopus* U8 RNA is shown. (ggg) T7 transcription is enhanced by 3 G nucleotides encoded on the templates for all exogenous U8 RNAs. Uppercase letters indicate positions conserved between *Xenopus* and mammals. Lowercase letters are nucleotides that have diverged. Below, the mutants generated by site-directed mutagenesis (Kunkel et al. 1987). For each mutant, only the relevant region of the U8 RNA is shown and the altered nucleotides are shaded.

capable particles, and function was analyzed. Clearly, if a mutant RNA was not stable in the cytoplasm, or could not accumulate within the nucleus, its function in pre-rRNA processing could not be assessed.

The amount of U8 RNA in the nuclear compartment of hand-dissected oocytes was compared with that remaining in the cytoplasm 6 hr after injection. Figure 4A shows the results of a localization assay (summarized in Table 1). The A-cap mutant RNA accumulates in the nucleus as efficiently as the G-cap wild-type RNA (Fig. 4A, lanes 1,2,18,19), and all other mutants are interpreted relative to these two RNAs. Most RNAs efficiently translocated to the nuclear compartment, with the exception of the Lp3M, C boxM, and D boxM mutants; these RNAs do not accumulate in the nucleus and

degrade completely during a 14-hr incubation (data not shown), although at the 6-hr time point the C box and D box mutant RNAs are still detected in the cytoplasm (Fig. 4, A, lanes 11 and 28, 16 and 33, 17 and 34; C, lanes 12 and 31, 17 and 36, 18 and 37). When injected into isolated nuclei, Lp3M, C boxM, and D boxM are likewise unstable.

The ability of each mutant to become hypermethylated was assayed by immunoprecipitation with anti-TMG antibodies. Six hours after injection of 32 P-labeled mutant RNAs into the cytoplasmic compartment, RNA was isolated from whole oocytes. The G-cap RNA was immunoprecipitated efficiently, whereas the A-cap RNA was not precipitated, as expected (Fig. 4B, lanes 1–4). The efficiency of immunoprecipitation by the anti-TMG an-

Figure 4. Assembly and stability of U8 RNA injected into U8-depleted oocytes. (A) Total RNA pooled from hand-dissected nuclei (*bottom*) and the corresponding cytoplasms (*top*) of oocytes that had received cytoplasmic injections of 32 P-labeled in vitro-synthesized U8 RNA or mutant RNA indicated. (B) Immunoprecipitations from extracts of whole oocytes injected with G-cap or A-cap U8 RNA (lanes 1–4) and of enucleated oocytes (lanes 5,6) or isolated nuclei (lanes 7–10) coinjected with G-cap U8 and U1 RNAs. (Lanes 1–8) Anti-TMG precipitations. (Lanes 9,10) Anti-fibrillarin immunoprecipitations from isolated nuclei identical to those used in lanes 7 and 8. Supernatants and pellets are indicated. (U8-deg) The stable 3' end fragment of U8 RNA. (C) Anti-fibrillarin immunoprecipitations from extracts of whole oocytes microinjected with the mutant U8 RNAs. (*Top*) The supernatants from anti-fibrillarin precipitations; (*bottom*) the corresponding pellets. (Lanes 1–18) supernatants from whole oocytes injected with the 32 P-labeled in vitro-transcribed mutant U8 RNA indicated; (lanes 20–37) the corresponding pellets. (Lanes 19,38) Supernatant and pellet, respectively, from enucleated oocytes injected with 32 P-labeled in vitro-transcribed G-cap U8 RNA.

tibodies varies from RNA to RNA (Krainer 1988). In our hands, precipitation of naked U8 RNA is ~50% efficient. Table 1 shows that all G-capped RNA mutants that were stable enough to be assayed also became hypermethylated. At shorter time points the C boxM and D boxM mutant RNAs present in the cytoplasm (Fig. 4A, lanes 16,17,33,34) were not detectably hypermethylated (data not shown).

To determine the cellular location of hypermethylation, we hand-dissected injected oocytes and assayed the nuclear and cytoplasmic compartments separately. We find that of the U8 RNA remaining in the cytoplasm, only ~10–20% is TMG-precipitable (data not shown) for both the wild-type (G-cap) and mutant RNAs. Conversely, 60–80% of U8 RNAs present within the nuclear compartment are TMG-precipitable. Whether this is because U8 RNPs typically are assembled in the cyto-

plasm, become hypermethylated, and translocate into the nucleus immediately or because the hypermethylation activity is largely nuclear cannot be distinguished by this assay.

Therefore, we microinjected U8 RNA directly into isolated nuclei and assayed hypermethylation. Figure 4B shows the supernatants and pellets from anti-TMG precipitations of RNA isolated from enucleated oocytes (lanes 5,6) or isolated nuclei (lanes 7,8) coinjected with U8 and U1 RNAs. The U1 RNA, which can become hypermethylated only after assembling with the Sm proteins in the cytoplasm (Mattaj 1986), serves as a control for cytoplasmic contamination of the isolated nuclei. The TMG pellet of the enucleated sample (Fig. 4B, lane 6) shows that 20% of the U8 RNA and 22% of the U1 RNA become hypermethylated in the cytoplasmic compartment. In contrast, typically 65–80% of G-cap U8 in-

Table 1. Summary of data on U8 mutants

Mutant	Nuclear accumulation ^a	TMG precipitation ^b	Fibrillarin association ^c	Rescue processing ^d
G-cap	++	++	+	+
A-cap	++	—	+	+
5'-end	++	++	+	—
St1M5'	++	(+)	(+)	—
St1M3'	+			—
St1Mc	+	(+)	(+)	—
Lp1M	++	++	+	—
Lp1-23	++	++	+	+
Lp1-45	++	++	+	—
St2M	+	+	+	—
St2M3'	+	(+)	(+)	+
St2C	+	+	+	+
Lp2M	+	+	+	—
Lp2-13	++	++	+	—
Lp2-24	++	++	+	—
Lp3M	+/-	ns	ns	ns
Lp4M	++	++	+	+
C-up	+	+	—	—
C-up-NC	++	++	+	+
C-up-conM	++	++	+	—
C boxM	+/-	ns	ns	ns
D boxM	+/-	ns	ns	ns

Summary of data on U8 mutants. U8 RNA mutants were examined for their ability to accumulate within the nucleus, be precipitated with anti-TMG antibodies, be precipitated with anti-fibrillarin antibodies, and rescue rRNA processing in U8-depleted oocytes. (++) Precipitates or accumulates to levels >85% that of G-cap; (ns) RNA not stable in cytoplasm after 12-hr incubation, <15% of G-cap remains. Data are averages from three to six independent experiments, except those indicated by (+), which are single determinations; the data shown in the corresponding figures are representative. All RNAs were labeled to the same specific activity and identical amounts injected into each oocyte. Quantitation was done on a Molecular Dynamics PhosphorImager.

^a(+) 75–85% of G-cap level; (+/-) <30% that of G-cap level.

^b(+) 70–85% that of G-cap; (—) <3% of RNA precipitates.

^c(+) >80% of G-cap; (+/-) <80% of G-cap.

^d(+) The appearance of 28S and conversion of 32S to 32S; (—) inability of the mutant U8 RNA to alter the U8-depleted processing pattern; (ns) unstable RNA, ability to rescue cannot be determined.

jected into isolated nuclei is precipitated by anti-TMG antibodies, whereas <9% of U1 RNA is precipitated from these same nuclei (Fig. 4B, lanes 7,8). U3 snRNA becomes hypermethylated in both the cytoplasmic (Baserga et al. 1992) and nuclear compartment (Terns and Dahlberg 1994) of *Xenopus* oocytes. Similarly, we conclude that U8 RNA can become hypermethylated in the cytoplasm, but cap modification is far more efficient in the nucleus.

Assaying the assembly of mutant U8 RNAs into particles

The ability of the mutants to assemble into RNP complexes was assayed indirectly by immunoprecipitation with anti-fibrillarin antibodies. Mutant ³²P-labeled U8 RNAs were microinjected into the cytoplasmic compartment of oocytes. After a 4-hr incubation, whole oocyte extracts were prepared and the fraction of fibrillarin-precipitable mutant U8 RNA determined. Most of the mutant RNAs were precipitated efficiently with anti-fibrillarin antibodies (Fig. 4C). The major exceptions were the Lp3M, C-upM, C boxM, and D boxM mutant RNAs

(lanes 12 and 31, 14 and 33, 17 and 36, 18 and 37). It is important to note that the C-upM RNA is stable and accumulates in the nucleus (Fig. 4A, lanes 13,30), yet none of this RNA detectably associates with fibrillarin (Fig. 4C, lanes 14,33).

The C boxM and D boxM mutant RNAs are generally less stable in the cytoplasm than the G-cap RNA. By microinjecting larger quantities (up to twofold) of C box, and D box RNAs into the cytoplasm, and taking shorter time points (a 4-hr incubation), these RNAs could be assayed for fibrillarin association (Fig. 4C, lanes 17,36,18,37). Even then, only a very small fraction (<5%) of these two mutant RNAs was precipitable, indicating that boxes C and D are essential for fibrillarin binding in vivo, as concluded previously in vitro (Baserga et al. 1991). These results suggest further that fibrillarin binding and cytoplasmic stability may be linked.

Oocytes were also hand-dissected prior to analysis, and the fibrillarin-associated mutant U8 RNA present in the nuclear or cytoplasmic compartments was examined separately (data not shown). These experiments showed that fibrillarin-associated mutant U8 RNAs were present in both compartments, but the majority was in the nu-

cleus. This assay cannot distinguish between rapid import of fibrillarin-associated U8 RNA and fibrillarin binding occurring within the nucleus after import. The fact that there are fibrillarin-associated, intron-encoded nucleolar snRNAs that have not been shown to have a cytoplasmic phase (Liu and Maxwell 1990; Fragapane et al. 1993; Prislei et al. 1993; Tycowski et al. 1993; K. Tycowski, pers. comm.) is consistent with the observations of Caizerguez-Ferrer et al. (1991), who report a pool of fibrillarin in the oocyte nucleus.

To determine whether U8 RNA can associate with fibrillarin in separated nuclear and cytoplasmic compartments, we microinjected U8 RNA into enucleated oocytes and incubated them prior to making an extract for immunoprecipitation with anti-fibrillarin antibodies. Similar fractions of U8 RNA (typically ~35–50%) were precipitated in the absence of a nucleus (Fig. 4C, lanes 19,38) and from a whole oocyte (Fig. 4C, lanes 1,20), indicating that U8 RNA can associate with fibrillarin in the cytoplasm. Subsequently, U1 plus U8 RNAs were microinjected into isolated nuclei, incubated for 4 hr, and used to make extract. Typically, ~50% of the U8 RNA was fibrillarin precipitable (Fig. 4B, lanes 9,10), whereas U1 RNA, which does not associate with fibrillarin, failed to precipitate (<1%). We conclude that fibrillarin association can occur in the nucleus, but the C-up mutant (Fig. 4C, lanes 14,33) shows that fibrillarin binding is not necessary for nuclear accumulation of U8 RNA.

The anti-fibrillarin immunoprecipitations revealed two forms of U8 RNA stably associated with fibrillarin in the nucleus of U8-injected oocytes (Fig. 4B, lanes 9,10; C). One RNA was full length, whereas the other band was a stable fragment apparently corresponding to the 3' end of U8 [as it is not anti-TMG precipitable (Fig. 4B, lanes 1–8)]. The length of the fragment (~100 nucleotides) suggests that it is produced by cleavage of U8 RNA within the single-stranded region upstream of box C.

Some mutant U8 RNAs can rescue rRNA processing

Each in vitro-synthesized ³²P-labeled G-cap mutant RNA was microinjected into the cytoplasmic compartment of U8-depleted oocytes, followed 4 hr later by [³²P]UTP to label nascent RNAs. As indicated in Table 1, some mutants were able to rescue rRNA processing while others were not. Figure 5, A and B, shows the rescue of rRNA processing by some of the mutants, compared with the A-cap (lanes 3) and the G-cap (lanes 4) wild-type U8 RNAs.

The 5' end mutant (altered in nucleotides 2–5 of U8 RNA) is defective in rRNA processing (Fig. 5A, lane 12). Likewise, the Lp1M mutant, which has all 5 of the nucleotides in the first loop of U8 altered, does not function (lane 13). The same is true of Lp1-45 in which only 2 of these 5 nucleotides are changed (lane 15). However, the Lp1-23 mutant (lane 5), in which the other 2 of the 5 nucleotides in the first loop of U8 RNA are changed, can function in rRNA processing. In contrast, mutations in

the fourth loop (Lp4M mutant) did not affect rescue of rRNA processing (lane 8) even though 7 of 10 nucleotides were altered, including 5 that are conserved between mammals and *Xenopus*.

There is scattered conservation of sequence in the single-stranded region of U8 RNA upstream of box C. Changing all 10 nucleotides in this region renders U8 nonfunctional, as does changing only the nucleotides conserved between mammals and *Xenopus* [C-up and C-up-conM (Table 1), respectively]. However, changing 3 of the 4 nucleotides not conserved between these two widely diverged vertebrates (C-up-NC) yields a functional RNA (Fig. 5A, lanes 9,18).

The first and second stems predicted for U8 RNA are conserved in structure, and partly in sequence, between *Xenopus* and mammals (Kato and Harada 1984; Reddy et al. 1985; Tyc and Steitz 1989; Peculis and Steitz 1993). In the mutants St1M5' and St1M3' (Fig. 3) this conserved structure is destroyed and the RNA is not functional (data not shown). The compensatory mutant, St1C, restores the base-pairing but remains nonfunctional. We conclude that the positions of the evolutionarily conserved sequences within this structure are critical.

The sequence of the 5' side of U8 RNA's second stem is evolutionarily conserved while the 3' side is not (Fig. 3). St2M alters the conserved nucleotides and is not functional (Fig. 5A, lane 6). St2M3' alters the nonconserved nucleotides and does function, but less efficiently (data not shown). The compensatory mutant, St2C, restores the structure of the stem and functions, but less efficiently (lane 7) than the wild type (lane 4). Therefore, we conclude that the evolutionarily conserved sequence on the 5' side of the second stem is important, but not critical, for function. In its absence, some function can be provided by creating a secondary structure. Thus, both the sequence and structure of stem 2 are important for optimal activity.

Figure 5B analyzes the lower molecular weight RNAs from the same oocytes as those shown in Figure 5A. The in vivo-labeled 5S rRNA serves as a control for loading while the 12S pre-rRNA can be seen in untreated oocytes (lane 1) as well as in those lanes containing RNA from oocytes injected with mutant U8 RNAs that can rescue processing (lanes 3,4,5,7,8,9,14,18). In addition, each lane shows the quantity of ³²P-labeled mutant U8 RNA present in the nuclei of the oocytes analyzed in Figure 5A. Generally, the amount of U8 RNA present correlates with the ability of each mutant to rescue processing. The A-cap, G-cap, Lp4M, and C-upNC RNAs are abundant in the nuclei of oocytes in which rRNA processing is rescued (Fig. 5A,B, lanes 3,4,8,9; also 14,18). Mutant St2M RNA is an exception in that it does accumulate within the nucleus (albeit at lower levels), but does not rescue pre-rRNA processing.

Because a mutant RNA that does not accumulate within the nucleus cannot be expected to function, titration studies were performed to establish the minimum amount of U8 RNA necessary to rescue processing. Figure 6 shows the titration curve for the G-cap U8 RNA, along with two mutants that do and two that do

Figure 5. Rescue of rRNA processing with mutant U8 RNAs. In vivo-labeled RNAs isolated from nuclei of oocytes preinjected with deoxyoligonucleotide and then with in vitro ^{32}P -labeled RNA as indicated. RNAs were resolved on 1% agarose-formaldehyde (A) or 8% denaturing polyacrylamide (B) gels. Precursor and mature rRNAs, and the in vitro-synthesized U8 RNA (and degradation product, U8 deg.) are indicated. In A, mature 18S rRNA has run just off the bottom of the gel in lanes 6–9. Longer exposures of lanes 11, 13, and 17 indicate that 18S levels relative to longer precursors are normal.

not rescue rRNA processing. Controls show the “normal” rRNA processing pattern in untreated oocytes (Fig. 6A, lane 1) and the U8-depleted pattern (lane 2). Figure 6 (lanes 3–6) contains RNA from U8-depleted oocytes subsequently injected with 50 nl of G-cap U8 RNA solution at concentrations representing $1\times$, $\frac{1}{2}\times$, $\frac{1}{4}\times$ and $\frac{1}{10}\times$ the amount of RNA used in Figure 5. Even at the lowest concentration of G-cap U8 RNA, rRNA processing was rescued; 28S and 12S rRNAs accumulated (Fig. 6A,B, lanes 5,6). The acrylamide gel (Fig. 6B) confirms that as less U8 RNA is injected into the cytoplasm, less accumulates in the nucleus (Fig. 6B, lanes 3–6).

Figure 6A (lanes 7–10) shows RNA from the nuclei of oocytes injected with 50 nl of four different mutant RNAs, all at the $1\times$ concentration. Lp1M RNA does not rescue rRNA processing (Fig. 6A, lane 7), although Figure 6B shows that a sufficient quantity of RNA is present in the nucleus. Much less Lp1-23 RNA is present in the nucleus (about one-fifth); however, this RNA can rescue rRNA processing (lanes 8). A large quantity of St2C RNA

accumulates in the nucleus and does function (lanes 9). Finally, there should be sufficient St2M RNA present in the nucleus (lanes 10), but rRNA processing is not rescued.

Discussion

Processing of 5.8S and 28S rRNAs is inhibited in *Xenopus* oocytes in the absence of U8 RNA, a hypermethylated, fibrillarin-associated, nucleolar snRNA. rRNA processing can be restored or rescued in U8-depleted oocytes by subsequent injection of in vitro-synthesized U8 RNA into the oocyte cytoplasm. We have examined nucleotides and structures within U8 RNA that are essential for its nuclear localization, assembly, and function in *Xenopus* oocytes.

U8 functions without a TMG cap

Transcription in the presence of an A-cap dinucleotide

Figure 6. Titration of U8 RNA to examine function of mutant U8 RNAs. Rescue of rRNA processing was examined in U8-depleted oocytes microinjected with decreasing concentrations of ^{32}P -labeled G-cap U8 RNA or mutant U8 RNAs (at $1\times$ concentration) as indicated. In vivo-labeled RNAs isolated from the nuclei of treated oocytes, as indicated, were resolved on 1% agarose-formaldehyde (A) or 8% denaturing polyacrylamide (B) gels. Precursor and mature rRNAs, in vitro-synthesized U8 RNA, and a degradation product (U8 deg.) are indicated. Titration of the A-cap U8 RNA (not shown) gave a nearly identical processing pattern; partial rescue was seen at the $1/4\times$ amount of RNA. The slightly altered migration of mature 28S in lane 3, compared with 4 and 5 is attributable to a loading difference [note that other bands in lane 3 are also shifted slightly upwards].

produces RNAs with a cap that is not a substrate for hypermethylation (Baserga et al. 1992). Yet, microinjection of the A-cap U8 RNA into oocytes produces fibrillar-precipitable RNAs that can rescue rRNA processing (Fig. 1). Titration experiments using as little as $0.1\times$ RNA showed that the A-cap RNAs function as effectively as the G-capped RNA (data not shown). The A-cap RNA must contain the A-cap dinucleotide (rather than G-cap) at its 5' end, as it does not become precipitable by the TMG antibodies (Fig. 4B, lanes 1–4). Also it is as stable as the G-capped RNA, whereas U8 RNAs synthesized in the absence of a cap analog are rapidly degraded ($<5\%$ remains after 6 hr) when injected into the cytoplasmic compartment of oocytes (data not shown). We conclude that the TMG cap is not necessary for assembly or function of the U8 RNP.

Although previous work examined the requirement of a TMG cap for nuclear import of Sm snRNAs (Mattaj 1986, 1988; Fischer and Lührmann 1990; Hamm et al. 1990a; Baserga et al. 1992; Fischer et al. 1993; Marshall and Lührmann 1994), our analyses are the first to demonstrate that a normally hypermethylated snRNA can function without a TMG cap. This is not to imply that the TMG cap plays no role in the U8 snRNP. The hypermethylated cap may be needed to regulate nuclear import, or prevent cytoplasmic retention after mitosis, as was seen with a G-capped U6 RNA (Fischer and Lü-

hrmann 1990). Likewise, the TMG cap may be dispensable for U8 function in the *Xenopus* oocyte, but essential in somatic cells. Marshall and Lührmann (1994) showed recently that U1 can be imported into the nucleus of a somatic cell independent of a TMG cap, which is required for import in oocytes (Mattaj 1988; Fischer and Lührmann 1990).

Location of particle assembly and hypermethylation of U8 RNA

In most of our experiments we microinjected in vitro-synthesized U8 snRNA into the cytoplasmic compartment of oocytes. This procedure was chosen because cytoplasmic injections produce more uniform results and, unlike nuclear injections, cause no mechanical damage that may complicate interpretation of the data because of leakage from the nucleus. U6 snRNA, the only RNA polymerase III transcript in the functional spliceosome (Dahlberg and Lund 1988), does not leave the nucleus after transcription, whereas the other spliceosomal snRNAs do (Hamm and Mattaj 1989; Vankan et al. 1990). Likewise, the biogenesis of the U3 snRNP does not appear to have a cytoplasmic phase (Terns and Dahlberg 1994). Yet, when U6 or U3 RNA is injected into the cytoplasm of *Xenopus* oocytes, it can localize to the nucleus (Hamm and Mattaj 1989; Baserga et al. 1992), pre-

sumably mimicking the relocation that occurs following mitosis. We argue that assembly, import, and function of U8 RNA introduced via cytoplasmic injections likewise utilize normal physiological pathways. All mutant U8 RNAs that were stable accumulated in the nucleus (Table 1), allowing their function to be assessed.

Microinjection of in vitro-transcribed RNAs into either enucleated oocytes or into isolated nuclei indicated that fibrillarin association can occur in both compartments, yet the site of fibrillarin binding is not necessarily that of U8 particle assembly as other U8-specific proteins (that have not yet been identified) may be required to bind before fibrillarin. The C-upM mutant is a stable RNA that does not bind fibrillarin, suggesting that while the stability of U8 may be enhanced by fibrillarin binding, the presence of other (perhaps U8-specific) proteins can stabilize this mutant RNA in the absence of fibrillarin. This would contrast with the situation for the Sm snRNPs, where partial particle assembly with the common Sm proteins occurs prior to nuclear import, with additional specific proteins (that enter the nucleus independent of the RNP) binding after translocation (Hamm et al. 1990b; Kambach and Mattaj 1992; Jarmolowski and Mattaj 1993). A steady-state pool of fibrillarin has been identified in the nucleus of hand-dissected *Xenopus* oocytes (Caizergues-Ferrer et al. 1991). However, because these analyses were performed by Western blot, it is not known how much of this pool represents RNA-associated protein or where a pool of free fibrillarin might reside in the cell. Mutants C boxM and D boxM are unstable over long (>6 hr) incubations. We have shown that at 6 hr, when some RNA is still present, fibrillarin does not detectably associate with these RNAs, although we do not know whether the instability is the result of an absence of other proteins that normally bind boxes C and D directly. These data constitute the first in vivo indications that boxes C and D are necessary for fibrillarin binding in vertebrates.

Like fibrillarin binding, hypermethylation of G-cap U8 RNA occurs after microinjection into either enucleated oocytes or isolated nuclei, although cap modification is more efficient in the nucleus. Previous work with U3, a relatively more abundant hypermethylated fibrillarin-associated nucleolar snRNA, indicates that it also can become hypermethylated in both the cytoplasmic (Baserga et al. 1992) and nuclear (Terns and Dahlberg 1994) compartments of oocytes.

The 5' domain of U8 RNA is essential for function

The 5' one-third (40 nucleotides) of U8 RNA is 90% identical between *Xenopus* and mammals. Such a high degree of conservation through evolution is often indicative of selection based on function. For example, the most highly conserved regions in U1, U2, U5, and U6 snRNAs are those involved in snRNA–snRNA or snRNA–substrate interactions (for review, see Moore et al. 1993). Our data indicate that most of the U8 mutations

in the 5' domain (including the first two stem–loops) of the RNA were deleterious; only 4 of the 14 mutants affecting the first 39 nucleotides could function (see Table 1). The addition of the three guanosine residues at the 5' end of U8 RNA (to enhance T7 transcription in vitro) did not prevent the RNA from functioning, although we cannot be sure that efficiency was not affected.

Mutations in most of the predicted single-stranded regions in the 5' domain of U8 RNA gave processing negative phenotypes. These include the extreme 5' end of the molecule as well as the loops of the first two stems. One mutant (Lp1-23) identified two conserved nucleotides in the first loop of U8 that could be changed without affecting function, but alteration of additional nucleotides in this loop resulted in a nonfunctional RNA. Extensive mutational studies on U1 and U2 have likewise identified sequence elements essential for specific steps in splicing (Hamm et al. 1988, 1989, 1990b; Ares and Igel 1990; Pan and Prives 1989; Zavanelli and Ares 1991; McPheeters and Abelson 1992).

Mutation of the first two stem structures of U8 demonstrates that they both contain essential elements for efficient functioning. In stem 1, altering either the 5' or 3' side abolishes function; restoring the structure is not sufficient to restore rRNA processing. However, for the second stem of U8, altering the 3' side (St2M3'), which destroys the structure but leaves intact the evolutionarily conserved nucleotides, gives a functional (albeit less efficient) RNA. Interestingly, the compensatory mutation, which restores the base-pairing but eliminates the conserved sequence (St2C), also functions (about as well as St2M3') showing that a stem structure can somehow substitute for the missing conserved sequence. Similarly, in U2 snRNA, mutation of critical secondary structures produced nonfunctional RNAs, whereas compensatory mutations restoring structure also restored function (Hamm et al. 1989; Ares and Igel 1990; Zavanelli and Ares 1991). In particular, stem 1 of U2 snRNA (McPheeters and Abelson 1992) is similar to stem 2 of U8 RNA in that structure is important but not absolutely essential for function. In U8 RNA the evolutionarily conserved nucleotides on the 5' side of this stem also affect efficiency.

The remaining two-thirds of U8 RNA is not evolutionarily conserved and was not mutated as extensively for these studies. Several of the mutations yielded RNAs that were unstable upon injection into either the cytoplasm or isolated nuclei (data not shown); these RNAs (Lp3M, C boxM and D boxM) could not be assessed for function. The only mutations that did affect the function of stable, nuclear accumulated RNAs affected conserved nucleotides in the single-stranded region just upstream of box C (nucleotides 42–50). Less than 40% of the eighty 3'-most nucleotides are conserved between *Xenopus* and mammals. The stable 100-nucleotide 3' U8 RNA fragment that remains after oligonucleotide targeting of the endogenous U8 (Peculis and Steitz 1993) is anti-fibrillarin precipitable but nonfunctional. Thus, in the absence of the 5' end of U8, fibrillarin binding is not sufficient for function. These results argue that the 5' do-

main of U8 RNA is probably the most critical for function.

How does the 5' end of U8 function in pre-rRNA processing?

It is possible that base-pairing between U8 RNA and *Xenopus* pre-rRNA sequences directs the U8 snRNP to its sites of action. However, no complementarity longer than 6 nucleotides exists within 100 nucleotides of either site 3 or T1 to a region in U8 available to RNase H cleavage (thus, exposed on the U8 particle). Because even a critical interaction might be only a few base pairs long, other types of experiments [e.g., cross-linking, used to identify U3 binding within the 5' external transcribed spacer (ETS) (Stroke and Weiner 1989; Tyc and Steitz 1992)] will be needed to analyze U8-pre-rRNA interactions.

There is an intriguing complementarity (13/17 nucleotides) between *Xenopus* U8 RNA and pre-rRNA nearly 200 nucleotides downstream of T1:

This interaction would predict that all loop 2 mutants

(Lp2M, Lp2-13, and Lp2-24) would be nonfunctional, and the data (Table 1) indicate that they are. However, changes in the 3' portion of stem 2 (nucleotides 37-42) should also be nonfunctional, as this region forms the core of the above complementarity. Rather, the ability of the St2M3' and St2C mutant RNAs to function (Table 1) shows that this sequence is nonessential and argues against this computer-identified complementarity as a site of U8-pre-rRNA interaction. Moreover, a corresponding complementarity cannot be identified between U8 and pre-rRNA at a conserved distance from the 3' end of 28S in mammals (Kato and Harada 1984; Reddy et al. 1985; Tyc and Steitz 1989).

An alternative explanation for the processing-negative phenotypes of the mutations affecting the 5' domain of U8 is that they interfere with protein binding, either directly or by inducing structural changes. For instance, the processing defects caused by mutations in loops 1 and 2 of U8 RNA are reminiscent of mutations in loop sequences of U1 RNA (Hamm et al. 1988, 1990b) or U2 RNA (Wells and Ares 1994) that direct specific protein binding. Further investigation will be required to establish the identity of such U8 proteins and their roles in U8 function.

The U8 RNP affects processing at two sites in the pre-rRNA

In *Xenopus* pre-rRNA processing, cleavage at site 2 or 3 determines which precursors to 28S rRNA will accumu-

late (Fig. 2B). Cleavage at site 2 produces mature 18S and 36S rRNAs, the latter of which is processed to yield 12S and 28S rRNAs, whereas cleavage at site 3 yields 20S and 32S rRNAs, the latter of which is processed to 12S and 28S rRNAs. In U8-depleted oocytes, no detectable cleavage occurs at T1 (Fig. 1A and 5A), which is normally a very rapid event (Labhart and Reeder 1986). This leaves a 3' extension present on all precursors of 28S rRNA (thereby producing shifts in mobility of the 32*S and "36S" rRNAs). Moreover, in U8-depleted oocytes, cleavage is very inefficient at site 3, increasing the amount of "36S". We do not know whether the 5' end of the 32*S has been produced in the normal way or by exonucleolytic action on "36S." When U8-depleted oocytes are rescued by injection of U8 RNA, normally migrating 32S and 28S rRNAs appear (see Fig. 2B), indicating that all RNAs cleaved at site 3 have also been processed at T1. However, the "36S" species remains, suggesting that cleavage near site 2 precludes subsequent processing at site T1. Perhaps if cleavage at T1 and site 2 do not occur in the normal order, an alternate RNA secondary/tertiary conformation forms that makes T1 unrecognizable, or unavailable, to the processing machinery.

In the titration experiments, the inability to completely rescue coordinated processing at T1 and site 3 by injection of U8 may be the result of a lack of sufficient U8 RNA. Decreasing the amount of wild-type G-capped U8 RNA injected by fourfold resulted in a partial rescue phenotype, where some mature 28S rRNA accumulated. However, the aberrant pre-rRNAs were also present (Fig. 6). Specifically, the conversion from 32*S to 32S was not complete, resulting in the appearance of four bands ("36S", 32*S, 32S, and 28S; see Fig. 2B) in this region of the gel (Fig. 6A, lanes 4,5). If only enough U8 is added to restore processing at some T1 sites, then only these pre-rRNAs will undergo normal cleavage at site 3. Because U8 and U3 (and possibly several other) RNPs affect pre-rRNA processing at site 3 (Savino and Gerbi 1990; Hughes and Ares 1991; Peculis and Steitz 1993), they may act together; depleting the system of one of these components would be expected to alter the efficiency at which that site is utilized and could indirectly determine which of the alternative processing pathways are used.

The inability to restore completely normal migration to "36S" rRNA, yet produce normal 32S RNA, indicates that in *Xenopus*, like yeast, cleavages at sites 3' of 28S and 5' of 5.8S (site 3, see Fig. 2B) are in some way linked (Veldman et al. 1981; for review, see van Nues et al. 1993). Because eukaryotic 5.8S rRNA corresponds to the 160 nucleotides at the 5' terminus of prokaryotic 23S rRNA (Brosius et al. 1979), the coordinate processing that occurs at the 3' end of 28S and the 5' end of 5.8S also parallels the cleavages that are made by RNase III in *Escherichia coli*. There, processing is directed by extensive base-pairing of the pre-rRNA flanking both 16S and 23S sequences (Young and Steitz. 1979; Bram et al. 1980). No extensive base-pairing of this type can be discerned in eukaryotic pre-rRNA sequences. Yet, the 5' end of 5.8S and the 3' end of 28S rRNAs could be brought into close proximity by base-pairing between the flanking se-

quences in yeast pre-rRNA (Veldman et al. 1981). No U8 RNA homolog has yet been identified in yeast.

Relative to the splicing snRNPs (for review, see Baserga and Steitz 1993; Moore et al. 1993), little is known about how the nucleolar snRNAs interact with each other and with the pre-rRNA to achieve efficient processing (for review, see van Nues et al. 1993). Correct folding and processing requires an ordered assembly with specific ribosomal proteins (Finley et al. 1989; Raue and Planta 1991), nonribosomal proteins (Jansen et al. 1991; Tollervey et al. 1991; Girard et al. 1992), and presumably nucleolar snRNPs. The role of nucleolar snRNPs in pre-rRNA processing is slowly being unveiled. By learning more about each nucleolar snRNA, how it assembles, and what is important for function, we hope to define the interactions that produce mature ribosomal subunits in vertebrates.

Materials and methods

Antibodies

Anti-TMG antibodies (Krainer 1988) were obtained from Oncogene Science (Manhasset, NY). Anti-fibrillarin monoclonal antibody (Reimer et al. 1987) was a gift from Eng Tan and Michael Pollard (Scripps Clinic, La Jolla, CA).

Preparation of oocytes for immunoprecipitations

Preparation of oocytes was essentially as described (Peculis and Steitz 1993) except that whole oocytes, enucleated oocytes, or isolated nuclei were disrupted in NET200 (40 mM Tris at pH 7.5, 200 mM NaCl, 0.05% NP-40) with the final NaCl concentration adjusted to 400 mM. Disruption was achieved by pipetting 8–10 oocytes, cytoplasm, or nuclei, five times through a P-200 pipette tip. Following centrifugation for 10 min at 15,000 rpm, the cleared extract was used for immunoprecipitation with anti-fibrillarin antibodies. For anti-TMG immunoprecipitations, the cleared extract was extracted with phenol/chloroform/isomyl alcohol (25:24:1) twice, precipitated with ethanol, and redissolved in NET200. All precipitations were performed in NET200, using protein A–Sepharose (Pharmacia) incubated previously with the appropriate antibody. The recovered RNAs were resolved on 8% denaturing polyacrylamide gels.

Deoxyoligonucleotides

Deoxyoligonucleotides, provided by John Flory (Yale University, New Haven, CT) were synthesized on an Applied Biosystems DNA synthesizer. The following deoxyoligonucleotides were used for site directed mutagenesis: U8-5' end, 5'-GATTAACCTCACTGTGTCTCCCTATAGTGAG-3'; U8-St1M5', 5'-GATTAATACGACTCACTATAGGGATCGTGTCATTCCTTAATCCTTACCTGTTCTCTCC-3'; U8-St1M3', 5'-GATTAATACGACTCACTATAGGGATCGTCAGTGAGGTTAATGGAGTCGACTTCCTCTCCGGG-3'; U8-St1C, 5'-GATTAATACGACTCACTATAGGGATCGTGTCATTCCTTAATGGAGTCGACTTCCTCTCCGGG-3'; U8-Lp1M, 5'-GGAA-CAGGTAAGGTAATTCCTCACTGACG-3'; U8-Lp1-23, 5'-GGAACAGGTAAGGATATACCTCACTGACG-3'; U8-Lp1-45, 5'-GGAACAGGTAAGGGGTAACCTCACTGACG-3'; U8-St2M, 5'-CATCATGTGCTGTTTCTCCCGGAGCCCTTCAAGGTAAGG-3'; U8-St2M3, 5'-CATCATGTGCTGTTTAAAGGGGAGAGGAACAGGTAAGG-3'; U8-St2C, 5'-CAT-

GTGCTGTTTAAGGGGAGCCCTTCAGGTAAGGATT-3'; U8-Lp2M, 5'-GCTGTTTCTCCCACTCAGGAACAGG-3'; U8-Lp2-13, 5'-GCTGTTTCTCCCAATAGGAACAGG-3'; U8-Lp-24, 5'-GCTGTTTCTCCCACTGAGGAACAGG-3'; U8-Lp3M, 5'-GGACCGGAGGACAGAGCTTAGTCTTACTCAGTCTCCTCC-3'; U8-Lp4M, 5'-GCCTGGTAAGACAA-CAGGCACCAGAC-3'; U8-C Boxup, 5'-GCGACTCAGTC-TCCCTCCATTCATCAATAGGATTTTCTCCCGGAGGAGG-3'; U8-Cup-NC, 5'-CCTCCATTCATCATGTACTATGTCT-CCCGGAG-3'; U8-Cup-conM, 5'-CCTCCATTCATCATGT-TAGCATACTCCCGGAG-3'; U8-C BoxM, 5'-GCGACTC-AGTCTCCTCGGACGTAAGTTGTGCTGTTTCTCCCGGA-GGAGG-3'; U8-D BoxM, 5'-CCCCGGGTTGAAAGTCTTA-GATCCTGCC-3'. Mutants were generated by site-directed mutagenesis (Kunkel et al. 1987) using the *E. coli* strain CJ236 (*dut⁻ ung⁻*) or via PCR (Peculis and Steitz 1993) (for St1 mutants).

Oocyte injections

Oocytes for injection were isolated, prepared, injected, and treated essentially as described (Peculis and Steitz 1993). RNA isolations and analysis of *in vivo*-labeled RNAs were performed as described (Peculis and Steitz 1993). Enucleated oocytes were prepared according to Ford and Gurdon (1977). Isolated nuclei were prepared as described by Lund and Paine (1990). U8-depleted oocytes were generated as described (Peculis and Steitz 1993), receiving a single injection of deoxyoligonucleotide U8 39–48. U8 RNA was synthesized *in vitro* in the presence of G-cap or A-cap dinucleotides (Pharmacia) and [α -³²P]UTP and [α -³²P]ATP. After transcription, RNAs were purified by phenol extraction, spun on a G-50 column (Sigma), and precipitated with ethanol. RNAs were dissolved in distilled water at a concentration of 15 ng/ μ l, as determined by specific activity. For the titration rescue experiment, wild-type G-cap RNA was diluted to 7.5 ng/ μ l ($\frac{1}{2}\times$), 3.75 ng/ μ l ($\frac{1}{4}\times$) and 1.5 ng/ μ l ($\frac{1}{10}\times$). Cytoplasmic injections (enucleated oocytes and whole oocytes) were at a volume of 50 nl/oocyte. Injections into isolated nuclei were 14 nl/nucleus. Oocytes were typically incubated for 14 hr after the last injection prior to analysis. For import studies and anti-fibrillarin precipitations (Fig. 4, A and C, respectively) oocytes were incubated for 6 hr prior to analysis. Isolated nuclei were injected then incubated for 4 hr before assaying. Anti-TMG precipitations were performed on RNAs isolated from eight enucleated oocytes 4 hr after injection. For examination of *in vivo*-labeled RNA, nuclei or the corresponding cytoplasmic compartments were collected and total RNA isolated (Peculis and Steitz 1993). Four oocyte (or nuclear) equivalents were loaded per lane and resolved on 1% agarose–formaldehyde gels or 8% denaturing polyacrylamide gels.

S1 nuclease protection assays

Four hours after microinjection of U8 39–48 deoxyoligonucleotide, the U8-depleted oocytes were injected with G-cap U8 RNA, incubated another 3 hr, and injected with 40 nl of [³²P]UTP at 5 μ Ci/ μ l. After a 14-hr incubation at 18°C, nuclei were hand isolated and total RNA extracted. The *in vivo*-labeled RNAs were ethanol precipitated along with unlabeled S1 probe, resuspended in S1 nuclease hybridization buffer (Maniatis et al. 1982), and incubated at 80°C for 15 min, then at 60°C for 3 hr. S1 nuclease (300 units; Pharmacia) was added with the reaction buffer and incubated for 1 hr at 37°C. Products were resolved on a 5% polyacrylamide gel. The S1 probe consisted of the 2.7-kb *EcoRI*–*PstI* fragment from pXlr12 plasmid (Dawid and Wellauer 1976).

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Sequence and structural elements critical for U8 snRNP function in *Xenopus* oocytes are evolutionarily conserved.

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