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The Sequence of the 5′ End of the U8 Small Nucleolar RNA Is Critical for 5.8S and 28S rRNA Maturation

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Ribosome biogenesis in eucaryotes involves many small nucleolar ribonucleoprotein particles (snoRNP), a few of which are essential for processing pre-rRNA. Previously, U8 snoRNA was shown to play a critical role in pre-rRNA processing, being essential for accumulation of mature 28S and 5.8S rRNAs. Here, evidence which identifies a functional site of interaction on the U8 RNA is presented. RNAs with mutations, insertions, or deletions within the 5′-most 15 nucleotides of U8 do not function in pre-rRNA processing. In vivo competitions in *Xenopus* oocytes with 2′*O*-methyl oligoribonucleotides have confirmed this region as a functional site of a base-pairing interaction. Cross-species hybrid molecules of U8 RNA show that this region of the U8 snoRNP is necessary for processing of pre-rRNA but not sufficient to direct efficient cleavage of the pre-rRNA substrate; the structure or proteins comprising, or recruited by, the U8 snoRNP modulate the efficiency of cleavage. Intriguingly, these 15 nucleotides have the potential to base pair with the 5′ end of 28S rRNA in a region where, in the mature ribosome, the 5′ end of 28S interacts with the 3′ end of 5.8S. The 28S-5.8S interaction is evolutionarily conserved and critical for pre-rRNA processing in *Xenopus laevis*. Taken together these data strongly suggest that the 5′ end of U8 RNA has the potential to bind pre-rRNA and in so doing, may regulate or alter the pre-rRNA folding pathway. The rest of the U8 particle may then facilitate cleavage or recruitment of other factors which are essential for pre-rRNA processing.

Ribosome biogenesis is a complex process in eucaryotes involving many components. The ribosomal DNA repeat transcribed by polymerase I yields a single long precursor molecule (pre-rRNA), which is processed and modified to yield mature 18S, 5.8S, and 28S rRNAs (Fig. 1A). These events occur within the nucleolus, where the pre-rRNA assembles, or transiently associates with, ribosomal proteins, nonribosomal proteins, and small nucleolar ribonucleoprotein particles (snoRNPs), which results in mature, folded, processed, and modified rRNAs within the assembled ribosomal subunits (reviewed in references 10 and 22). Very little is known about the signals which direct the endonucleolytic cleavages and exonucleolytic activities that yield the mature 18S, 5.8S, and 28S rRNAs. We are beginning to learn more about the roles that the various nucleolar components play in ribosome biogenesis, particularly the roles of some of the snoRNPs.

Many of the snoRNAs associate with a common protein, fibrillarin, which itself is essential for pre-rRNA processing (13) and is evolutionarily conserved (1, 6, 8, 32). Most snoRNA genes do not exist as independent transcription units; they are encoded within introns of host genes, and their production is dependent upon splicing of the host gene in which they are encoded (reviewed in reference 22). It was observed by many groups that several of the intron-encoded, fibrillarin-associated snoRNAs contain relatively large stretches of perfect or near-perfect complementarity with rRNA, in some cases up to 22 nucleotides of perfect complementarity (reviewed in references 2 and 22). Kiss-Laszlo et al. (16) recently showed that base pairing between the snoRNA and rRNA directs the site of 2′O methylation of a nucleotide in rRNA based on the alignment of the conserved box D element in the snoRNA. Thus, each of the snoRNAs in this class is thought to direct the placement of specific 2′*O*-methyl modifications in rRNA. Interestingly, neither the intron-encoded, fibrillarin-associated snoRNAs nor the methylation events that they direct appear to be critical for pre-rRNA processing (16).

In contrast to the intron-encoded, fibrillarin-associated snoRNAs which are not required for pre-rRNA processing, there are a few snoRNAs which are essential for accumulation of mature rRNAs. These include U3, U8, U14, and U22. U3, the most abundant and ubiquitous snoRNA, is essential for the accumulation of mature 18S rRNA; *Saccharomyces cerevisiae* cells genetically depleted of U3 snoRNA fail to accumulate 18S rRNA (12). *Xenopus* oocytes depleted of U3 by RNase H-mediated degradation show altered pre-rRNA processing (28), and extracts depleted of U3 will not process exogenous pre-rRNA (14). In addition, U3 has the potential to base pair with pre-rRNA (4, 11) and has been cross-linked in vitro to sites within the 5′ external transcribed spacer (5′ ETS) (Fig. 1A) found upstream of 18S in pre-rRNA (31); processing at this site requires the presence of U3 (3). Mutagenesis of this region in rRNA and compensatory changes in U3 in yeast demonstrated that base pairing of U3 to pre-rRNA is essential for efficient accumulation of 18S rRNA (3). U3 has been implicated in facilitating the formation of a pseudoknot structure which exists in mature 18S rRNA (11). U14 is a less abundant, intron-encoded, but still highly conserved, snoRNA (17, 20, 30, 35) which is also essential for accumulation of mature 18S rRNA (18). U14 has the potential to base pair with pre-rRNA at a site within the mature 18S sequence. The ability to bind has been correlated with pre-rRNA processing (19).

In addition to these two evolutionarily conserved snoRNAs, there are two other snoRNAs, U8 and U22, which are also essential for pre-rRNA processing but whose evolutionary distribution is less well known at this point. U22, identified in humans and *Xenopus laevis*, has been shown to be essential for accumulation of 18S pre-rRNA (34). U8 has been character-

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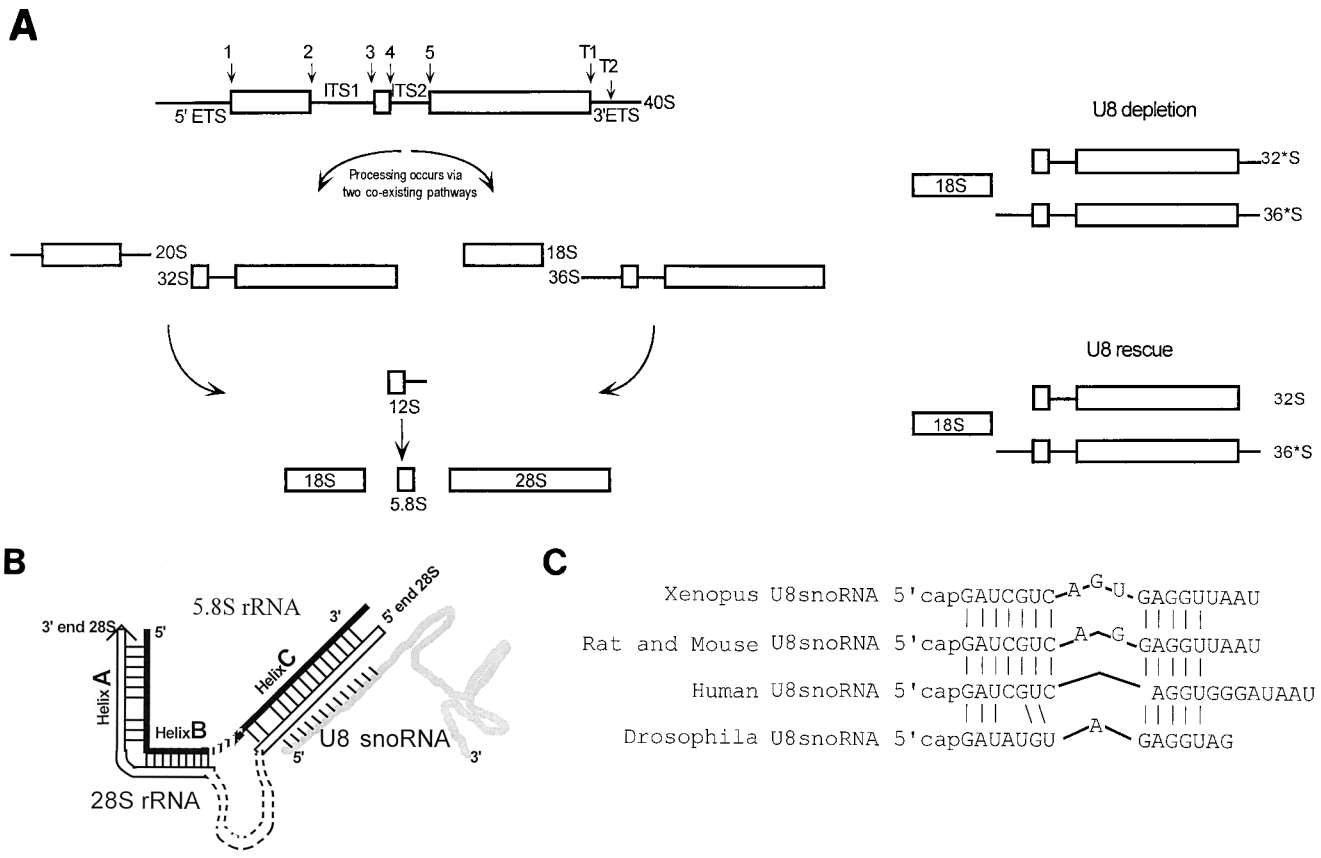


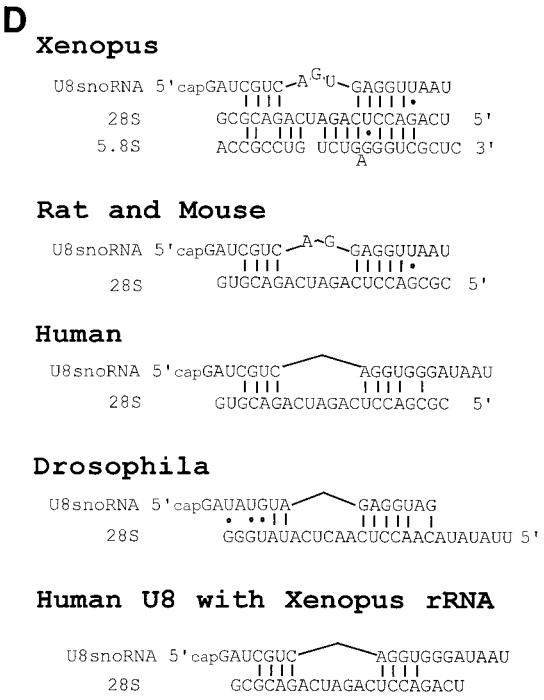


FIG. 1. U8 RNA can base pair with rRNA. (A) Pre-rRNA processing in *Xenopus* occurs by two coexisting pathways in oocytes; both yield mature 18S, 5.8S, and 28S rRNAs by removing the 5′ and 3′ ETSs, ITS1, and ITS2. Numbered arrows indicate the mature termini which result from the various processing cleavages. Shown on the right are the precursors which accumulate in U8-depleted and U8-rescued oocytes. The 32*S and 36*S are abnormal precursors of 32S and 36S rRNAs. (B) A schematic is shown of the three regions in 5.8S which base pair to 28S rRNA, forming the three helices labeled A, B, and C. The longest interaction (helix C) occurs at the 3′ end of 5.8S and the 5′ end of 28S rRNA and is the region of 28S to which U8 snoRNA has complementarity. RNAs are not shown to scale, and dashed lines represent structures omitted for simplicity. Helix coordinates in *Xenopus* rRNA are as follows: helix A contains 5.8S nucleotides 7 to 16 and 28S nucleotides 416 to 426, helix B contains 5.8S nucleotides 27 to 36 and 28S nucleotides 337 to 348, and helix C contains 5.8S nucleotides 142 to 157 and 28S nucleotides 4 to 19. (C) Alignment of the 5′ ends of U8 homologs. The 5′ ends of U8 RNAs were aligned to demonstrate the strong sequence conservation at the 5′ end of the RNA. (D) U8 snoRNA interacts with 28S rRNA via two short helices separated by a 6-nucleotide bulge in 28S rRNA. The panel for *Xenopus* shows U8 RNA as it base pairs with 28S and the 28S-5.8S rRNA interaction, as depicted in helix C. The interaction between 5.8S and 28S is an imperfect 14-nucleotide helix in *Xenopus*. Alignment of the three sequences is for clarity and is not intended to imply triple-helix conformation.

ized in several mammals, including mice (15), rats (27), and humans (33), and in an amphibian, *Xenopus* (24). In *Xenopus*, U8 was shown to be essential for accumulation of both 5.8S and 28S rRNAs by RNase H-mediated degradation of U8 RNA (24).

The focus of this work is the U8 snoRNP. Previously, I demonstrated that depletion of U8 RNA affects and coordinates processing at two distant sites in pre-rRNA, 3 and T1 (Fig. 1A) (24). Cleavage must occur at sites 3 and T1 for subsequent processing events at sites 4 and 5 (24). The inhibition of processing which results from U8 depletion in oocytes can be reversed, and normal rRNA processing can be rescued by microinjection of in vitro-synthesized U8 snoRNA. This result indicates that exogenously supplied U8 RNA can assemble into a functional RNP, which is transported into the nucleus, most likely accumulating within nucleoli, where it participates in processing of pre-rRNA. This finding allowed for extensive functional analysis of mutated U8 RNAs (25). While the mutagenesis studies provided some interesting information

about the structure of the RNP particle, they yielded little information concerning the mechanism by which U8 RNP is able to facilitate processing.

Here I describe a functional site of a base-pairing interaction on the U8 snoRNA. Using 2′*O*-methyl oligoribonucleotides for in vivo competition studies, I show that the 5′ end of U8 must be accessible and able to base pair with a target molecule to allow for correct maturation of 5.8S and 28S rRNAs. Sequence alignment of the 5′-most 15 nucleotides of U8 RNA with pre-rRNA indicated a potential site of interaction between U8 and the 5′ end of 28S rRNA. Mutated U8 RNAs and cross-species U8 hybrid molecules were generated to demonstrate that the potential base pairing between U8 and pre-rRNA is necessary but not sufficient to promote pre-rRNA processing. The base pairing between U8 and 28S in the pre-rRNA would inhibit an interaction between mature 28S and 5.8S, an evolutionarily conserved structure which exists in the mature ribosomes of all organisms (9). Together, these data imply that the 5′ end of U8 acts as an anchor, tethering U8 to 28S and thereby modulating the timing of the 5.8S-28S interaction, which must occur prior to completion of ribosome maturation.

MATERIALS AND METHODS

Deoxyoligonucleotides. Deoxyoligonucleotides provided by George Poy (National Institutes of Health) were synthesized on an Applied Biosystems DNA synthesizer. The following deoxyoligonucleotides were used for injections: U8 39 DNA (5′GCUGUUUCUCCC3′ [2 nucleotides longer than U8 39-48, as described in reference 24]) and U8 5′ DNA (5′AACCUCACUGACG3′, based on the U8 sno-RNA sequence [24]).

Oocyte injections. Oocytes, deoxyoligonucleotides, and RNAs for injection were isolated, prepared, injected, and treated essentially as previously described (24). RNA isolations and analysis of in vivo-labeled RNAs were as described before. U8-depleted oocytes were generated as described previously (24), receiving a single 40-nl cytoplasmic injection of either deoxyoligonucleotide U8 5′ DNA or U8 39 DNA at a concentration of 8 μM as indicated below. U8 RNAs were synthesized in vitro in the presence of GpppG dinucleotides (Pharmacia) and [^{32}P]UTP (Amersham). After transcription, RNAs were purified by phenol extraction and free nuleotide was removed with a G-50 spin column (Sigma) and by ethanol precipitation. RNAs were dissolved in distilled water at a concentration of 15 ng/μl, as determined by specific activity, and 40 nl was injected into the cytoplasm of each oocyte, followed by the injection of [^{32}P]UTP 2 h later. Oocytes were typically incubated for 10 to 14 h after the last injection prior to analysis. For examination of in vivo-labeled RNAs, nuclei, or their corresponding cytoplasmic compartments, were collected and total RNA was isolated (24). Four oocyte (or nuclear) equivalents were loaded per lane and resolved on 1% agarose–formaldehyde gels or 8% denaturing polyacrylamide gels.

In vivo competition experiments. 2′*O*-methyl RNA oligonucleotides were synthesized by George Poy (National Institutes of Health). Oligonucleotide designations and sequences were as follows: U8 5′ sense 2′*O*-methyl oligonucleotide, 5′CGUCAGUGAGGUUbbb3′; U8 5′c 2′*O*-methyl oligonucleotide, 5′AACCUCACUGACGbbb3′; 28Sc 2′*O*-methyl oligonucleotide, 5′GUCUGAUCUGAGGUbbb3′; 5.8Sc 2′*O*-methyl oligonucleotide, 5′GACCCUCAGACAGGCbbb3′; and U8 39 2′*O*-methyl oligonucleotide, 5′GCUGUUUCUCCCbbb3′. The bbb refers to three biotin residues conjugated to the oligonucleotide during synthesis which were used in streptavidin-agarose selections to examine the specificity of an oligonucleotide for the target RNAs. Oocytes received 30 nl of a 2′*O*-methyl RNA oligonucleotide at 5 μM in the cytoplasmic compartment. After 2 h, oocytes were injected with [^{32}P]UTP and incubated for 10 to 14 h at 18°C. Nuclei were hand isolated, and total RNA was isolated and resolved in denaturing agarose-formaldehyde gels or polyacrylamide gels.

Mutant U8 RNAs. The 5′-end-shortened U8 RNAs and the U8 strong (Str) mutants were generated via PCR with the wild-type U8 cDNA as the template and the U8-31 3′ oligonucleotide (24). The 5′ DNA oligonucleotides used were U8PCRG4 (5′GATTAATACGACTCACTATAGTCAGTGAGGTTAATC3′), U8PCRG8 (5′GATTAATACGACTCACTATAGTGAGGTTAATCC3′), U8PCRStr3 (5′GATTAATACGACTCACTATAGGGATCGTCAGATCTGAGGTTAATC3′), U8PCRStr3+3 (5′GATTAATACGACTCACTATAGGGATCGTCAGATCTGAGGTTAATCCTTAGATCCTGTT3′), U8 5′ Str (5′GATTAATACGACTCACTATAGGCCGCGTCAGTGAGG3′), U8 StrLp1 (5′GATTAATACGACTCACTATAGGGATCGTTCAGTGAGGTCTGACCTTACC3′), and U8St1c(4dst) (5′GATTAATACGACTCACTATAGGGATCGTCAGTCUCCTTAATGGAGACCTGTTCC3′).

Human and hybrid U8 RNAs. Human U8 cDNA was synthesized by reverse transcription-PCR with RNAs which coprecipitated with fibrillarin (24) and oligonucleotides complementary to the 5′ and 3′ ends of the human U8 RNAs designated 5′hU8 (GGCGCCTAATACGACTCACTATAGGGATCGTCAGGTGGGATA3′) and 3′hU8 (5′CCCGGGTTCGAATCAGATAGGAGCAATCAG3′). The 5′ oligonucleotide contained a synthetic T7 promoter followed by three G residues to enhance transcription in vitro. The 3′-end oligonucleotide contained a *Bst*BI site which would allow perfect sense-strand RNAs to be synthesized in vitro by T7 run-off transcription. The 5′hybU8X oligonucleotide (5′GGCGCATAATACGACTCACTATAGGGATCGTCAGTGAGGTTAATCCTTACC3′) and the 5′hybU8H oligonucleotide (5′GGCGCATAATACGACTCACTATAGGGATCGTCAGGTGGGATAATCCTTACC3′) were used to generate the human-*Xenopus* and *Xenopus*-human hybrid RNAs by PCR. With the appropriate cDNA template, 5′- and 3′-end-oligonucleotide (hU8 [described above] and U8-31 3′ [24]) PCR products which contained a T7 promoter, three exogenous G residues, the first 40 nucleotides of U8 RNA, and the remainder of the molecule from the species producing the hybrid molecule were obtained. PCR was performed as previously described (24), and the products were cloned into a pSP64 vector, linearized with *Bst*BI, and used as templates for in vitro transcription.

RESULTS

Evolutionary conservation of the 5′ end of U8 snoRNA. In an attempt to learn more about the molecular mechanisms by which U8 snoRNA acts, I identified and sequenced putative U8 homologs in additional species phylogenetically diverged from mammals and amphibians. A U8 homolog in *Drosophila melanogaster* was identified by coimmunoprecipitation with antifibrillarin antibodies and subsequent immunoprecipitation with antibodies directed against the trimethylguanosine cap present on the vertebrate U8 homologs. As with the results previously obtained with vertebrate cell extracts (24), only three RNAs were enriched for by this method. Direct enzymatic RNA sequencing of the other two RNAs identified them clearly as U3 and U13 homologs (23a). Sequencing of the putative U8 homolog of *Drosophila* revealed limited sequence conservation compared to that of the U8 RNAs from vertebrates, with the exception of readily identifiable C and D boxes, the conserved elements found in all snoRNAs that associate with fibrillarin (reviewed in reference 22). In addition, the 5′-most 15 nucleotides of the putative U8 RNA in *Drosophila* bore strong similarity to those of *Xenopus* and mammalian U8 RNAs (Fig. 1C), although the rest of the RNAs show little sequence identity (23a). Thus, the evidence that the *Drosophila* RNA is a true U8 homolog includes fibrillarin association, the presence of a hypermethylated 5′ cap, conserved C and D box sequences, and a 5′ terminus with similarity to U8 in other species.

The high conservation of 5′-end sequences between vertebrate and *Drosophila* U8 homologs implicated the 5′ end of U8 RNA in playing a functional role; evolutionarily conserved regions often reflect functional domains. The first 15 nucleotides of *Xenopus* U8 snoRNA were aligned with *Xenopus* pre-rRNA to identify a potential site of direct interaction between U8 RNA and pre-rRNA. Several potential sites of interaction were identified, but only one maintained the complementarity across the evolutionary diverged species from which U8 sequences were available. U8 in *Xenopus* has the potential to base pair with the 5′ end of 28S rRNA in a region where 28S normally base pairs with the 3′ end of 5.8S rRNA in the mature ribosome (see helix C in Fig. 1B and D). The proposed *Xenopus* U8-28S rRNA interaction consists of 10 of 16 nucleotides of complementarity (relative to 28S) forming two short helices separated by a bulge. The bulged nucleotides in rRNA, AGAUCA, are conserved among the vertebrates examined. The potential for U8 to base pair with and maintain the bulged nucleotides in rRNA is conserved in every species for which the U8 sequence is known: mouse, rat, human, *Xenopus*, and *Drosophila* (Fig. 1D) (15, 23a, 24, 27, 33). The number of nucleotides in U8 which oppose the rRNA bulge varies between species, but the ability for U8 to base pair is maintained.

The relatively weak interaction between U8 and 28S is replaced by an imperfect 14-bp helix between 5.8S and 28S in the mature ribosome in *Xenopus*. Potential implications of this alignment and the role of the bulged nucleotides are presented in Discussion.

5′ end of U8 RNA is accessible in U8 RNP. The interaction between U8 and pre-rRNA described here suggests that the 5′ end of U8 RNA may be accessible in vivo in the endogenous U8 RNP particle. If this is the case, the U8 RNP should be targeted for degradation by endogenous RNase H if a deoxyoligonucleotide complementary to the 5′ end of U8 RNA is injected into oocytes. Previously, three different antisense DNA oligonucleotides complementary to U8 RNA were microinjected into *Xenopus* oocytes in an attempt to deplete the cell of the U8 RNP (24). The efficiency with which each deoxyoligonucleotide was able to target the endogenous U8 RNA varied significantly (24), presumably because not all regions on U8 RNA are equally accessible in the RNP in vivo. To test whether the 5′ end of U8 RNA is accessible in the RNP, *Xenopus* oocytes were microinjected with a deoxyoligonucleotide complementary to U8 nucleotides 3 to 15 (U8 5′ DNA), which span the region in U8 complementary to 28S. As a positive control for U8 depletion, a different set of oocytes was injected with the U8 39 DNA oligonucleotide previously shown to be very efficient at depleting the endogenous U8 RNA. Total nuclear RNA was extracted from injected or uninjected oocytes, and U8 RNA was examined by Northern blot analysis (Fig. 2A) and primer extension (data not shown). No full-length U8 RNA was detected in oocytes injected with either the U8 39 DNA oligonucleotide (lanes 2) or the U8 5′ DNA oligonucleotide (lane 3), whereas control (no oligonucleotide) oocytes had a band corresponding to the endogenous full-length U8 RNA (Fig. 2A, lane 1).

Oocytes depleted of U8 RNA by the U8 5′ DNA oligonucleotide were examined for effects upon pre-rRNA processing. Following injection of the U8 39 DNA or U8 5′ DNA oligonucleotides, oocytes were injected with [^{32}P]UTP and incubated for approximately 10 h. In vivo-labeled RNAs from oocytes injected with the U8 5′ DNA oligonucleotide showed a pattern of inhibited pre-rRNA processing identical to that obtained with the U8 39 DNA oligonucleotide previously described (Fig. 2B, lanes 2 and 3) (24). Sites 3, 4, 5, and T1 were not used, resulting in an absence of mature 28S rRNA and an accumulation of the abnormal precursors 32*S and 36*S (Fig. 1A). This inhibition could be released and rRNA processing could be rescued by subsequent injection of in vitro-synthesized *Xenopus* U8 RNA, demonstrating that the effect on processing was due to targeted destruction of U8 RNA and not a nonspecific effect of the injection or the oligonucleotide (see Fig. 5A and data not shown).

The oligonucleotide complementary to the 5′ end of U8 is very similar in sequence to 28S and thus potentially complementary to 5.8S rRNA as well. However, due to the lack of perfect complementarity between U8 and 28S, the interaction between the U8 5′ DNA oligonucleotide and 5.8S rRNA would be, at most, 5 of 15 bp, making it a very unlikely target for RNase H activity. Since the DNA oligonucleotides have a fairly short half-life in vivo, the specificity of the U8 5′ DNA oligonucleotide was examined by injecting the oligonucleotide into oocytes which had already accumulated in vivo-labeled ^{32}P-rRNA. After a 1-h incubation, the rRNA was examined. Under these conditions, there was no indication that the U8 5′ DNA oligonucleotide was affecting 5.8S or 28S rRNAs; however, U8 depletion was nearly complete in this period (data not shown and Fig. 2A and B).

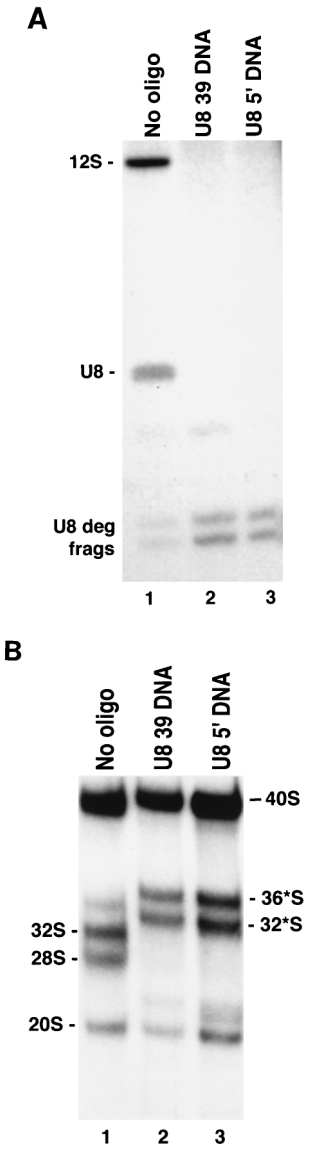
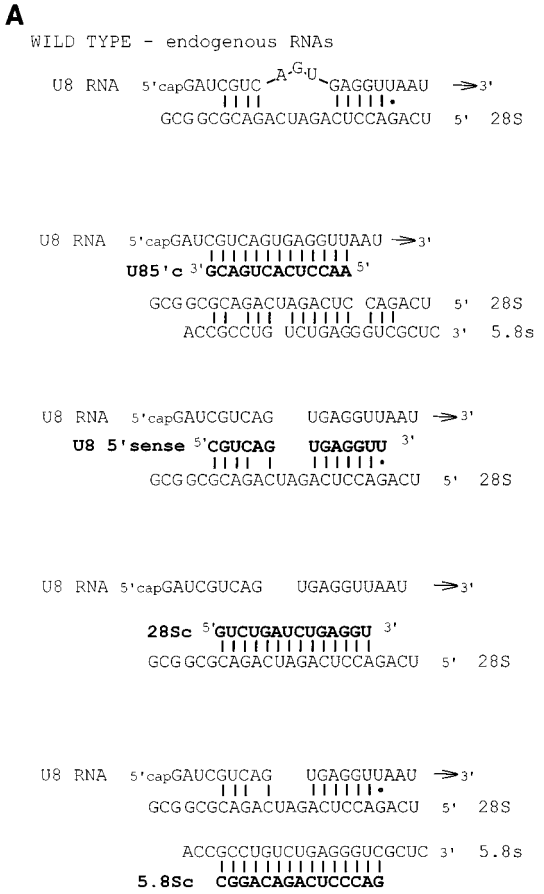


FIG. 2. The 5′ end of U8 snoRNA is accessible in vivo. Oocytes were microinjected with an antisense DNA oligonucleotide complementary to nucleotides 3 to 15 of *Xenopus* U8 RNA (U8 5′ DNA) or with an antisense DNA oligonucleotide complementary to an internal region within U8 (U8 39 DNA) and then with [^{32}P]UTP to in vivo label RNA. (A) Northern blot probed for U8, showing that the endogenous U8 RNA was very efficiently depleted by both the 5′ and internal oligonucleotides (oligo). In vivo-labeled 12S rRNA accumulated only in the no-oligonucleotide sample, when pre-rRNA processing was inhibited (Fig. 1). U8 deg frags, U8 degradation fragments. (B) In vivo-labeled RNAs resolved on a denaturing agarose gel. Total RNA was isolated from oocytes depleted of their U8 via either the U8 5′ DNA oligonucleotide or the internal U8 39 DNA oligonucleotide. The 18S rRNA was run off the bottom of the gel shown, but other experiments showed that mature 18S accumulated normally. Lanes 1, no oligonucleotide; lanes 2, U8 39 DNA oligonucleotide; lanes 3, U8 5′ DNA oligonucleotide. 32*S and 36*S, 32S and 36S abnormal precursors.

In vivo competition studies using 2′*O*-methyl oligoribonucleotides. In vivo competition studies using 2′*O*-methyl oligoribonucleotides were carried out to test whether U8 RNA interacts with its in vivo target via base pairing. Since 2′*O*-methyl oligoribonucleotides are not substrates for either RNA- or DNA-specific nucleases, their greater stability makes them more effective than natural RNA antisense molecules for studying small nuclear RNA (snRNA)-RNA base pairing interactions in



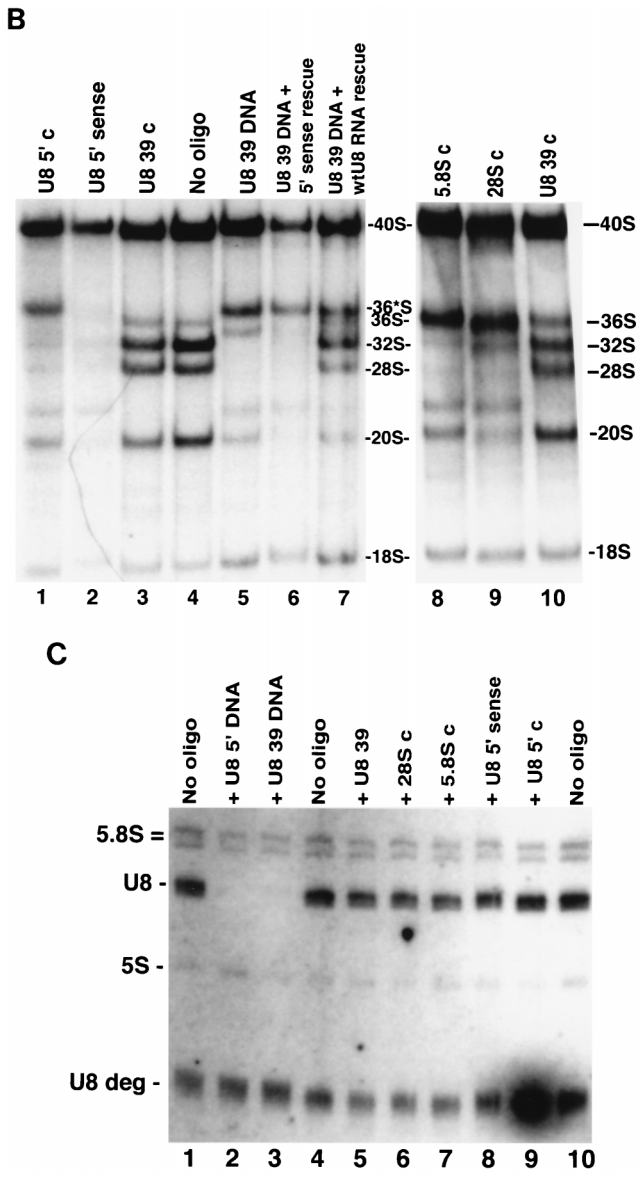


FIG. 3. The 5′ end of U8 is a functional site of interaction. In vivo competitions designed to examine the functional site of interaction between U8 and pre-rRNA are shown. (A) Base pairings in U8. The 2′*O*-methyl oligoribonucleotides (in boldface type) and the interaction each was designed to disrupt are depicted. First pairing, U8 snoRNA base paired to 28S rRNA; second pairing, U8 5′c oligonucleotide base paired to U8 RNA; third pairing, U8 5′ sense oligoribonucleotide base paired to 28S rRNA; fourth pairing, 28Sc oligoribonucleotide base paired to 28S rRNA; fifth pairing, 5.8Sc oligoribonucleotide base paired to 5.8S rRNA. (B) Oocytes were injected with the 2′*O*-methyl oligoribonucleotide (lanes 1 to 3 and 8 to 10) or DNA oligonucleotide (lanes 5 to 7) indicated and then injected with [^{32}P]UTP. In vivo-labeled RNAs resolved on a denaturing agarose gel are shown. Lanes 6 and 7 show the results of rescue experiments where the U8-depleted oocytes received injections of either U8 5′ sense 2′*O*-methyl oligoribonucleotides or full-length wild-type (wt) U8 RNA prior to the injection of [^{32}P]UTP. The band running just above 20S is of unknown identity but is probably a stable pre-rRNA degradation fragment. (C) Northern blot of in vivo-labeled RNAs from some of the oocytes shown in panel B, probed for both 5.8S and U8 RNAs. The in vivo-labeled 5S rRNA is seen and serves as a control for equal loadings of lanes. Prior to probing, this 5S band, which was in vivo labeled to low specific activity, was the only band seen in this region of the gel. The degradation fragment labeled U8 deg is a stable 3′ 100-nucleotide fragment of U8 RNA which has previously been described (25). oligo, oligoribonucleotide.

vitro (7, 29). The 2′*O*-methyl oligoribonucleotides were used here to compete with and for U8 binding to test if the proposed U8-28S interaction exists in vivo. The use of 2′*O*-methyl oligoribonucleotides for in vitro competition studies using HeLa cell extracts has previously been described. Seiwert and Steitz (29) used 2′*O*-methyl oligonucleotides complementary to the 5′ end of U1 snRNA to block binding of U1 snRNA at the 5′ splice site and inhibit splicing. Cotten et al. (7) used 2′*O*-methyl oligoribonucleotides complementary to U7 snRNA to inhibit processing of histone pre-mRNAs. Thus, this approach has previously shown that 2′*O*-methyl oligonucleotides are useful for defining functional sites of interaction via base pairing.

For the in vivo competition studies, several 2′*O*-methyl oligoribonucleotides were designed (Fig. 3A), each with a different target and specificity. To test if the 5′ end of U8 snoRNA is involved in a base-pairing interaction, a 2′*O*-methyl oligoribonucleotide complementary to the 5′ end of U8 snoRNA (U8 5′c) was designed to block the 5′ end of U8 RNA and prevent it from binding its target. Since the target of U8 RNA binding is proposed to be the 5′ end of 28S rRNA, two different 2′*O*-methyl oligoribonucleotides complementary to the 5′ end of 28S and varying in specificity were generated. The U8 5′ sense oligoribonucleotide was identical in sequence to the 5′ end of U8 RNA and competed with U8 RNP for binding to pre-rRNA. The 28Sc 2′*O*-methyl oligoribonucleotide was perfectly complementary to the 5′ end of 28S rRNA and should also bind 28S rRNA, preventing the U8 RNP from binding. Use of a 2′*O*-methyl oligoribonucleotide complementary to the 3′ end of 5.8S rRNA (5.8Sc) addressed whether the 5.8S-28S interaction was necessary for processing to proceed. Finally, a 2′*O*-methyl oligoribonucleotide complementary to an internal, accessible region of U8 RNA (U8 39c) was synthesized to

examine the specificities of the 2′*O*-methyl oligoribonucleotides and their interaction.

The 2′*O*-methyl oligoribonucleotides were microinjected into oocytes, and then [^{32}P]UTP was injected. After an incubation which served as the in vivo labeling period, total RNA was obtained from hand-isolated nuclei and resolved in denaturing gels. The newly synthesized rRNAs were detected by autoradiography (Fig. 3B). Northern blot analysis was performed to show that, as expected, the various 2′*O*-methyl oligoribonucleotides affected neither the level nor the integrity of U8 RNA (Fig. 3C) or other targeted RNAs (i.e., 5.8S or 28S rRNA [data not shown]).

U8 5′c 2′*O*-methyl oligoribonucleotide. Injection of 2′*O*-methyl oligonucleotides complementary to the 5′ end of U8 RNA (U8 5′c) (Fig. 3A, second pairing) resulted in efficient inhibition of pre-rRNA processing (Fig. 3B, lane 1). The 36*S precursor, characteristic of U8 depletion, was present, but neither mature 28S, the 32S precursor, nor the 32*S precursor accumulated. The appearance of only the 36*S precursor was previously seen with a very efficient depletion of U8 RNA and is typically seen after two sequential injections of the U8 39 DNA oligonucleotide (24). With a single DNA oligonucleotide injection, a small amount of residual processing at or near site 3 was observed (Fig. 1A), resulting in an accumulation of a small amount of 32*S (Fig. 3B, lane 5). Thus, the U8 5′c 2′*O*-methyl oligoribonucleotide inhibits pre-rRNA processing, producing an accumulation of intermediates similar to that seen in oocytes depleted of U8 RNA. Since the U8 RNP was not degraded with the 2′*O*-methyl oligoribonucleotide, processing is presumably inhibited because the pairing of U8 snoRNA with its target was blocked.

U8 5′ sense 2′*O*-methyl oligoribonucleotide. 2′*O*-methyl oligoribonucleotides identical to the 5′ end of U8 (U8 5′ sense) (Fig. 3A, third pairing) were generated to compete for binding with the endogenous U8 RNP. The U8 5′ sense oligoribonucleotide had a slightly different effect upon pre-rRNA processing. Again, no mature 28S rRNA was observed, but no precursors to 28S accumulated with this oligoribonucleotide either (Fig. 3B, lane 2). Maturation of 18S did not appear to be significantly affected (lane 2 is slightly underloaded). The U8 5′ sense oligoribonucleotide, being identical to the 5′ end of U8, could base pair with the 5′ end of 28S with an affinity similar to that of U8 RNA itself (Fig. 3A, second pairing). This prevented 5.8S (and U8 RNA) from binding to 28S. With the U8 5′ sense oligonucleotide, the 36*S and 32*S intermediates did not accumulate and presumably were degraded. This instability of pre-rRNA is apparently induced by the U8 5′ sense 2′*O*-methyl oligoribonucleotides, since the intermediates do accumulate in the absence of U8 RNA and when the 5′ end of U8 is blocked with a complementary 2′*O*-methyl oligoribonucleotide (U8 5′c; see above).

28Sc 2′*O*-methyl oligoribonucleotide. To further test whether U8 base pairs with the 5′ end of 28S in pre-rRNA, a 2′*O*-methyl oligoribonucleotide complementary to the 5′ end of 28S rRNA (28Sc) (Fig. 3A, fourth pairing) was examined. This oligonucleotide has perfect complementarity to 28S rRNA and should bind 28S with greater affinity than U8 RNA (or the U8 5′ sense oligoribonucleotide) (Fig. 3A, first pairing). Since the 28Sc oligoribonucleotide is nearly identical to the 3′ end of 5.8S, it was also expected to inhibit the 5′ end of 28S from base pairing with the 3′ end of 5.8S during ribosome biogenesis.

The presence of the 28Sc oligoribonucleotide very effectively inhibited pre-rRNA processing (Fig. 3B, lane 9). The effect was similar to that observed in U8-depleted oocytes: no 28S rRNA was formed, as evidenced by the accumulation of 32*S and 36*S. However, the presence of the 28Sc oligoribonucleotide was not as destructive to pre-rRNA as the U8 5′ sense oligoribonucleotide, perhaps because of the absence of a bulge in the paired 28S structure. The only precursor to 28S which accumulated with the 28Sc oligoribonucleotide injections was 36S, again reflecting the pattern seen with a very efficient RNase H-mediated depletion of the endogenous U8 RNP.

5.8Sc 2′*O*-methyl oligoribonucleotide. To examine whether the interaction between 5.8S and 28S is necessary for pre-rRNA processing, a 2′*O*-methyl oligoribonucleotide complementary to the 3′ end of 5.8S (5.8Sc) was injected into oocytes. This oligonucleotide, which is nearly identical to the 5′ end of 28S rRNA (Fig. 3A, fifth pairing), efficiently inhibits pre-rRNA processing (Fig. 3B, lane 8). The pattern of pre-rRNA which accumulates is similar to that seen when the endogenous pool of U8 RNA is depleted from oocytes via RNase H activity (Fig. 3B, lane 5). This result implies that U8 may bind 28S in pre-rRNA but that the resulting helix-bulge-helix structure must be replaced by an interaction between 5.8S and 28S which must occur before processing can proceed (see Discussion).

There are four lines of evidence demonstrating that the effects on pre-rRNA processing observed in the presence of the 2′*O*-methyl oligoribonucleotides were due to competition at functional sites of base pairing interactions. First, the specificities of the 2′*O*-methyl oligoribonucleotides were examined by injection of DNA oligonucleotides of identical sequences into oocytes and by analysis of results at early time points. The 5.8Sc DNA oligonucleotide did not affect endogenous U8 RNA levels, the U8 5′c DNA oligonucleotide did not make 5.8S a target for RNase H, and the 28Sc DNA oligonucleotide did not affect 5.8S or U8 RNA (data not shown). Second, to rule out any nonspecific effects of the 2′*O*-methyl oligoribonucleotides on pre-rRNA processing, a 2′*O*-methyl oligonucleotide complementary to an internal region of U8 (U8 39c), which is not proposed to be involved in any direct RNA-RNA interaction, was injected into oocytes. This oligonucleotide did not inhibit rRNA processing (Fig. 3B, lane 3). This internal region is accessible in the U8 RNP in vivo; a deoxyoligonucleotide complementary to this region very efficiently directs RNase H-mediated degradation of U8 RNA (U8 39 DNA) (Fig. 3C, lane 3). Third, the 2′*O*-methyl oligonucleotides were further examined and shown to be specific for their targets by selection of the biotin-conjugated oligonucleotide and associated RNAs from extracts with streptavidin-agarose (data not shown).

Finally, results at longer time points were examined after microinjection of the corresponding DNA oligonucleotides (identical in sequence to the 2′*O*-methyl oligoribonucleotides) into oocytes. The results with the 5.8Sc oligoribonucleotide were of particular interest because of its potential complementarity to U8 RNA. Injection of the 5.8Sc DNA oligonucleotide did not affect endogenous U8 RNA levels, nor did it inhibit pre-rRNA processing at the 10-h time point (data not shown). Since DNA oligonucleotides have a short half-life in vivo, the oligonucleotide may target 5.8S rRNA, but after all of the oligonucleotide degrades (within 3 to 4 h) (23, 24, 26), newly transcribed 5.8S rRNA can again accumulate. However, if U8 RNA was unintentionally targeted, the significantly lower rate of U8 RNA synthesis in vivo (24) would prevent replacement of U8 RNA, so no mature 28S rRNA or 12S precursor to 5.8S would accumulate. Since mature 28S rRNA did accumulate after injection of the 5.8Sc oligonucleotide, the U8 RNA must not be targeted. Likewise, injection of DNA oligonucleotides complementary to 28S or identical to the 5′ end of U8 RNA affected neither pre-rRNA processing nor the endogenous U8 RNA at the longer incubation time point, suggesting that the inhibition of pre-rRNA processing seen in the presence of

2′*O*-methyl oligoribonucleotides was due to in vivo competitions of base paired interactions and not an indirect effect on U8 RNA.

Pre-rRNA processing is not directed by U8 RNA–pre-rRNA interactions alone. The data described above suggest that although the 5′ ends of both U8 and 28S must be accessible in vivo, a simple RNA-RNA interaction is not sufficient to facilitate processing. Pre-rRNA processing was inhibited in oocytes which received injections of the U8 5′ sense oligoribonucleotide (Fig. 3B, lane 2). This result is significant, because this RNA molecule should have very closely mimicked and could have substituted for U8 if a simple RNA-RNA interaction was sufficient to direct processing. This can additionally be demonstrated by the observation that the rRNA processing events inhibited in U8-depleted oocytes could not be rescued by a subsequent injection of the U8 5′ sense 2′*O*-methyl oligoribonucleotide (Fig. 3B, lane 6) but rather required the intact full-length U8 RNA (lane 7). This implies that there are proteins or additional RNA structures (in the U8 RNP and/or the pre-rRNP) which may mediate the interaction between U8 and pre-rRNA, facilitate cleavage, or enhance U8 RNA dissociation to allow 5.8S rRNA access to 28S.

Delineation of a region of U8 RNA essential for function. Because the sequence of the 5′ end of U8 RNA was implicated in an intramolecular base-pairing interaction, the role of the 5′ end of U8 in pre-rRNA processing was more directly examined by introducing mutations in this region (Fig. 4). Previously, a series of U8 RNAs were generated and examined for RNA stability, particle assembly, and functionality (25). Seven of these contained mutations within the first 25 nucleotides of U8 RNA. Most of these altered U8 RNAs, including those with mutations in the first loop of U8 (Lp1M) (Fig. 5, lane 8), the 5′ end of U8 (U8 5′ M) (Fig. 5, lane 4), and the sequence of the first stem, were shown not to function in vivo (25). The functional capabilities of U8 RNAs with mutations in this region were tested more rigorously by altering U8 RNA at sites within the proposed region of interaction. The altered U8 RNAs were injected into U8-depleted oocytes and assayed for the ability to rescue pre-rRNA processing. Figure 4 diagrams some of the additional mutations generated in U8 and the predicted interactions with pre-rRNA which would result. Deletion of the first 4 nucleotides of U8 RNA (RNAs that initiate at the G at position 5 in the U8 RNA [Δ4U8 RNA]) resulted in an RNA that was functional in vivo (Fig. 5, lane 9). This RNA was stable and associated with fibrillarin (data not shown) and could efficiently rescue rRNA processing in U8-depleted oocytes. The Δ4U8 RNA deleted only 1 bp from the proposed interaction (Fig. 4). However, deletion of 8 nucleotides from the 5′ end of U8 (RNAs that initiate at the G at position 9 in the Δ8U8 RNA) resulted in a nonfunctional RNA (Fig. 5, lane 10), although this RNA was stable in vivo and associated with fibrillarin (data not shown). The Δ8U8 RNA eliminated one-half of the proposed interaction, as only one of the two short helices could form.

The U8St1c(4dst) mutation alters the 4 terminal nucleotides at the distal end of the first stem (GAGG to CUCC) to prevent formation of one of the short helices proposed (Fig. 4). This mutation also contains compensatory changes on the other side of that stem which maintain the ability of the U8 stem to form but allow only one-half of the proposed interaction with 28S to occur (Fig. 4). This RNA failed to function in vivo (data not shown), indicating that both the 5′ and the 3′ helix must form to facilitate pre-rRNA processing.

The importance of the internal bulge was addressed by the U8 Str3 and U8 Str3+3 mutations, which were designed to reduce the size of the internal bulge (Fig. 4). In the U8 Str3 RNA, 3 nucleotides which base pair with 28S were added to U8 within the region of the bulge, thus partially closing the bulge. Because the addition of 3 nucleotides could potentially destabilize the U8 RNP structure, the U8 Str3+3 mutation was generated. This mutation added the same 3 nucleotides within U8 as the U8 Str3 mutation, along with 3 more nucleotides to the opposite side of the proposed first stem in U8, thus lengthening the stem but not disrupting the structure of the U8 RNA (Fig. 4). Both RNAs were stable in vivo and associated with fibrillarin, but neither could rescue pre-rRNA processing (Fig. 5, lane 6).

To examine whether the strength of the proposed U8-28S interaction is important, independent of the internal bulge, two other mutations which also strengthened the interaction between U8 and 28S RNAs were generated without altering the bulged nucleotides. These substitution or insertion mutations provided U8 RNA with 3 additional nucleotides of complementarity to 28S, at either at the 5′ (U8 5′ Str) or the 3′ (U8 Lp1Str) end of the proposed interaction (Fig. 4). In neither

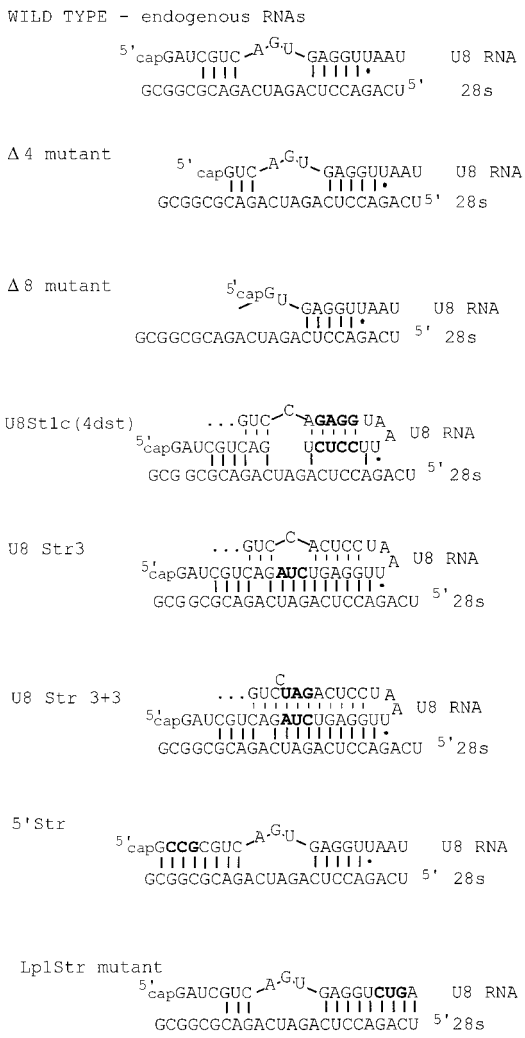


FIG. 4. U8 RNA mutants can base pair with 28S rRNA. Schematics of various mutated U8 RNAs designed to assay the requirement of U8-28S base pairing for processing and shown. The wild-type endogenous interaction is shown at the top. Other panels show the sequence alterations in U8 RNA (in boldface type) and the predicted changes in 28S interactions. Nomenclature for the mutants is described in the text.

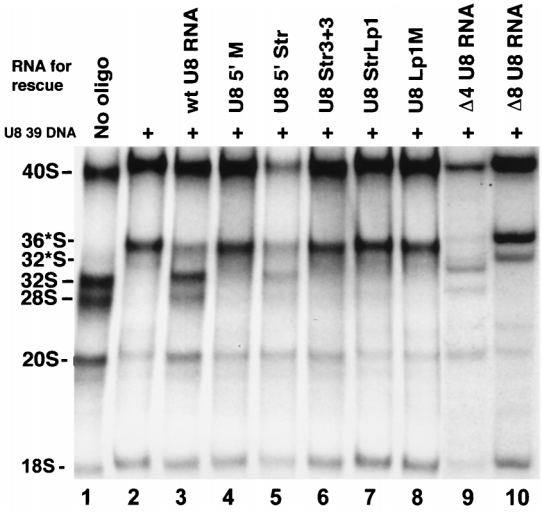


FIG. 5. *Xenopus* U8 RNA can rescue processing when the 5′ end of U8 RNA base pairs to 28S. In vivo-labeled RNAs resolved on a denaturing agarose-formaldehyde gel are shown. Total RNA was isolated from oocytes treated as indicated above the lanes. The + designation indicates that the oocytes were depleted of their endogenous pool of U8 RNA by antisense U8 deoxyoligonucleotide injection prior to the injection of the U8 RNA variant indicated. Note that lane 9 is underloaded. oligo, oligonucleotide; wt, wild-type; 32*S and 36*S, 32S and 36S abnormal precursors.

case did RNA from these U8 Str mutations function efficiently in vivo (Fig. 5, lanes 5 and 7, and data not shown). These data suggest that a transient interaction with 28S appears essential for U8 function, but if the interaction between U8 and 28S is too strong, U8 might effectively prevent 5.8S from binding to 28S. Studies directly addressing this theory are under way.

The U8 5′ Str construct had limited function in vivo (Fig. 5, lane 5); a very small amount of mature 28S rRNA was observed. However, many replications of this experiment showed that rescue is typically incomplete in that the amount of mature 28S was reduced and there was still an accumulation of 32*S, as is typical of the U8-depleted pathway. Injecting more of the U8 5′ Str RNA did not enhance the efficiency of the rescue (data not shown). A possible explanation for the limited activity of this mutation is presented in Discussion.

The sequence of the 5′ end of U8 RNA is necessary but not sufficient for U8 RNP function. Examination of the base-pairing interaction between human U8 RNA and *Xenopus* 28S rRNA (Fig. 1D) reveals that the human U8 RNA should be about as effective at directing U8 to its in vivo target as *Xenopus* U8. However, the human U8 RNA does not function in *Xenopus* (Fig. 6B, lane 3).

A series of U8 hybrid molecules were designed to determine the basis for the inability of human U8 RNA to function in *Xenopus* (Fig. 6A). Since the 5′-most 40 nucleotides are highly conserved (~90% identity among vertebrates), differences in sequence or structure at the 3′ end of the U8 RNA (only ~45% identity) were believed to be responsible. To further analyze these differences, hybrid RNAs were generated by replacing the 5′-most 40 nucleotides of *Xenopus* U8 with the human U8 RNA sequence, and vice versa, essentially replacing the first two stems in each RNA (Fig. 6A). Hybrid U8 RNAs synthesized in vitro were injected into U8-depleted oocytes. After an incubation, the stability, fibrillarin association, and ability of the hybrid RNAs to function in vivo were assayed.

The human-*Xenopus* hybrid RNA (5′ end of human U8 and 3′ end of *Xenopus* U8) was as stable in vivo as the wild-type *Xenopus* U8 RNA (Fig. 6C, lanes 2 and 4) and coprecipitated with fibrillarin as efficiently (data not shown). In addition, this hybrid molecule functioned in pre-rRNA processing as effectively as the wild-type U8 RNA (Fig. 6B, lanes 4 and 6), indicating that the proposed 8 of 15 bp may be sufficient to direct U8 RNA to the 5′ end of 28S in pre-rRNA and facilitate processing (Fig. 1B). Therefore, the failure of the human U8 RNA to rescue pre-rRNA processing in *Xenopus* was not simply an inability to base pair with *Xenopus* pre-rRNA.

The *Xenopus*-human hybrid RNA (5′ end of *Xenopus* U8 and 3′ end of human U8) was neither as stable nor as readily coprecipitable with fibrillarin as the wild-type *Xenopus* U8 (Fig. 6C, lane 3, and data not shown). In addition, this hybrid molecule did not function as efficiently in vivo as the *Xenopus* wild type or the human-*Xenopus* hybrid (Fig. 6B, compare lanes 5 with lanes 4 and 6). Although there was a slight accumulation of 28S, the rescue was not complete, as demonstrated by the presence of 32*S, normally seen in U8-depleted oocytes. This indicates that while 10 of 16 base pair interactions between *Xenopus* U8 and pre-rRNA may be able to direct U8 to the proper site in pre-rRNA, the ability to base pair is not sufficient for efficient processing.

These data indicate that the human U8 and the *Xenopus*-human hybrid RNAs do not function in *Xenopus* due to differences in the sequences or structures of the 3′ ends of U8 RNAs or in the proteins associating with, or recruited by, this region. This is supported by the observation that the 3′ end of *Xenopus* U8 (in the wild-type *Xenopus* and human-*Xenopus* RNAs) can exist as a nonfunctional stable degradation product of approximately 100 nucleotides in length (Fig. 6C, lanes 2 and 4) (see also references 24 and 25). This fragment corresponds to the 3′ 100 nucleotides of U8 RNA resulting from a cleavage within the single-stranded region in the middle of the molecule. The *Xenopus* U8 degradation fragment is relatively stable and fibrillarin precipitable but does not function in pre-rRNA processing (24, 25). However, when the 3′ end of the molecule consisted of the human sequence (human or *Xenopus*-human RNAs), no stable degradation product accumulated, as the human RNA is generally less stable in *Xenopus* oocytes. Evidence for the difference in stability can be seen in the full-length U8 RNAs (Fig. 6C, compare lanes 1 and 3 to lanes 2 and 4). Because equivalent amounts of the different U8 RNAs were injected into each oocyte, the dramatic difference in the quantity of full-length U8 RNA present in each lane is due to the inherent instability of the hybrid RNAs in oocytes. These results, plus the observation that U8 RNAs containing human U8 3′ ends do not coimmunoprecipitate with fibrillarin as efficiently as the *Xenopus* 3′ end RNAs (25), indicate that the 3′ end of human U8 RNA may assemble inefficiently in *Xenopus* in vivo or have alterations in structure or protein composition which prevent the human molecule from functioning. The proteins which comprise the U8 particle in *Xenopus* are currently being examined to directly address these and other issues.

DISCUSSION

Previously, U8 was demonstrated to be essential for pre-rRNA processing in *Xenopus* oocytes, facilitating and coordinating processing at both the 5′ end of the 5.8S and the 3′ end of the 28S rRNA (24). Until these two cleavages occur, processing does not occur within the second intervening transcribed spacer (ITS2) in U8-depleted oocytes, resulting in the appearance of 32*S and 36*S, abnormal precursor of 28S which accumulate in oocytes only upon depletion of U8 snoRNA (25) (Fig. 1A). Here, I demonstrated that the sequence and availability of the 5′ end of U8 RNA is important

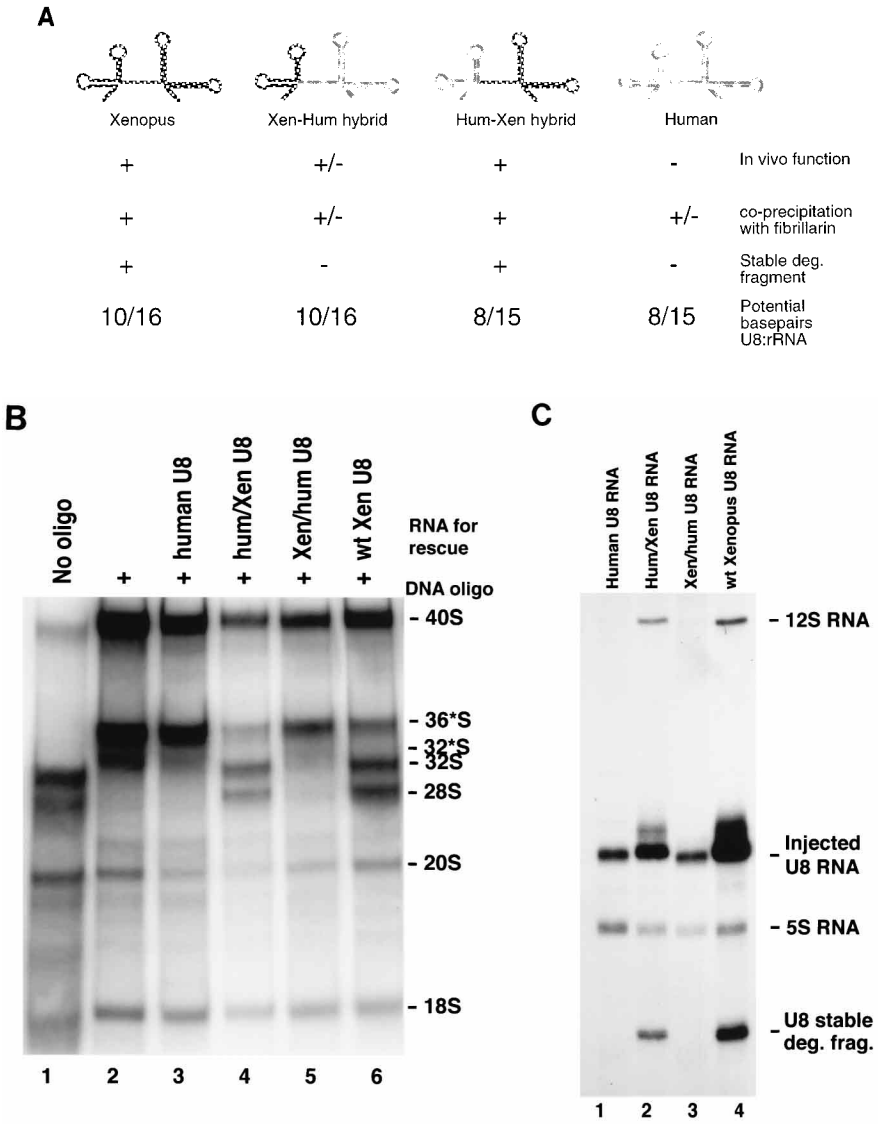


FIG. 6. Hybrid U8 RNAs show that the 3′ end of U8 RNP modulates efficiency of processing. (A) U8 RNA hybrid molecules were generated by exchanging the 5′-most 40 nucleotides of each RNA. The human-*Xenopus* (Hum-Xen) and *Xenopus*-human (Xen-Hum) hybrid constructs along with the wild-type human and *Xenopus* U8 RNAs were assayed for stability, fibrillarin association, and ability to function in vivo. deg., degradation. (B) Oocytes depleted of U8 by prior injection of the U8 5′ DNA oligonucleotide were injected with in vitro-synthesized U8 hybrid RNAs and then injected with [^{32}P]UTP. In vivo-labeled RNAs were resolved on a denaturing agarose-formaldehyde gel. oligo, oligonucleotide; hum, human; Xen, *Xenopus*, wt, wild-type; 32*S and 36*S, 32S and 36S abnormal precursors, respectively. (C) RNAs from the same oocytes shown in panel B were resolved in an acrylamide gel to examine U8 RNA levels present for each hybrid RNA. When the 3′ end of the molecule is of *Xenopus* origin, the RNA is generally much more stable and a stable degradation product (U8 stable deg. frag.) accumulates.

for function in vivo. U8 homologs have strong conservation of the first 15 nucleotides, but the remainder of the molecule is not well conserved (<45%). Alignment programs show that this conserved region in *Xenopus* U8 RNA has the potential to base pair with pre-rRNA at a few positions in pre-rRNA. One of the strongest interactions, and the only one conserved among the species examined, is between the 5′ end of U8 and the 5′ terminus of 28S, in the region which base pairs with the 3′ end of 5.8S, forming a stem structure present in the mature ribosome of every organism. Thus, these data strongly implicate the 5′ end of U8 RNA as an anchor point for the U8 RNP; once it has bound to pre-rRNA, U8 may recruit other cleavage or modification factors or additional snoRNPs which together facilitate processing. Interestingly, the site at which U8 appears to bind inhibits the longest of the three 5.8S-28S rRNA interactions which must occur prior to processing. Binding of U8 itself may modulate the timing at which 5.8S rRNA binds and cleavage occurs. However, since the 3′ end of U8 is also necessary, the structure or proteins comprising the U8 RNP are also critical for function.

Intermolecular interactions between rRNAs. In the mature ribosomes of eucaryotes, 5.8S rRNA base pairs with 28S rRNA in three regions, tethering these two RNAs (Fig. 1B). The three helices formed by these interactions are evolutionarily conserved and have structural counterparts in procaryotes. The helix formed by the pairing of the 5′ end of 28S and the 3′ end of 5.8S rRNA (labeled helix C in Fig. 1B) is the longest of the three interactions, forming an imperfect 10- to 16-nucleotide stem, depending on species (Fig. 1B and C and 7 and see reference 9).

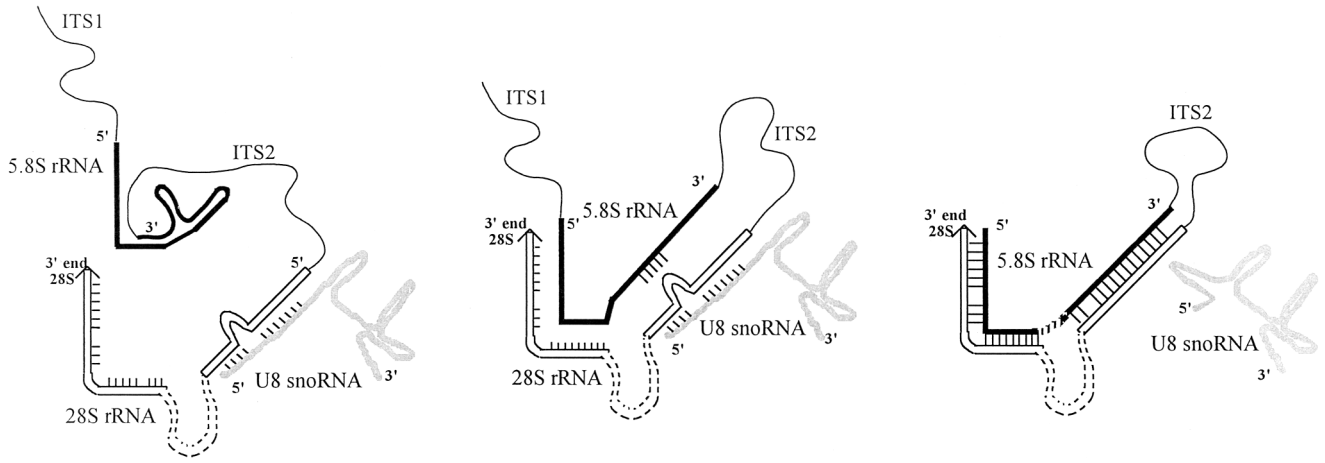


FIG. 7. Model for the 5′ end of U8 snoRNA modulating the timing of the 5.8S-28S interaction. Shown is a schematic of the 5′ of U8 snoRNA as it base pairs with the 5′ end of 28S in pre-rRNA, thereby potentially inhibiting an interaction essential for the completion of ribosome biogenesis. The binding of U8 to 28S produces a 6-nucleotide bulge of 28S sequence which may serve as the nucleation site for the 5.8S-28S interaction. It is not known whether the U8 snoRNP is still associated with the processing complex at the time of cleavage.

The results from the 2′*O*-methyl 5.8Sc oligoribonucleotide injections support the notion that 28S must interact with 5.8S in order for processing to occur. In the presence of the 5.8Sc oligoribonucleotide, no mature 28S rRNA accumulates. Microinjection of a DNA oligonucleotide with a sequence identical to that of the 5.8Sc 2′*O*-methyl oligoribonucleotide demonstrates that the inhibition of processing is not an indirect effect caused by the 5.8Sc oligoribonucleotide binding to U8 RNA, as the 5.8Sc DNA oligoribonucleotide does not target U8 for RNase H-mediated degradation. Thus, the inhibition of pre-rRNA processing in the presence of the 5.8Sc 2′*O*-methyl oligoribonucleotide is presumably due to binding of the oligonucleotide to 5.8S, thereby preventing it from interacting with 28S. The inability of 5.8S to interact with 28S may itself be the basis for the inhibition of processing, as this interaction does occur in the mature ribosome (Fig. 1B and 7), and formation of helix C is likely to be required for correct folding, which may allow the subsequent processing of pre-rRNA at sites 3, 4, 5, and T1 (Fig. 1A).

The functional site of interaction for the 5′ end of U8 snoRNA may be 28S rRNA. Taken together, the simplest explanation for these data is that the 5′ end of 28S rRNA is a functional site of a base pairing interaction with the U8 RNP. The interaction between U8 and 28S rRNA described for *Xenopus* is phylogenetically conserved; similar structures can be formed between pre-rRNA and the respective U8 homologs in mammals, amphibians, and insects. Although the overall sequence of U8 is poorly conserved among the species examined here, the 5′-most 15 nucleotides of U8 RNA are fairly well conserved and maintain the ability to form this interaction.

The structure formed by the base pairing of U8 and 28S is conserved in the various species examined. The unpaired region of the helix-bulge-helix conformation between U8 and 28S centers on a conserved 5′AGAUCA3′ sequence in 28S rRNA, where the underlined positions are absolutely conserved in the species examined (Fig. 1D). The bulge is opposed by 0 to 3 nucleotides in U8 RNA, indicating that it is not so much the structure of U8 that is critical as the structure induced in the rRNA. The two short helices allow U8 to span the same region of 28S that base pairs with 5.8S in the mature ribosome (see Fig. 1B for the *Xenopus* alignment).

There are four lines of evidence which support the existence of this interaction. First, RNase H-mediated degradation of U8 with a deoxyoligonucleotide directed against the 5′ end of U8 RNA (U8 5′ DNA) indicated that this region of U8 was accessible in the U8 RNP particle in vivo and thus is available to base pair with pre-rRNA as proposed. The resulting inhibition of processing caused an accumulation of pre-rRNA precursors typical of U8 depletion.

Second, in vivo competition studies using 2′*O*-methyl oligoribonucleotides demonstrated that the 5′ end of U8 RNA was a functional site of a base-pairing interaction. 2′*O*-methyl oligoribonucleotides which could bind the 5′ end of U8 RNA (U8 5′c) or could block access of U8 to the site of interaction in 28S (U8 5′ sense and 28Sc) inhibited pre-rRNA processing and yielded intermediates typical of a U8 RNA depletion, demonstrating the critical role this region has in pre-rRNA processing. Although the use of 2′*O*-methyl oligoribonucleotides has been described for in vitro competition experiments in cell extracts (7, 29), this study is the first demonstration of their use with in vivo competition studies. The data presented here demonstrate the effectiveness of this technique and the versatility of the oocyte system for in vivo assays. These types of experiments are particularly useful for pre-rRNA processing, because there exists no in vitro system capable of yielding mature 28S rRNA. These studies must be pursued in vivo, and the *Xenopus* oocyte is an excellent vertebrate model system.

Third, the 5′ end of U8 RNA must be present and intact for processing; disruption of this region in U8, whether through insertions, deletions, or mutations, inhibits pre-rRNA processing. The Δ8U8 mutation, which eliminated one entire helix of the helix-bulge-helix interaction between U8 and 28S, failed to rescue pre-rRNA processing. However, the Δ4U8 mutation, which removed only 1 bp from one helix, could function in vivo, indicating the need for the presence of both helices to facilitate processing.

Finally, the data from the cross-species hybrid molecules supported the importance of the U8-28S base-pairing interaction. These constructs demonstrated that the snoRNA–pre-rRNA interaction was necessary but not sufficient for efficient pre-rRNA processing. The human-*Xenopus* hybrid, like the Δ4U8 mutation, eliminated 1 bp from one helix, and the RNA functioned in vivo. However, the *Xenopus*-human hybrid, which forms the full bulge-helix-bulge interaction, could not function, presumably because the hybrid U8 RNP could not efficiently assemble in vivo. Additional sequences, structures or

protein components present in the U8 RNP are needed for efficient processing in vivo.

U8 must be displaced from pre-rRNA before processing can occur. Previously, there was evidence that the U8 snoRNA was displaced prior to the completion of ribosome biogenesis. The most apparent evidence was that U8 RNA was not part of the mature ribosome in the cytoplasm. In situ hybridization in HeLa cells showed that U8 RNA localized to a very specific subcompartment of the nucleolus, corresponding to the site of rDNA transcription (21). A prior in vivo study with *Xenopus* showed that U8 must be present (24), and this study has shown that U8 must have the potential to base pair with its target for mature 28S rRNA accumulation. Thus, the 5.8S-28S interaction in the mature ribosome requires that U8 RNA be displaced by 5.8S before ribosome biogenesis is completed.

The results from experiments with the 2′*O*-methyl 5.8Sc oligoribonucleotide support the notion that U8 base pairing with 28S must be disrupted prior to the processing event. In the presence of the 5.8Sc oligoribonucleotide, the intact U8 RNP can interact with 28S in pre-rRNA, although no mature 28S rRNA accumulates. The inability of 5.8S to interact with 28S is proposed to be the basis for the inhibition of processing.

Additional evidence supporting the need to displace U8 and allow 5.8S to bind prior to processing is provided by the U8 Str mutations. These constructs indicated that the relatively weak interaction between U8 and pre-rRNA is essential for U8 function. Strengthening this interaction by decreasing the size of the internal bulge, or addition of nucleotides capable of extending the base pairing at the 5′ or 3′ end of the interaction, dramatically decreased or eliminated pre-rRNA processing.

Increasing the region of complementarity between U8 and 28S by altering nucleotides within the first loop of U8 (U8 StrLp1) extended the double-stranded region nearer the mature 5′ terminus of 28S. This mutant could not function in vivo. However, altering nucleotides at the 5′ end of U8 (U8 5′ Str) extended the region of complementarity into a region in 28S normally single stranded, beyond the stem which forms between 5.8S and 28S. This mutant functions in vivo but much less efficiently than the wild type. These data support the theory that U8 RNA transiently base pairs with 28S rRNA and is displaced by an interaction between 28S and 5.8S which must occur for processing to proceed.

Previous work demonstrated that 5.8S rRNA has the ability to interact with itself, and several alternative secondary structures have been proposed for 5.8S in *Xenopus* (5). In every case, the 3′ end of 5.8S rRNA is base paired to an internal region. Since it is transcribed before 28S, the 5.8S rRNA may form temporary intramolecular base pairs which are stable but which must be broken before the 28S-5.8S intermolecular interactions can occur. Since the interaction between U8 and 28S is significantly weaker than the interaction between 5.8S and 28S, 5.8S should be quite efficient at competing 28S away from U8. The 6 unpaired nucleotides which comprise the bulge in the U8-28S interaction are conserved in all species examined. This bulge might serve a variety of functions, including acting as the nucleation point for displacement of U8 by 5.8S rRNA in the maturation pathway (Fig. 7).

The 5′ end of U8 anchors the U8 RNP to pre-rRNA and facilitates processing. Evidence presented here suggests that an essential base-pairing interaction occurs between U8 and 28S, which must be displaced by 5.8S prior to processing. In binding to 28S, the 5′ end of U8 RNA may alter the configuration and folding of pre-rRNA, preventing 5.8S interactions with 28S. The bulge in the U8-28S interaction may facilitate the interaction between the 5′ end of 28S and the 3′ end of 5.8S. If this theory is true, the base pairing between the 5′ ends of U8 and 28S may direct pre-rRNA folding along a specific pathway by preventing the formation of inappropriate 28S interactions.

Functional analyses of cross-species hybrid U8 RNA molecules suggest that while the 5′ end of U8 is essential, it is not sufficient for pre-rRNA processing. The proteins present on, or recruited by, U8 RNA are critical for function. Acting alone, or in combination with other snoRNAs, U8 may also participate more directly in folding and processing by bringing the 3′ end of 5.8S into proximity with 28S in a structure capable of base pairing with 28S. At present it is not clear when the U8 RNP disassociates from the assembling rRNP or if other U8-rRNA base-pairing interactions exist which may additionally tether U8 to pre-rRNA before or after the 5′ end of U8 RNA has been released. These issues are currently being addressed via more direct assays. It is possible that the U8 RNP constitutes or recruits the catalytic machinery and remains associated with the pre-rRNA in a large multicomponent complex when the processing event occurs or that additional intermediate structures, with or without the U8 RNP, are formed.

U8 RNA function requires an intramolecular base-pairing interaction. The 5′ end of U8 snoRNA may function by anchoring the U8 RNP to pre-rRNA, modulating the timing of an intermolecular interaction in the pre-rRNA. A similar snoRNA-rRNA interaction may occur between the 5′ end of 5.8S and U19 snoRNA. U19, identified in mouse and human, has a region of complementarity with the 5′ end of 5.8S rRNA (16) in a region involved in a different intermolecular interaction with 28S (Fig. 1B, helix A). Although the nature of the U19 interaction with 5.8S rRNA appears to very closely parallel that of U8, neither the actual existence or evolutionary conservation of the proposed interaction nor the in vivo role of U19 in pre-rRNA processing, if any, has yet been determined.

Several dozen snoRNAs have been identified, and the functions of a few have recently been elucidated (12, 16, 18, 24, 34). Of the snoRNAs essential for processing, U3, U14, and now U8 appear to base pair with pre-rRNA to facilitate processing.

In yeast, U3 base pairs in the 5′ ETS and is essential for cleavage within the 5′ ETS, but cleavage does not occur at the binding site (14). There is additional evidence that U3 may be acting at a site within mature 18S, facilitating the formation of a pseudoknot, which is present in the mature ribosome (11). In the U14 snoRNA, each of two separate sequences in U14 base pairs with a region in 18S. One of the two sequences in U14 is essential for processing (19). It is not known whether the U3 and U14 snoRNAs are catalytic, but in both, the cleavage sites in pre-rRNA are not at the sites of snoRNA base pairing.

Many of the fibrillarin-associated, intron-encoded snoRNAs with long regions of complementarity with rRNA are thought to play a role in 2′*O*-methylation of specific sites in rRNA (16). Although it is clear that the base pairing of snoRNA directs the specific nucleotide in rRNA that is to be methylated, it is not known whether the methylase activity is part of the snoRNP or is an activity recruited to, or directed by, snoRNA binding. Surprisingly, the snoRNAs which direct methylation are neither essential for pre-rRNA processing nor required for cell viability in yeast; the biological relevance of the methylated nucleotides in rRNA is not well understood at this time.

The in vivo study presented here implies that *Xenopus* U8 base pairs with pre-rRNA and inhibits an rRNA-rRNA interaction which not only occurs in the mature ribosome but must occur for processing to proceed. Little is known about pre-rRNA cleavage mechanisms in vertebrates. It is equally plausible that the snoRNAs are catalytic, that the snoRNPs contain a catalytic protein, or that the snoRNP-pre-rRNA complex recruits the cleavage activity. In addition there are still several snoRNAs whose roles have not yet been identified. It may be

that some of the remaining uncharacterized snoRNAs play a pivotal role in pre-rRNA cleavage, but those whose roles have been elucidated seem to play more cooperative roles by coordinating and orchestrating the folding, processing, modification, and biogenesis of the ribosome, perhaps in a structure that works like, and is as dynamic as, the spliceosome.

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