Nucleotide sequence determination and secondary structure of *Xenopus* U3 snRNA

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ABSTRACT

Using a combination of RNA sequencing and construction of cDNA clones followed by DNA sequencing, we have determined the primary nucleotide sequence of U3 snRNA in *Xenopus laevis* and *Xenopus borealis*. This molecule has a length of 219 nucleotides. Alignment of the *Xenopus* sequences with U3 snRNA sequences from other organisms reveals three evolutionarily conserved blocks. We have probed the secondary structure of U3 snRNA in intact *Xenopus laevis* nuclei using single-strand specific chemical reagents; primer extension was used to map the positions of chemical modification. The three blocks of conserved sequences fall within single-stranded regions, and are therefore accessible for interaction with other molecules. Models of U3 snRNA function are discussed in light of these data.

INTRODUCTION

All eukaryotes examined so far have a distinctive set of uridine-rich small nuclear RNAs [U snRNAs; (1-2)] that are coupled with proteins in ribonucleoprotein particles [snRNPs]. Most U snRNAs are products of RNA polymerase II transcription (3-5). They are distinct from typical RNA polymerase II transcripts in that most snRNAs (except U6) have a unique trimethylguanosine cap at their 5' end (2), and they lack a poly (A) tail at their 3' end.

What roles are played by snRNPs? snRNPs containing U1, U2, U4, U5 and U6 snRNAs are required for splicing messenger RNA precursors [reviewed in 6-9]. Early in splicing, the U1 snRNP binds to the 5' splice site of the intron. RNase H cleavage has shown that the 5' end of U1 snRNA is necessary for splicing at the 5' end of the intron (10-12), and a compensatory mutation near the 5' end of U1 snRNA is able to suppress a mutation at the 5' splice site (13), showing that base-pairing between U1 snRNA and the 5' splice site is an important feature of the recognition. Although this implies that the base-pairing of U1 snRNA to the 5' splice site is necessary, it is not sufficient in itself, as U1 snRNP proteins are needed

to impart specificity to U1 snRNP binding (14). RNase H cleavage has shown that the snRNA molecules of U2, U4 and U6 are also required for splicing (11-12, 15-16). It has been proposed that the yeast analogue to U2 snRNA (17) hydrogen-bonds to the conserved branch site within yeast introns; this has been supported by compensatory mutations in the yeast U2 snRNA analogue that can suppress mutations in the intron branch site (18). snRNPs may also play other roles in RNA processing in addition to splicing. For example, the U7 snRNP is involved in processing the 3' end of histone mRNA, and compensatory mutation experiments support the proposal that U7 snRNA base-pairs with a 3' end processing signal in the histone mRNA precursor (19).

A function has not been identified for the U3 snRNP. Does U3 snRNA also play a role in RNA processing? The U3 snRNP is different from the other snRNPs because it is localized in the nucleolus (1, 2), where it is associated with pre-ribosomal particles greater than 60S (20). Because of these features, it seemed possible that U3 snRNA might be important for pre-rRNA processing (a nucleolar event), perhaps in removal of the internal transcribed spacer 2 (ITS 2) during the processing of 32S pre-rRNA to 28S rRNA (21-23). This processing event releases 5.8S RNA, the counterpart of the 5' end of prokaryotic 23S rRNA (24-26). The notion that the 5.8S gene became separated from the main body of the 28S gene during evolution by the insertion of the ITS 2 prompted an analogy between ITS 2 and introns, including the speculation that a region within U3 snRNA base-pairs with a sequence at the 5' end of the ITS 2 of vertebrates (22, 23). At the time this model was proposed, the only species in which both the ITS 2 and U3 snRNA sequences were known was the rat. Although the list has now grown slightly (Table I), there is a taxonomic gap between mammals and lower eukaryotes. Moreover, the 5' end of the ITS 2 is not conserved in the latter group (23). To test the hypothesized interaction of U3 snRNA with ITS 2, we have extended the available data base by examining a non-mammalian vertebrate. We chose the amphibia Xenopus laevis and Xenopus borealis because previously Furlong and Maden (32) had compared the ITS 2 sequences from these two species. They identified conserved tracts interspersed among divergent sequences. We predicted that if any of the conserved tracts in ITS 2 base-pair with U3 snRNA, then both X. laevis and X. borealis should have an identical sequence in U3 snRNA that is complementary to the conserved tract of ITS 2. Alternatively, if mutation has occurred, then compensatory base changes should be found between a non-conserved stretch

TABLE I: Sequence References

vertebrates	ITS 2 of pre-rRNA	U3 snRNA
human	C.A. Chambers, I.L. Gonzalez and R.D. Schmickel, personal communication	(27)
rat	(28)	(29,30)
<u>Xenopus laevis</u>	(31)	this paper
<u>Xenopus borealis</u>	(32)	this paper
<u>lower eukaryotes</u>		
<u>Dictyostelium discoideum</u>	(33)	(34)
<u>Saccharomyces cerevisiae</u>	(35, 36*)	(37)

* S. carlsbergensis is a strain isolate of S. cerevisiae (38)

of ITS 2 sequence and a complementary region of U3 snRNA. In any case, for testing models of U3 snRNA interaction with pre-rRNA, Xenopus provides a good system because its entire rDNA repeat unit has been sequenced (Table I of ref. 39). The only other eukaryotic rDNA entirely sequenced is yeast rDNA (ETS: 35; 18S rRNA: 40; ITS: 35, 36; 26S rRNA: 41; NTS: 42). We now present our findings on the primary sequence and secondary structure of Xenopus U3 snRNA, and discuss models of U3 snRNA function in light of these results.

MATERIALS AND METHODS

Purification of U3 snRNA

X. laevis or X. borealis (obtained from Nasco, Fort Atkinson, Wis.) was killed by decapitation; the liver was homogenized in 2.5% cold citric acid (43, 44) and the nuclei were sedimented at 600g for 10 minutes. The nuclei were resuspended in 1.5% citric acid, 0.25M sucrose and layered over 1.5% citric acid, 0.88M sucrose for centrifugation at 900g for 10 minutes. Nuclei were resuspended in RSB (10 mM Tris pH 7.4, 10 mM NaCl, 3 mM MgCl₂), and an equal volume of 0.2 M Tris pH 7.5, 2% SDS, 5 mM EDTA was added at 65°C, followed by phenol:chloroform extractions at room temperature. After ethanol precipitation, DNA was removed by RNase-free DNase I (Worthington) treatment (50 µg/ml in 50 mM Na acetate pH 6.5, 10 mM MgCl₂, 2 mM CaCl₂) at 37°C for 30 minutes, followed by phenol:chloroform extraction and ethanol precipitation. The RNA was subjected to preparative gel electrophoresis in a 5% polyacrylamide 7M urea gel run in TBE (50 mM Tris-base pH 8.0, 50 mM boric acid, 2.5 mM EDTA) at 250 volts. After ethidium bromide staining the U3 snRNA band was excised from the gel, eluted in 0.5M NH₄ acetate, 0.1%

SDS, 0.1 mM EDTA) at 37°C overnight, and ethanol precipitated to use for cDNA cloning.

U3 snRNA for RNA sequencing was obtained from *X. laevis* cell line A110. The harvested cells were homogenized in RSB, the nuclei were isolated by centrifugation, resuspended in 2 ml RSB and the outer nuclear membrane removed by vortexing after the addition of 0.3 ml 6.7% NP-40, 3.3% Na deoxycholate. The nuclei were pelleted again, resuspended in HSB (10 mM Tris pH 7.4, 0.5 M NaCl, 50 mM MgCl₂) and treated with 20 µg/ml DNase I at 37°C for 30 minutes. The mixture was then made 75 mM in EDTA, 0.5% in SDS, and 1 mg/ml in proteinase K, and incubation was continued for 1 hour. RNA was isolated by extraction with phenol:chloroform and precipitated with ethanol.

RNA Sequencing

RNA sequencing of *X. laevis* U3 snRNA was performed by Dr. Manuel Ares, Jr. Total nuclear RNA or gel purified U3 snRNA was labeled at the 3' end by incubation with 5' [³²P], 3' cytidine bisphosphate (pCp) and RNA ligase, according to England and Uhlenbeck (45). After phenol extraction and ethanol precipitation, trimethylguanosine-capped RNA was immunoprecipitated using the procedure of Chabot *et al.* (46). Anti-trimethylguanosine antibodies were the kind gift of Drs. Doug Black and David LeMaster. Immunoprecipitated, 3'-end labeled U3 snRNA was gel purified and sequenced enzymatically using an RNA sequencing kit from Pharmacia P-L Biochemicals in accordance with the instructions provided with the kit.

Cloning of cDNA to U3 snRNA from *X. laevis* and *X. borealis*

First Strand cDNA Synthesis: The 17-mer

5' - GGCTTGTGTTCTCTCCC - 3', complementary to residues 7-23 from the 3' end of *X. laevis* U3 snRNA (determined by RNA sequencing), was synthesized on a Biosearch 8600 synthesizer. Full length product was isolated by electrophoresis on a 20% polyacrylamide, 8 M urea gel, excised and eluted from the gel, bound to a DE-52 column, eluted and then desalted on a P4 column.

About 1 µg of gel purified U3 snRNA was annealed with 1.25 µg of the 3'-end primer in 22 µl 50 mM Tris pH 7.5, by heating to 75°C for 3 minutes followed by cooling on ice. 25 µl 2x RT buffer (0.1 M Tris pH 8.3, 0.2 M KCl, 16 mM MgCl₂) containing 2 mM DTT, 4 mM dNTPs, α-³²P dATP, 80 units RNasin (Promega) and 56 units AMV reverse transcriptase (Life Sciences) was added, and the reaction incubated at 40°C for 2 hours. The reaction was

stopped by addition of 2 μ l 0.5 M EDTA pH 8.0, followed by 25 μ l 150 mM NaOH and incubation at 37°C overnight to hydrolyze the RNA. The cDNA was recovered by addition of 400 μ l 0.3 M Na acetate pH 5.0 and 2 μ g carrier tRNA followed by ethanol precipitation. In order to separate cDNA from the primer and insure cloning of full length cDNA, the synthesized DNA was subjected to electrophoresis on a 5% polyacrylamide, 8 M urea gel. After ethidium bromide staining of the gel and autoradiography, the band corresponding to full length cDNA was excised and eluted in 0.5 M NH₄ acetate, 1 mM EDTA; 50 ng full length U3 cDNA was obtained after ethanol precipitation.

Poly (A) Tailing of First Strand U3 cDNA: A poly (A)_N tail needed for priming of second strand cDNA was added to the U3 cDNA using terminal deoxynucleotidyl transferase (TdT). The reaction was carried out at 37°C for 2 hours in 18 μ l TdT buffer (67 mM K cacodylate pH 7.2), 5 mM MgCl₂, 1 mM DTT) containing 30 units TdT (Pharmacia P-L) and 0.1 mM dATP:ddATP (30:1). Addition of ddATP as chain terminator allows the use of high amounts of enzyme with prolonged incubation time (ensuring a high efficiency of the reaction), while the tail-length can be controlled by the dATP:ddATP ratio. After incubation at 37°C for 2 hours, the mixture was phenol extracted and ethanol precipitated.

Second Strand Synthesis and Cloning of cDNA: The poly (A) tailed cDNA was annealed with 1.5 μ g oligo (dT)₁₂₋₁₈ (Pharmacia P-L) in 25 μ l 2x RT buffer, heated to 75°C, cooled and held at 20°C for 1 hour. The second strand reaction was started by increasing the reaction volume to 50 μ l containing 2 mM dNTPs, 1 mM DTT, and 56 units reverse transcriptase. After incubation at 37°C for 20 minutes and then 40°C for 60 minutes, the reaction was diluted with 50 μ l of a buffer consisting of 0.1 M Tris pH 7.5, 5 mM MgCl₂ followed by addition of 25 units Klenow DNA polymerase (N.E. BioLabs). The reaction was incubated at room temperature for 5 hours to insure blunt end formation. Double stranded cDNA was separated from the oligo (dT) primer by passage over an Elutip column (Schleicher and Schuell). The cDNA was coprecipitated from the elution buffer with 100 ng Sma I digested pUC8 DNA. Following kination and ligation the DNA was transformed into E. coli strain JM 103 (47).

Sequencing of positive clones: The positive clones derived from X. laevis and X. borealis U3 cDNA are referred to as pX1U3 and pXbU3, respectively. These clones have short poly (A) tails (about 25 nt). They were sequenced from both ends by the Maxam and Gilbert technique (48),

using the Eco RI and Bam HI sites of the pUC8 polylinker for 3'-end labeling. The sequence was later verified by dideoxy sequencing (49) in the secondary structure probing experiments described below.

Secondary Structure Probing by Chemical Modification

Purification of Nuclei: For isolation of nuclei (43), livers of mature *X. laevis* females were dissected and homogenized in a cold solution of 2.2 M sucrose and 10 mM MgCl₂. The homogenate was layered over 2.5 volumes of the same solution and centrifuged at 40,500g at 5°C, 1 hour in a Beckman SW-27 rotor. Conditions for subsequent chemical treatment of nuclei were adapted from published procedures (50-52). In each case, purified nuclei were resuspended in the appropriate chemical modification buffer at approximately 3 ml/g original tissue.

Chemical Modification: (a) DMS treatment: Nuclei were gently resuspended in 6 ml cold CMK (80 mM Na cacodylate, pH 7.2, 0.1 M KCl, 5 mM MgCl₂) and allowed to equilibrate at 25°C for 5-10 minutes. 30 µl DMS (dimethyl sulfate) was added and incubation continued at 25°C for 1, 2, 5 or 20 minutes. The reaction was stopped with 1.5 ml cold DMS stop buffer (1.5 M Na acetate, pH 5.2, 1 M β-mercaptoethanol, 1 M Tris pH 7.5, 0.1 mM EDTA), and nuclei were ethanol precipitated. As a control to define any strong pauses of reverse transcriptase reading unmodified U3 snRNA template, untreated nuclei in CMK buffer were kept on ice for 20 minutes, followed by ethanol precipitation.

(b) Kethoxal treatment: Kethoxal (gift from Upjohn; 37 mg/ml in 20% EtOH) was added at a final dilution of 1/20 and 1/40 final volume (3 ml) to nuclei in CMK pre-equilibrated at 25°C and then incubated for 15 minutes at 25°C. The reaction was stopped by adding 1.25 volumes of cold kethoxal stop buffer (0.5 M K borate, pH 7.0), and the samples were ethanol precipitated. A control reaction received stop buffer containing the equivalent of the highest concentration of kethoxal.

(c) CMCT treatment: Nuclei were resuspended in cold BMK (80 mM K borate pH 8.1, 0.1 M KCl, 5 mM MgCl₂) and warmed to 25°C as above. CMCT [1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluene sulfonate from Sigma; 42 mg/ml in BMK] was added to nuclei at a dilution of either 1/6, 1/3 or 2/3 of the final volume of 3 ml, and samples were incubated at 25°C for 15 minutes. The reaction was stopped with 1.25 volumes of cold CMCT stop buffer (0.5 M K borate pH 6.1), left on ice 5 minutes, and ethanol precipitated. As a control to monitor the efficiency of stopping and removing CMCT, 1 ml of CMCT and 3.75 ml cold CMCT stop buffer were

pre-mixed and added to nuclei, which were then ethanol precipitated.

Following chemical treatment all samples and controls were resuspended in 10 mM Tris, pH 7.4, 0.5 M NaCl, 50 mM MgCl₂. Fresh vanadyl ribonucleoside complex (VRC; N.E. Biolabs) was added to a final concentration of 10 mM, and samples were incubated with 0.1 mg/ml DNase I at room temperature for 2 hours. Proteinase K (Sigma) and SDS were then added to a final concentration of 0.25 mg/ml and 0.1%, respectively, and the samples were left at room temperature overnight. Samples were extracted with phenol:chloroform and ethanol precipitated.

Purification of Modified U3 snRNA: The RNA was resuspended in 10 mM Tris pH 7.4, 0.1 M NaCl, 1 mM MgCl₂ and centrifuged through a 5-40% sucrose gradient in the same buffer in a Beckman SW-41 rotor at 24,000 rpm, 16 hours at 5°C. Fractions were collected from the top in 2.5 ml volumes and were ethanol precipitated. Fractions were resuspended in 50 mM Na acetate pH 6.5, 10 mM MgCl₂, 2 mM CaCl₂ containing 50 µg/ml DNase I and incubated 45 minutes at 37°C. After phenol:chloroform extraction, samples were centrifuged through a Sephadex G-50 column to remove the remaining VRC and contaminating oligonucleotides, and then ethanol precipitated.

Detection of Chemically Modified Nucleotides: Synthetic primers were kindly prepared by Bettina Franz using a Biosearch 8600 DNA synthesizer (phosphoramidite method). Primer 103 is a 19-mer with the sequence 5' - CCACTCAGCTTGTGTTCTC - 3'; it is complementary to nucleotides 201-219 at the 3' end of *X. laevis* U3 snRNA. Primer 105 is a 20-mer with the sequence 5' - CTTACGCTCAGGAGAAAAC - 3'; it is complementary to nucleotides 96-115 in the middle of *X. laevis* U3 snRNA.

Primer extension was used for detection of chemically modified nucleotides, as originally described by Qu *et al.* (53). Synthetic DNA primers were 5'-end labeled with γ -³²P ATP and kinase (47). Approximately 10 ng U3 snRNA were dried down with 1 pmole labeled primer, resuspended in 2 µl annealing buffer (0.1 M Tris pH 8.3, 0.1 M KCl, 10 mM DTT) and heat denatured at 90°C for 3 minutes and quickly chilled. To this was added 3 µl extension buffer (375 µM dNTPs, 0.1 M Tris pH 8.3, 0.1 M KCl, 10 mM MgCl₂, 10 mM DTT) and reverse transcriptase at a final concentration of 1 unit/µl. Annealing and extension proceeded at 42°C for 30 minutes, and was stopped by addition of 2 volumes loading solution (95% deionized formamide, 0.1% xylene cyanol, 0.1% bromophenol blue, 10 mM EDTA pH 8.0).

For dideoxy sequencing (49), 1 µg uncut pX1U3 (cloned U3 cDNA) was dried down with 1 pmole of labeled primer, resuspended in 10 µl annealing

buffer and heat denatured at 100°C for 3 minutes. It was then immediately quenched on ice, and 2 µl aliquots were put into 4 separate tubes, each containing 3 µl of a different dideoxy mixture (extension buffer with 100 µM of any one of the four ddNTPs; Pharmacia P-L) and reverse transcriptase for extension as described above.

Primer extension and dideoxy sequencing reactions were heat denatured and quenched on ice prior to electrophoresis on a 0.5 mm x 40 cm gel of 8% polyacrylamide (20:1 bis), 7 M urea, and TBE that was run at 25 mAmp 2.5-5 hours.

RESULTS

Primary Sequence of *Xenopus* U3 snRNA

Because pseudogenes occur for many other snRNAs (30, 54) we decided to base the sequence determination of U3 on the snRNA transcript itself. The length of the molecule (219 nt) and its relatively low abundance would have made direct RNA sequencing difficult; therefore, we chose to clone and sequence the cDNA.

SnRNAs do not have poly (A) tails, so we tried to add a poly (A) tail at the 3' end of *X. laevis* U3 snRNA using poly (A) polymerase in order to prime first strand cDNA synthesis with oligo (dT). Repeated attempts failed, perhaps due to strong base-pairing in the secondary structure of the 3' end of the U3 snRNA molecule (Figure 7) that could not be easily denatured. In a control experiment, *X. laevis* U2 snRNA was successfully polyadenylated (data not shown). Therefore as an alternate route Dr. Manuel Ares, Jr. offered us the sequence of the 3' end of *X. laevis* U3 snRNA determined by direct RNA sequencing; this sequence was used to make a complementary synthetic oligonucleotide to use as a primer for cDNA synthesis. U3 snRNA, like most of the other snRNAs, has a trimethylguanosine cap at its 5' end (2), and is precipitated by anti-trimethylguanosine antibody as shown in Figure 1A. To purify U3 snRNA for sequencing, nuclear RNA was fractionated by gel electrophoresis and material in the size range of U3 snRNA was eluted, labeled at its 3' end and immunoprecipitated using an antibody to the trimethylguanosine cap (Figure 1B). The immunoprecipitate was fractionated on a gel, and the purified 3' end labeled U3 snRNA was eluted for use in RNA sequencing. One of several RNA sequence determinations of the 3' end of *X. laevis* U3 snRNA is shown in Figure 1C. A complementary synthetic primer was made slightly inward from the 3' end of U3 snRNA (residues 7-23 from the 3' end); G was chosen as the 5' most base

Figure 1. Purification and 3' end sequence of *X. laevis* U3 snRNA
 (A) A trimethylguanosine capped RNA the size of U3 snRNA is present in *X. laevis*. Total nuclear RNA from *X. laevis* A110 cells was pCp labeled and immunoprecipitated with antitrimethylguanosine antibodies. Lane T is total nuclear RNA, and lane P is the immunoprecipitated RNA which includes a band at the size expected for U3 snRNA. (B) Purification of U3 snRNA for sequencing. Nuclear RNA from A110 cells was fractionated on a 5% acrylamide - 8M urea gel, and a fraction in the size range of U3 snRNA was eluted from the gel, pCp labeled, and precipitated with antitrimethylguanosine antibodies. Lane T contains one tenth of the total reaction, lane S has the immunosupernatant, and lane P has the immunoprecipitate that is enriched for U3 snRNA. The band of U3 snRNA was eluted from lane P and used for RNA sequencing. (C) 3' end sequence of *X. laevis* U3 snRNA. Purified *X. laevis* U3 snRNA was digested as follows: lane 1 = RNase T1 (G specific), lane 2 = RNase U2 (A specific), lane 3 = RNase PhyM (A+U specific), lane 4 = RNase *B. cereus* (C+U specific), lane 5 = partial alkaline cleavage products. Y indicates a pyrimidine that we deduce from other experiments to be U rather than C. The bracket shows the region complementary to a synthetic oligonucleotide that was prepared for cDNA cloning.

of the primer so that either a G·C or G·U base pair could form with the pyrimidine at position 7 from the 3' end of U3 snRNA that had been difficult to determine.

Using this oligonucleotide primer, we constructed cDNA clones for U3 snRNA from *X. laevis* and *X. borealis* as described in Materials and Methods; these clones are named pXIU3 and pXbU3 respectively. The clones were

Figure 2. Mapping the 5' end of U3 snRNA. The 5' end of U3 snRNA is shown in the left-most lane. ^{32}P -labeled primer 105 was extended with reverse transcriptase using gel-purified U3 snRNA as template and run on an 8% sequencing gel. The sequence to the right was generated by dideoxy sequencing using reverse transcriptase, the same primer and cloned U3 cDNA (pX1U3) as the template. The RNA sequence complementary to the determined DNA sequence is indicated; lanes G, A, U and C contain sequencing reactions done in the presence of ddC, ddT, ddA and ddG, respectively. Regions labeled "tail" and "vector" correspond to the poly(A) tail added to U3 cDNA in the cloning procedure and the Sma I cloning site of pUC8, respectively. The 5' end of U3 snRNA (left lane) aligns with the last base of cloned U3 sequence (A_1).

sequenced on both strands using the Maxam and Gilbert technique (48), and the sequence was later confirmed by dideoxy sequencing using *X. laevis* U3 cDNA (pX1U3) or *X. laevis* U3 snRNA as a template (49). The 23 nucleotides at the 3' end were deduced from RNA sequencing of *X. laevis* U3 snRNA. These 23 nucleotides were not experimentally determined for *X. borealis* U3 snRNA, but are likely to be the same as in *X. laevis* because the *X. laevis* primer hybridized efficiently to *X. borealis* U3 snRNA during cDNA clone construction. The clones were shown to be full length by primer-extension on a purified U3 snRNA template (Figure 2).

Figure 3 shows the U3 snRNA sequence from *X. laevis* and *X. borealis* aligned with other published U3 sequences. Our alignment is based on secondary structure considerations to ensure that nucleotides at similar

Figure 3. U3 snRNA Sequence Alignment. U3 snRNA sequences from *X. laevis* and *X. borealis* (this paper) have been aligned with U3 snRNA sequences from rat U3B (29), human (27), *Dictyostelium discoideum* (from the DNA sequence determination in Fig. 2 of ref. 34; this differs in a few positions from the sequence presented in Fig. 6 of the same ref.) and snR17A from the yeast *Saccharomyces cerevisiae* (37). The trimethylguanosine cap added post-transcriptionally to the 5' end is not shown. A dot indicates that the nucleotide is the same as for *X. laevis*, and a dash indicates a residue not present in that organism but present in another organism at the same position. The ? indicates nucleotides at the 3' end of *X. borealis* U3 snRNA that were not determined. Yeast snR17A is substantially larger than the other U3 snRNAs shown, and the extra nucleotides in yeast are shown by a number in brackets with an arrow. Conserved Boxes A, B and C are indicated.

positions of the secondary structure will be aligned against each other in the primary sequence comparison. The alignment of Hughes *et al.* (37) of yeast U3 snRNA compared to other U3 snRNAs differs from ours for a large region of the molecule, and appears not to follow the secondary structure guidelines we used for primary sequence alignment. As can be seen in Figure 3, only 9 nucleotides out of the first 196 nucleotides differ between *X. laevis* and *X. borealis* U3 snRNA; the U3 snRNA sequence is 95% identical between these two species. A high degree of sequence similarity is observed when comparing *Xenopus* U3 snRNA to those of other vertebrates (73% and 74% of the *X. laevis* U3 nucleotides are identical to those from rat and human, respectively), but the amount of sequence similarity decreases over most of the molecule when compared to U3 snRNAs of the lower eukaryotes *S. cerevisiae* and *Dictyostelium*. However, even these lower eukaryotes keep a few blocks of sequence conservation, which are indicated as Boxes A, B and C in Figure 3. These are regions that retain sequence identity in U3 snRNA of all species in our alignment. These conserved regions were first detected by Wise and Weiner (34), and recently extended to other species by the U3 snRNA sequence alignment of Hughes *et al.* (37). However we show shorter stretches for Boxes A, B and C than others, as we have been more conservative and require 100% sequence match for all comparisons (except for yeast where a few mismatches occur in Boxes B and C). We do not include Box D of Hughes *et al.* (37) (*Xenopus* residues 207-216 on our alignment) since it has more mismatches when comparisons are made between species, as can be seen in Figure 3 (only 3/10 nucleotides from this region of *X. laevis* U3 snRNA are the same in yeast U3 snRNA).

Secondary Structure of *Xenopus* U3 snRNA

Given that other snRNPs interact with substrates by RNA-RNA base-pairing, it seemed possible that conserved Boxes A, B and/or C base-pair with a conserved complementary region in pre-rRNA. However, one cannot exclude *a priori* the coevolution of nonconserved regions in U3 snRNA with pairing partners in nonconserved regions of pre-rRNA. Computer searches for complementarity involving any region within U3 snRNA and any region within pre-rRNA generated an overwhelming number of possibilities. It was therefore necessary to experimentally define which regions of U3 snRNA contained in the snRNP particle are single-stranded and hence potentially capable of base-pairing with pre-rRNA. Therefore we developed a protocol whereby the secondary structure of RNA molecules can be probed *in situ* in purified nuclei (see Materials and Methods). Isolated *X. laevis* liver nuclei

Figure 4. DMS Modification (A > C). Nuclei were treated with DMS for 1, 2, 5 and 20 minutes. The 0' control received no DMS. Total nuclear RNA was extracted, fractionated on a 5-40% sucrose gradient, and subsequently purified on a 5% polyacrylamide denaturing gel. Modified U3 snRNA was used as a template for primer extension with reverse transcriptase (lanes 0' through 20'). Bands that increase with intensity as exposure to DMS increases reflect base modification and are indicated with a dot and also labeled on the left. Symbols for degree of modification are: ● (strong), ○ (moderate), ◐ (weak). The sequence of U3 snRNA is shown on the right and was generated by dideoxy sequencing of pX1U3. Note that modified nucleotides are identified by locating the base in the sequencing lanes that is just above the designated band in the DMS lanes.

were treated with chemicals that react with exposed single-stranded nucleotides at a base-pairing position: DMS modifies A(N1) > C(N3), kethoxal modifies G(N1 and N2) and CMCT modifies U(N3) > G(N1) (52). The modifications were carried out under buffer conditions where the nuclei remained intact, as judged by light microscopy; since the U3 snRNA is still

Figure 5. Kethoxal Modification (G). Nuclei were treated with 1/20 and 1/40 volumes kethoxal. The 0 control received simultaneously the stop buffer and the highest concentration of kethoxal at the end of the incubation period. The kethoxal modified U3 snRNA was used as a template for primer extension with reverse transcriptase, and the modified G's are marked on the right, with the same symbols as in Figure (4). The sequence of U3 snRNA is shown on the left and was generated by dideoxy sequencing of unmodified U3 snRNA.

part of a snRNP particle, proteins of the particle could block the reactivity of certain single-stranded nucleotides. Therefore, we are assaying only single-stranded residues that are exposed and available for interactions *in vivo* with this protocol. After chemical treatment of nuclei, RNA was extracted and separated on sucrose gradients into fractions where U3 snRNA was free (75-90%) or associated with high molecular weight material (10-25%). Chemically reactive nucleotides in samples of U3 snRNA from the free fractions were analyzed by hybridization with a synthetic oligonucleotide followed by primer extension using reverse transcriptase. Reverse transcriptase pauses one nucleotide before a modified residue (55, 56); therefore, positions of chemical modification can be read as bands on a sequencing gel. Examples of this procedure are shown in Figure 4 (DMS modification of Box A and of the C₇₂-A₇₅ region), Figure 5 (kethoxal modification of the G₉₀ loop and of Box B), and Figure 6 (CMCT modification of the U₁₃₄-U₁₃₇ loop and of Box C). Only bands that were stronger than background bands of the control lane were scored. We could not score modifications 3' distal to A₂₀₀, as this was the region complementary to our 3' end primer used for extension.

Figure 7 summarizes our chemical modification data and shows a secondary structure model for *X. laevis* U3 snRNA. This model is based upon the chemical modification data just described, as well as upon

Figure 6. CMCT (U > G). Nuclei were treated with 2/3, 1/3 and 1/6 volumes CMCT. The O₂ control received the highest concentration of CMCT mixed with CMCT stop buffer; the O₁ control received no CMCT. Total nuclear RNA was fractionated by sucrose density centrifugation and U3 snRNA from the top fraction was isolated and used without further gel purification. CMCT modified U3 snRNA was used as a template for primer extension with reverse transcriptase (five lanes on right), and the nucleotides are indicated on the right, with the same symbols as in Figure (4). The sequence of U3 snRNA is shown on the left, based upon dideoxy sequencing using unmodified U3 snRNA as template.

phylogenetic evidence. A hairpin stem is considered phylogenetically "proven" if two or more compensatory base changes are found which retain base-pairing (57); if a bulge occurs in a stem, then the portion of the stem beyond the bulge must also have two or more compensatory base changes itself to qualify for phylogenetic proof. Our model depicts the minimal base-pairing; if a nucleotide is not part of a phylogenetically proven stem then it is depicted as unpaired. Although the 5' region of U3 snRNA could be drawn as base-paired (see refs. 30, 37, 54, 58), we believe it may be single-stranded. Several of the nucleotides in this region are chemically modified

(summarized in Figure 7), and there is no phylogenetic proof for a stem here, so we have drawn it as single-stranded. Furthermore, our chemical modification data do not support the base-pairing proposed by others for rat U3B snRNA of the residues matching *Xenopus* 82-85 (30, 54), 87-90 (58) and 106-109 (30, 54); we depict these regions as unpaired. Parker and Steitz (58) also report chemical modification for U3 snRNA (human), and we have included their data for comparison to ours in Figure 7. Generally, there is good agreement between the two sets of data. The differences in positions of modification may reflect differences in our methods (e.g., they used a sonicated cell extract and we homogenized cells to prepare nuclei), differences in sequences for human and *Xenopus* U3 snRNA, or different conformers of U3 snRNA. Finally, conserved Boxes A, B and C are shown by brackets in Figure 7, and it can be seen that they all contain nucleotides which are accessible to chemical modification that is specific for single-stranded nucleotides.

DISCUSSION

Since U3 snRNAs from *X. laevis* and *X. borealis* are nearly identical, we can rule out the possibility that U3 snRNA base-pairs with any of the nonconserved stretches of ITS 2: there are neither sequence complementarities nor compensatory base changes between U3 snRNA and the nonconserved stretches of ITS 2 in these two species of amphibia. None of the tracts of conserved sequence in ITS 2 of *X. laevis* and *X. borealis* show complementarity to *Xenopus* U3 snRNA except for the conserved tract at the 5' end of the ITS 2 that can base-pair with nucleotides 157-167 of U3 snRNA, many of which are included in Box C. Our data show that five residues within Box C are modified by single-strand specific chemicals and two other residues were additionally found to be modified by Parker and Steitz (58). Taken together, the data show that all but one residue in Box C can be modified, supporting the interpretation that Box C is single-stranded as depicted in our model (Figure 7). Since our chemical modification was performed on U3 snRNP in nuclei, we conclude that Box C is available for interactions even when complexed with protein in the native snRNP particle. In contrast to this, RNase A and T1 protection data lead Parker and Steitz to believe that Box C may be protected when U3 is within the snRNP particle (58). Furthermore they were unable to obtain RNase H cleavage after attempting to bind an oligonucleotide to this region of U3 snRNP. These differences could be explained by differences in the size of the probe used,

Figure 7. Secondary Structure of *X. laevis* U3 snRNA. Nucleotides which were strongly, moderately or weakly modified in our experiments are indicated by adjacent circles ●, ○, Δ, respectively. Adjacent triangles show the chemical modification data in human U3 snRNP by Parker and Steitz (58): ▲ (strong), Δ (moderate), ΔΔ (weak). An X indicates a nucleotide whose modification we could not determine because of a strong background band in the control lane. A plus is drawn superimposed on a base-pairing bar when a compensatory base change is seen in at least one other organism. Although base-pairing is theoretically possible for the 5' end of U3 snRNA (and complementary bases are drawn opposite one another in our figure), no base-pairing bars are shown here because there is no phylogenetic proof for pairing, and many residues in this region can be chemically modified. Phylogenetic proof for a stem is taken from the sequences depicted in the U3 snRNA alignment of Figure 3, but yeast was omitted from our phylogenetic proof because its exact alignment is less certain due to insertions of bases. Conserved Boxes A, B and C are indicated by brackets.

i.e., a small chemical molecule vs. a protein such as RNase. Finally, as we noted previously (23), although Box C of U3 snRNA is conserved in lower eukaryotes, the sequence of the conserved tract at the 5' end of the ITS 2 is

different in lower eukaryotes and cannot base-pair with Box C. Further experiments are required to determine clearly whether Box C of U3 snRNA may base-pair with the 5' end of ITS 2 even in vertebrates.

Do either conserved Boxes A or B of U3 snRNA base-pair with pre-rRNA? Like Box C, some residues within Boxes A and B can also be chemically modified and are drawn as single-stranded in our secondary structure model of U3 snRNA (Figure 7); therefore they are potentially available for interactions with other molecules, such as pre-rRNA. Rat U3 snRNA has been psoralen cross-linked to the external transcribed spacer (ETS) of pre-rRNA and the cross-links are found in or near the Box A region of U3 snRNA (I.L. Stroke and A.M. Weiner, personal communication). The cross-link is in a region of the ETS that contains a processing site (I.L. Stroke and A.M. Weiner, personal communication) homologous to processing sites in mouse (59) and human (60) pre-rRNA. However, processing cleavage has not been observed within the ETS of *Xenopus* (B. Sollner-Webb, personal communication). Moreover, there is no significant complementarity conserved between Box A of U3 snRNA and the ETS of *X. laevis* (61) or *X. borealis* (62). Therefore, the evidence is not compelling for base-pairing between Box A of U3 snRNA and the ETS in *Xenopus*.

Parker and Steitz (58) proposed that Box B in U3 snRNA might base-pair with a termination processing region downstream of the 3' end of 28S rRNA. Their proposed base-pairing for *X. laevis* :

beyond <i>X. laevis</i> 28S rRNA 3' end	⁺⁸³ CGCGCUC I • I I I I ⁺¹¹²	⁺⁸⁹ GUGCGAG ⁺¹⁰⁶
<i>X. laevis</i> U3 snRNA		

is weaker in *X. borealis* due to a base change in the homologous region (63) of the latter's pre-RNA:

beyond <i>X. borealis</i> 28S rRNA 3' end	⁺¹¹⁹ CGCGCCC I • I I I I ⁺¹¹²	⁺¹²⁵ GUGCGAG ⁺¹⁰⁶
<i>X. borealis</i> U3 snRNA		

Finally, our data (Figure 7) show much greater protection of Box B from chemical modification than was found by Parker and Steitz (58), thereby questioning its accessibility for base-pairing interactions.

The proposed interactions between Boxes A, B and C of U3 snRNA and pre-rRNA are not mutually exclusive, and it is conceivable that several of these interactions could occur. However, there is no strong evidence at

present that any of these interactions occur via base-pairing between U3 snRNA and pre-rRNA. Nonetheless, RNA molecules may interact with one another in ways other than base-pairing. For example, the conserved sequence near the 3' end of 16-18S rRNA (C₁₄₀₀ region of *E. coli* 16S rRNA) associates with tRNA when it is in the P site (64-66) or A site (67) of the ribosome; there is no conserved and complementary region of the various tRNAs to base-pair with this area of rRNA. In a similar way, perhaps the conserved sequences found in Boxes A, B and/or C of U3 snRNA may associate with pre-rRNA. RNA molecules can play enzymatic roles in RNA processing even without apparent base-pairing. For example, M1 RNA of *E. coli* RNase P can, in the absence of proteins, catalyze the cleavage of tRNA precursors to generate mature 5' termini (68), even though there is no obvious standard base-pairing between M1 RNA and pre-tRNA.

Although some residues within Boxes A, B and C can be chemically modified, the strongest areas of U3 snRNP modification were found in the C₇₂-A₇₅ region, the G₉₀ loop, and the U₁₃₄-U₁₃₇ loop. These highly accessible areas are not conserved in primary sequence between different organisms; if they interact with pre-rRNA by base-pairing, it must be with nonconserved regions of pre-rRNA whose sequence has coevolved with these areas of U3 snRNA to retain complementarity.

Proteins can play a major role in the binding of snRNPs to RNA substrates. It has been shown that 65% of total U3 snRNA in the nucleolus is bound to pre-rRNP, and 85% of this bound U3 snRNA can be removed by phenol extraction, suggesting the importance of protein interactions (69).

In fact, it is not even clear at present if U3 snRNP plays solely an enzymatic role in pre-rRNA processing. It is plausible that U3 snRNP might play a structural role for nucleolar organization. For example, U3 snRNP might bind pre-rRNP to hold it in the proper location and conformation while processing steps occur in the nucleolus. A fibrillar matrix can be seen even in the pseudonucleoli of anucleolate mutants of *Xenopus* which lack rDNA (70, 71), and this fibrillar matrix contains RNA (71). It is possible that this matrix RNA is maternally derived pre-rRNA, but if synthesis and processing has already occurred we would expect the maternal rRNA to be present in mature ribosomes in the cytoplasm. Does this matrix contain U3 snRNP? Several proteins are found within the nucleolus (summarized in ref. 72), including ribocharin (73) and B23 = NO38 (72) which are both in the granular portion of the nucleolus and associate with ribosomal sub-unit precursors, perhaps playing a role in the later maturation events and/or transport of the

ribosomal sub-units. Some of the proteins found in the fibrillar portion of the nucleolus include nucleolin which associates with pre-ribosomes and also with the rRNA genes (74), and fibrillarin which is implicated as part of the U3 snRNP complex (58). With the solid foundation laid for U3 snRNA sequence and secondary structure, experiments can now be designed to test the functions of U3 snRNA and its associations with other components of the nucleolus.

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