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A comparison of the solution structures and conformational properties of the somatic and oocyte 5S rRNAs of *Xenopus laevis*

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ABSTRACT

The secondary and tertiary structures of *Xenopus* oocyte and somatic 5S rRNAs were investigated using chemical and enzymatic probes. The accessibility of both RNAs towards single-strand specific nucleases (T_1, T_2, A and S_1) and a helix-specific ribonuclease from cobra venom (RNase V_1) was determined. The reactivity of nucleobase N7, N3 and N1 positions towards chemical probes was investigated under native (5 mM $MgCl_2$, 100 mM KCl, 20 °C) and semi-denaturing (1 mM EDTA, 20 °C) conditions. Ethylnitrosourea was used to identify phosphates not reactive towards alkylation under native conditions. The results obtained confirm the presence of the five helical stems predicted by the consensus secondary structure model of 5S rRNA. The chemical reactivity data indicate that loops C and D are involved in a number of tertiary interactions, and loop E folds into an unusual secondary structure. A comparison of the data obtained for the two types of *Xenopus* 5S rRNA indicates that the conformations of the oocyte and somatic 5S rRNAs are very similar. However, the data obtained with nucleases under native conditions, and chemical probes under semi-denaturing conditions, reveal that helices III and IV in the somatic 5S rRNA are less stable than the same structures in oocyte 5S rRNA. Using chimeric 5S rRNAs, it was possible to demonstrate that the relative resistance of oocyte 5S rRNA to partial denaturation in 4 M urea is conferred by the five oocyte-specific nucleotide substitutions in loop B/helix III. In contrast, the superior stability of oocyte 5S rRNA in the presence of EDTA is related to a single C substitution at position 79.

INTRODUCTION

5S ribosomal RNA is a ubiquitous component of the large ribosomal subunit. Experiments with immune electron microscopy have located the 5S rRNA near the interface region of the prokaryotic ribosome [1], where it may interact both with the 23S rRNA of the large subunit [2] and the 16S rRNA of the small subunit [3]. In the prokaryotic ribosome, 5S rRNA forms a specific complex with 2-3 ribosomal proteins [4,5], while in the eukaryotic ribosome, the RNA interacts specifically with one protein [6,7]. A number of proposals have been made regarding the functional role of 5S rRNA in the prokaryotic ribosome, including subunit association [3], tRNA binding [8], peptidyl-transferase activity [9] and GTPase activity [10]. However, the true functional role of the 5S rRNA in the ribosome and the translational process remains obscure.

A detailed understanding of the tertiary structure of 5S rRNA will contribute to an understanding of how this RNA interacts with specific proteins, and help to clarify its functional role in the ribosome. Sequence comparison has lead to the proposal of a universal secondary structure for all 5S rRNAs [11,12], which is based upon the original model proposed by Woese and Fox [13]. The fact that all 5S rRNA sequences can be folded into a common structure argues for highly conserved structural and functional roles in the ribosome. The solution structures of 5S rRNAs from both prokaryotic and eukaryotic sources have been studied with enzymatic or chemical probes [14-19]. A crystal structure of 5S rRNA has not yet been obtained. Several tentative tertiary structure models have been

proposed for a variety of 5S rRNAs [20-23], but the universality of any model is not supported by phylogenetic sequence comparison. There is also evidence that 5S rRNAs are flexible and undergo conformational switches [24-27], which may have a functional importance, but which also complicates studies on the structure of the molecule.

In eukaryotes, 5S rRNA is transcribed by RNA polymerase III. In *Xenopus*, the 5S rRNA is stored in the cytoplasm of immature oocytes bound to one of the proteins, TF IIIA, required for the transcription of the gene [28]. By binding to the internal control region of the 5S gene, TF IIIA initiates a process in which two other proteins (TF IIIB and TF IIIC) form a stable ternary transcription complex that directs the initiation of transcription by RNA polymerase III [29,30]. Therefore TF IIIA has the unusual ability to bind specifically to both a DNA target in a gene, and an RNA target in the transcript of the same gene. TF IIIA from *Xenopus laevis* has a stronger affinity for the somatic 5S rRNA gene compared to the oocyte 5S rRNA gene [31], and it has recently been shown that the protein similarly has a stronger affinity for the somatic *vs.* oocyte 5S rRNA [32].

Analysis of the somatic and oocyte 5S rRNAs from the loach *Misgurnus fossilis* indicated that the two 5S rRNAs have considerably different structures in their 5' regions, and also display different conformational properties towards partial denaturation in the presence of EDTA [33]. Given the differential affinity of TF IIIA for the somatic and oocyte 5S rRNAs of *Xenopus*, we have studied the solution structures of both RNAs. These studies were conducted using the single-strand specific nucleases T_1, T_2, A and S_1, and the helix-specific ribonuclease V_1. The accessibility of purine N7 positions was determined using the chemical probes diethylpyrocarbonate (DEPC) for adenines, and dimethylsulfate (DMS) for guanines, under native and semi-denaturating (absence of magnesium) conditions. The accessibility of Watson-Crick positions in both native and semi-denatured 5S rRNA were determined at A-N1 and C-N3 positions using DMS, and at G-N1 and U-N3 positions using N-cyclohexyl-N'-(2-morpholinoethyl-) carbodiimide metho p-toluene sulfonate (CMCT). The reactivity of phosphate residues to alkylation under native conditions was determined using ethylnitrosourea (ENU). The results of these experiments indicated that both somatic and oocyte 5S rRNAs of *Xenopus* adopt very similar conformations under the conditions used to study binding to TF IIIA, but the somatic 5S rRNA was found to have a less stable conformation. The secondary structure of the RNAs determined in solution is in good agreement with the universal secondary structure model, although the chemical modification data indicate that the interior loop E has an unusual conformation. The chemical modification data also identified several potential sites for the formation of tertiary interactions in the 5S rRNA structure. In addition, we have used both wild type and chimeric 5S rRNAs to determine the molecular basis for the differential sensitivity of oocyte and somatic 5S rRNA to partial denaturation by urea and EDTA.

MATERIALS AND METHODS

Materials

Ribonucleases used in the structural analysis were purchased from either Pharmacia or Sigma, T4 polynucleotide kinase was purchased from USB Biochemicals, and AMV reverse transcriptase was purchased from Life Sciences. DMS was purchased from Aldrich, aniline and CMCT were purchased from Merck, and hydrazine, ENU and DEPC were purchased from Sigma. [γ-^{32}P] ATP was purchased from Amersham and New England Nuclear. T7 RNA polymerase was purified from *E. coli* strain BL21/pAR1219 by a published procedure [34].

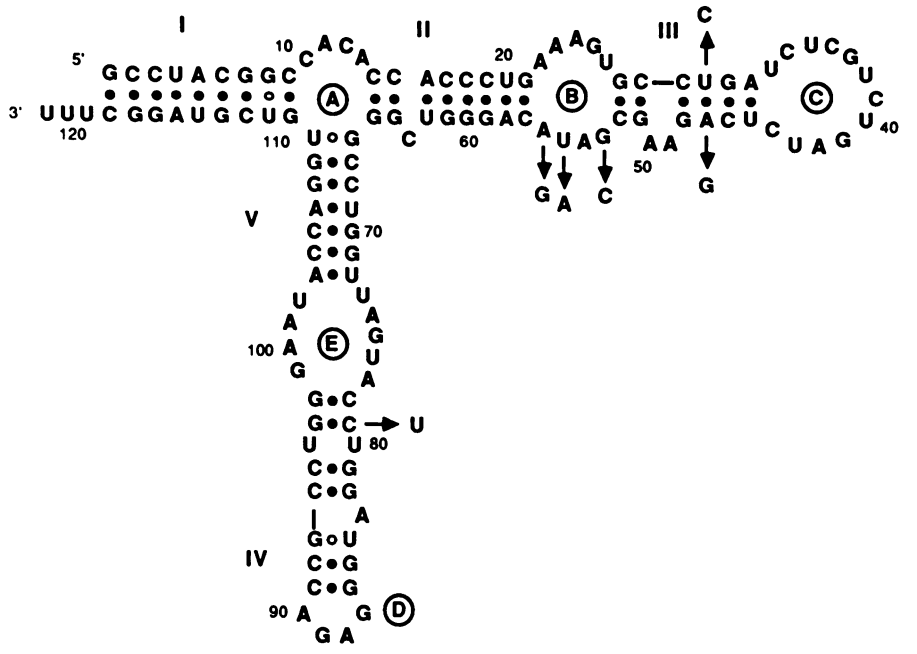

Figure 1. Secondary structure of *Xenopus* oocyte 5S rRNA, with somatic substitutions indicated. The 5'-Xls chimer has the first five substitutions, but maintains a C at position 79. The 5'-Xlo chimer has only the U substitution at position 79.

Preparation of 5S rRNAs

The wild type somatic and oocyte 5S rRNAs, and the chimeric 5'-Xls and 5'-Xlo 5S rRNAs (Figure 1) were obtained from cloned genes by *in vitro* transcription with T7 RNA polymerase, as previously described [33]. These 5S rRNA genes were constructed so that the T7 transcripts have 5' and 3' termini identical to those found in the natural 5S rRNA molecules [33]. The RNA transcripts were purified by gel permeation HPLC using a BioRad TSK-125 column (7.5 mm X 30 cm) and an elution buffer of 0.2 M sodium acetate, 1% methanol pH 6. Fractions containing 5S rRNA were concentrated, and the RNA recovered by ethanol precipitation. 5S rRNA was stored at -20 °C as an aqueous 1 mg/ml solution.

End Labeling of RNA

Before labeling RNA at the 5' terminus, the RNA was dephosphorylated with calf intestinal alkaline phosphatase using a published procedure [35], and was then purified on a 8M urea -12% polyacrylamide gel. The recovered RNA was 5' end labeled using [γ-^{32}P] ATP and polynucleotide kinase. 5S rRNA was labeled at the 3' terminus with [5'-^{32}P]pCp using T4 RNA ligase [36]. Labeled RNAs were repurified by denaturing polyacrylamide gel electrophoresis before use.

Limited Enzymatic Digestions of Labeled 5S rRNA

Before digestion, labeled 5S rRNA was renatured by incubating at 55 °C for 10 min in 100 µl of TMK buffer

(20 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 100 mM KCl) containing 10 µg carrier tRNA, followed by slow (60 min) cooling to 20 °C. The RNA was then digested for 20 min at 20 °C using the following nucleases: T_1 (0.1-3 units), T_2 (0.01-0.3 unit), A (0.1-10 ng), S_1 (22-110 units) and V_1 (0.22-0.7 unit). The reaction mixtures were then extracted with 100 µl of phenol:chloroform, and the RNA was recovered from the aqueous phase by ethanol precipitation. The RNA was redissolved in 5 µl of urea-dye buffer, heat denatured and analyzed on a 8 M urea-12% polyacrylamide sequencing gel.

Alkylation of 5S rRNA by Ethylnitrosourea

The procedure used was essentially that described by Vlassov *et al* [37]. Labeled 5S rRNA was supplemented with 2 µg of carrier tRNA. Native reaction: RNA was renatured as described above in 20 µl of buffer N-1 (300 mM sodium cacodylate pH 8, 5 mM $MgCl_2$, 100 mM KCl). A saturated solution of ethylnitrosourea in ethanol (5 µl) was added, and then the RNA was incubated for 3 h at 20 °C or 30 min at 37 °C. Denaturing reaction: Buffer D-1 (300 mM sodium cacodylate pH 8, 1 mM EDTA) was used, and incubation was 2 min at 80 °C. After alkylation, 2 µl of 3 M sodium acetate pH 6 was added, and the RNA was precipitated with 3 volumes of ethanol. After reprecipitation of the RNA from 0.3 M sodium acetate containing 1 mM EDTA, the RNA was washed with ethanol and cleaved by incubation in 0.1 M Tris-HCl pH 9. The reactions were then analyzed on a denaturing polyacrylamide gel, and the positions of the phosphates were determined by comparison to a RNase T_1 sequencing reaction [35]. The degree of phosphate alkylation at each position was determined by densitometry scanning of the autoradiogram with a Joyce-Loebl Scanning III densitometer (Gatherhead, England). Data were then processed and averaged on a PDP 11/44 minicomputer using a locally written program.

Chemical Modification of the Bases

In each experiment, 5S rRNA (labeled or unlabeled) was supplemented with 10 µg of carrier tRNA.

(i) Dimethylsulfate Modification

Native reaction: RNA was renatured as described above in 200 µl of buffer N-2 (50 mM sodium cacodylate pH 7.5, 5 mM $MgCl_2$, 100 mM KCl). DMS (0.5 µl) was added, and the reaction was incubated for 5 to 20 min at 20 °C with occasional stirring. Semi-denaturing reaction: the same procedure was used, except that renaturation of RNA was carried out in buffer D-2 (50 mM sodium cacodylate pH 7.5, 1 mM EDTA). After incubation, 100 µl of 0.3 M sodium acetate pH 6 was added, and the modified RNA was precipitated with 2.5 volumes of ethanol. RNA pellets were resuspended in 100 µl of 0.3 M sodium acetate pH 6, precipitated with ethanol, washed with 75% ethanol and vacuum dried.

(ii) Diethylpyrocarbonate Modification

Buffers and renaturation conditions were the same as those used for DMS modification. Modification was carried out by adding 20 µl of DEPC to each sample, and incubating for 30 min at 20 °C, with occasional mixing. After the incubation, RNA was recovered as outlined above.

(iii) Carbodiimide Modification

Native reaction: the RNA was renatured in 150 µl of buffer N-3 (50 mM potassium borate pH 8, 5 mM $MgCl_2$, 100 mM KCl). The reaction was started by the addition of 50 µl of CMCT (42 mg/ml), followed by incubation at 20 °C for 15, 30 or 60 min. Semi-denaturing reaction: buffer D-3 (50 mM potassium borate pH 8, 1 mM EDTA) was used, and incubation was at 20 °C for 5 or 15 min. RNA was then recovered as outlined above.

Table 1. Summary of Nuclease Cleavage Data for *Xenopus* oocyte and somatic 5S rRNAs

	T_1		T_2		A		S_1		V_1			T_1		T_2		A		S_1		V_1	
	Xlo	Xls	Xlo	Xls	Xlo	Xls	Xlo	Xls	Xlo	Xls		Xlo	Xls	Xlo	Xls	Xlo	Xls	Xlo	Xls	Xlo	Xls
U4									+++		A54						+				
A5									+++		U/A55				+	++					
C6									++		A58									+	+
G7										+++	G59									++	++
C9			++							+++	G60										
C10			+		+++	++				++	U69									+	++
A11			++	+							U72								+		
C12					++	++					U73					+	++		++		
C14						+					A74			+	++				++		
C15						+			+	++	G75	+	++						++		
A16			+						++	+++	C/U79									++	++
C17									++	++	U80									++	++
C18									++	++	G81									++	++
C19									+	++	G82									+	
U20				+		+			++	++	G87	+	+						++		
G21	+	+		+				+	+	+	A88			++	++				++		
A22			++	+				+		+	G89	+++	+++						++		
A23			++	+						+	C92									++	
A24			++	+							G93									+++	+
G25	++										C94									++	++
U26						+					C95										+++
C29									+	++	U96									+++	+
U/C30									+	++	G97									++	
G31										++	G98									+	
C34			+								G99		+							++	
U35			+								A100				++				+		
C36			++	++		+					A101				++				+		
G37	++	++	++	+				++			U102						++		+		
U38			++	++	+	++		++			C104									+	
C39			+++	+++	+++	+++		++			C105									+	
U40			+	++		++					A106									++	++
G41	+	+									G107									++	++
U45				+				++			G108									+	+
C46				++				++			U109									+	+
A/G47		+++		++				+++			U114									++	++
G48	+	+++						+++			A115									++	++
A49			++	+++				++			G116									++	++
A50			++	++							G117									++	++
G51	+	+									C118									++	++
G/C53	+					++					U119										+
											U120										+

Symbols: +++, strong cleavage; ++, moderate cleavage; +, weak cleavage. Where two nucleotides are shown, the first represents the sequence in the oocyte 5S rRNA, and the second represents the sequence in the somatic 5S rRNA.

Detection and Analysis of Modified Bases

(i) Cleavage of labeled 5S rRNA at Modified Positions

RNAs containing guanosines methylated by DMS were incubated for 5 min at 0 °C in 1 M Tris-HCl pH 8.2 containing 0.2 M sodium borohydride. To detect cytidines methylated by DMS, RNA was incubated in 10% hydrazine at 0 °C for 5 min. In both cases, the RNA was ethanol precipitated twice from 0.3 M sodium acetate pH 6, washed with 75% ethanol and vacuum dried. RNA modified at G-N7, C-N3 or A-N7 was then cleaved with aniline at 60 °C. The reactions were then analysed by electrophoresis on 8M urea-15% polyacrylamide sequencing gels.

(ii) Primer Extension with Reverse Transcriptase

The oligonucleotide primer AAGCCTACG, complementary to nucleotides 112-120 of the 5S rRNAs, was

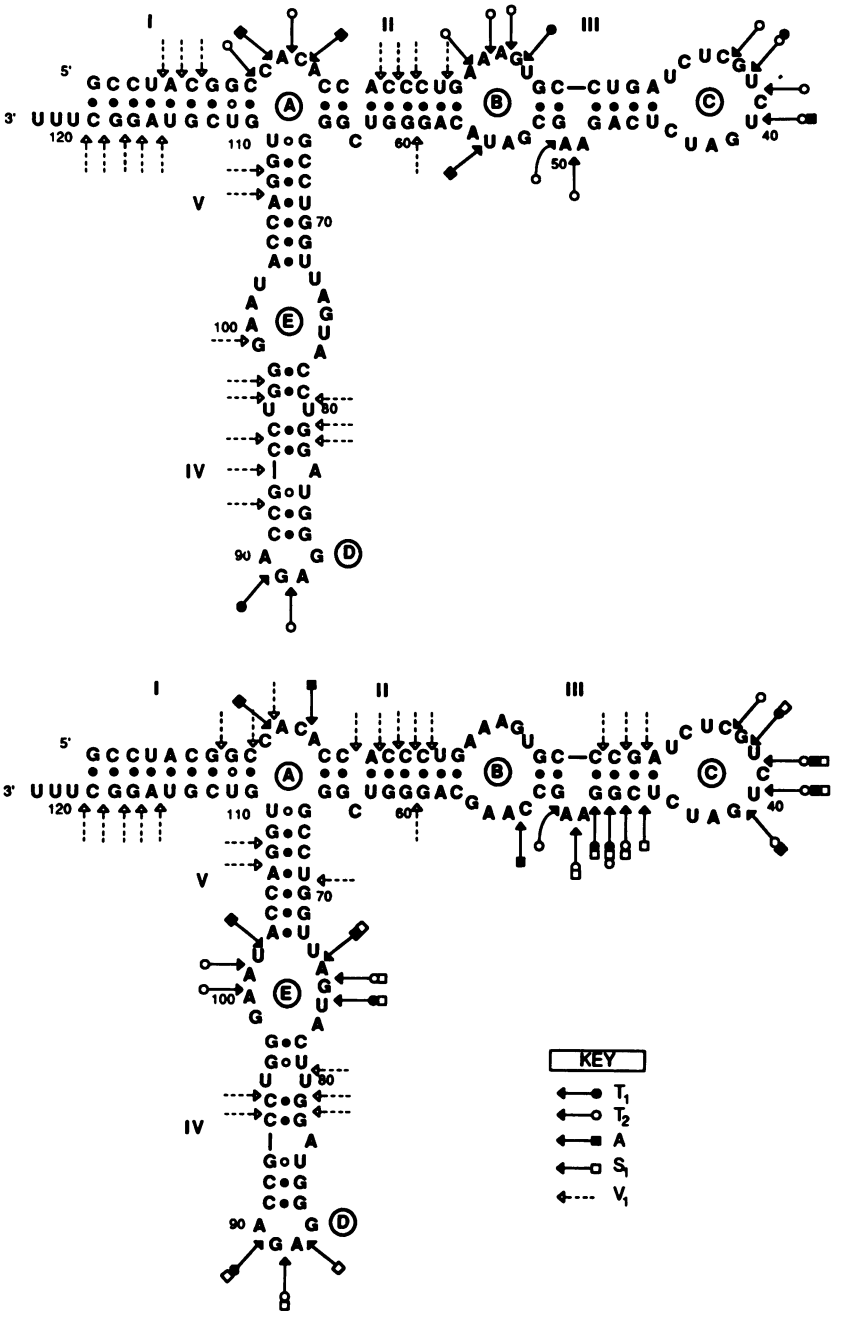

Figure 2. Summary of the nuclease digestion experiments, showing moderate and strong cleavages on the secondary structure models of oocyte (top) and somatic (bottom) 5S rRNAs.

synthesized by the phosphoramidite method using an Applied Biosystems instrument. The primer was labeled with ^{32}P at the 5' end according to Silberklang *et al.* [38]. For each extension reaction, the labeled primer (80,000 cpm) was hybridized to modified 5S rRNA by incubation at 65 °C for 5 min in 8 µl of water. Then 2 µl of 5X annealing buffer (250 mM Tris-HCl pH 8.3 [37 °C], 30 mM $MgCl_2$, 200 mM KCl) were added and incubation continued for a further 5 min at 65 °C. The annealing mixture was then slowly cooled to room temperature. The elongation reaction was performed in 15 µl of 1X annealing buffer for 35 min at 37 °C after the addition of 1 unit of reverse transcriptase, and 0.25 mM of each deoxyribonucleotide. The reaction was stopped by precipitating the nucleic acids by the addition of 200 µl of 0.3 M sodium acetate pH 6, and 600 µl ethanol. Pellets were reprecipitated from 100 µl of 0.3 M sodium acetate, washed with 80% ethanol and dried under vacuum. Each sample was redissolved in a formamide-dye sample buffer and analyzed by electrophoresis on 8M urea-10% polyacrylamide gels.

Analysis of the Conformational Stability of 5S rRNA

(i) Semi-denaturing Electrophoresis in the Presence of 4M urea

Each labeled 5S rRNA sample was analyzed by semi-denaturing 4M urea-polyacrylamide gel electrophoresis in the presence of ethidium bromide [39]. Gels were electrophoresed at 15 mA constant current until the xylene cyanol dye had run off the bottom of the gel.

(ii) Semi-denaturing Effect of EDTA

5S rRNAs were renatured in four different buffers: TK (20 mM Tris-HCl pH 7.5, 100 mM KCl), TMK (20 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 100 mM KCl), TMK + EDTA (20 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 100 mM KCl, 10 mM EDTA) and TEK (20 mM Tris-HCl pH 7.5, 10 mM EDTA, 100 mM KCl). Renaturation was carried out by incubating the labeled RNA in 4 µl of the appropriate buffer for 10 min at 55 °C, and then either slowly cooling (*ca.* 60 min) to room temperature before placing on ice, or immediately placing on ice. Before loading onto a non-denaturing mini-polyacrylamide gel (10% polyacrylamide in 1X TBE buffer), 2 µl of glycerol-dye buffer was added to each sample, and 2 µl of the sample were then applied to the gel. The gel was electrophoresed at 4 °C at constant voltage (100 V) until the xylene cyanol dye had just reached the bottom. The gel was then fixed for 10 min in 10% methanol/10% acetic acid (v/v), and dried before autoradiography.

RESULTS AND DISCUSSION

Nuclease Digestion

The solution structures of oocyte and somatic 5S rRNAs were probed with the single-strand specific nucleases T_1, T_2, A and S_1, and the helix-specific RNase V_1, using a range of enzyme concentrations. Cleavage patterns were determined with both 5' and 3' end labeled 5S rRNA to allow the discrimination of primary vs. secondary cuts. The primary cleavage sites for all of the enzymes observed with the oocyte and somatic 5S rRNAs are summarized in Table 1.

In order to study the native conformation of the RNA molecules, both 5S rRNAs were carefully renatured in the presence of Mg^{2+} ions prior to modification, and care was taken to use uniform conditions of salt concentration, pH and temperature for all of the enzymes. The conditions chosen are those used to study the interaction of these RNAs with TFIIIA [32]. Such considerations are extremely important. For example, structure probing with nuclease S_1 has been most commonly carried out at pH 4.5 in the absence of Mg^{2+} [40]. However, we find that treatment of

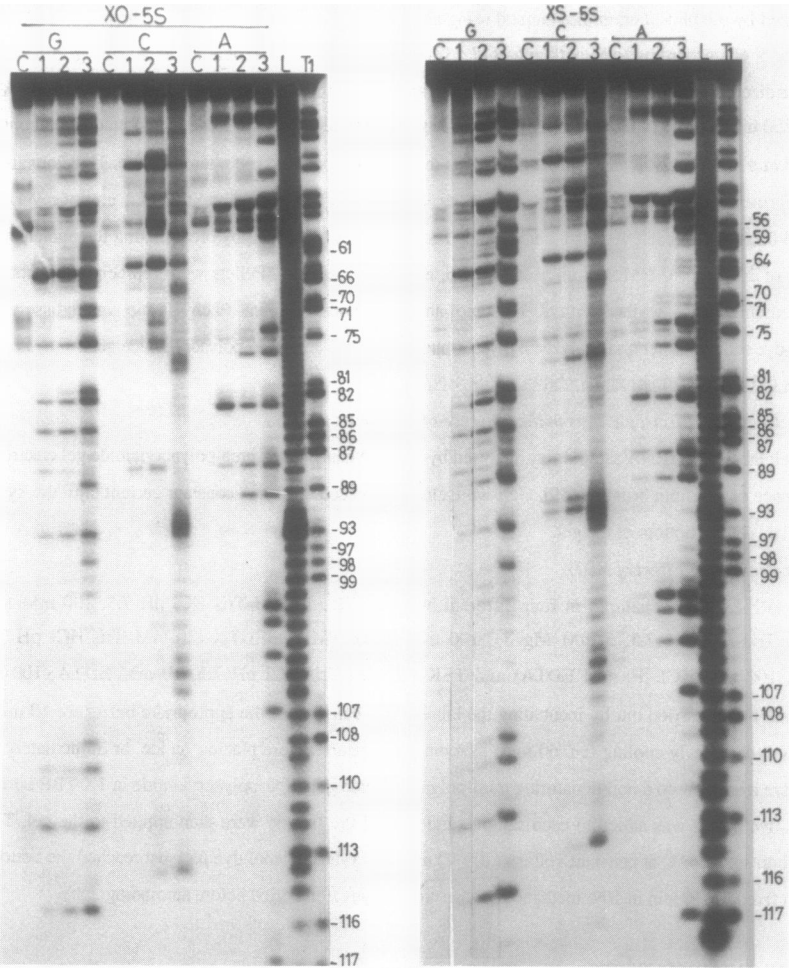

Figure 3. Autoradiograms of structural analysis gels showing the results of modifying the oocyte and somatic 5S rRNAs at the N-7 positions of G and A, and the N-3 position of C. Legend: C, control; 1, native conditions; 2, semi-denaturing conditions; 3, denaturing conditions; L, base hydrolysis ladder; T_1, T_1 sequencing reaction.

oocyte 5S rRNA under these conditions results in a completely different pattern of S_1 cleavage compared to that observed when the reactions are carried out in normal TMK buffer. At pH 4.5, loops B, C and E of *Xenopus* 5S rRNA become much more accessible to S_1 nuclease, and the U_{45}-C_{52} region of helix III is also more susceptible to cleavage (results not shown). This effect is presumably the result of a conformational change that is primarily pH driven, although addition of Mg^{2+} to a final concentration of 20 mM does reduce the reactivity in loops B and C. We have also found that raising the Mg^{2+} concentration to 20 mM in the TMK buffer results in the appearance of new

RNase V_1 cleavages at U_{33}, U_{35} and U_{72}. These additional sites of reactivity occur in apparent single stranded loops, and probably result from an increase in the stacking of nucleotides in the loop [41].

The sites of moderate and strong accessibilities towards the nucleases under native conditions in TMK buffer have been summarized on the secondary structure models of the oocyte and somatic 5S rRNAs (Figure 2). For the oocyte 5S rRNA, the most accessible regions for the single-strand specific nucleases are loops A and D and certain regions in loops B and C. Only a few minor cleavages are found in loop E (see Table 1). Strong RNase V_1 cuts are observed in each of the five helical stems. The oocyte 5S rRNA was generally resistant to cleavage by nuclease S_1, there being only a few minor cleavages under conditions in which a much stronger reactivity was observed with the somatic 5S rRNA. This difference is perhaps most striking when one compares the accessibility towards nucleases T_2 and S_1: in the somatic 5S rRNA, there is generally good correspondence between the cleavage pattern of both nucleases (Table 1), while the oocyte 5S rRNA is far more susceptible to T_2 than it is to S_1.

Comparison of the nuclease cleavage points for the somatic and oocyte 5S rRNAs shown in Figure 2 reveals some significant differences. For the somatic 5S rRNA, these differences include: a decrease in the sensitivity of loops A and B towards single-strand specific nucleases; an enhanced sensitivity of loops C and D towards nuclease S_1; and an increased sensitivity of loop E towards several single-strand specific nucleases. Helices I, II, IV and V are supported by the presence of RNase V_1 cleavages and the absence of cleavage by any single-strand specific nucleases. Only helix III contradicts this pattern. The 5' side of this helix is cleaved by RNase V_1, but the 3' side is susceptible to moderate cleavage by almost all of the single strand specific nucleases used. The decreased stability of helix III in somatic *vs.* oocyte 5S rRNA was unexpected, since one of the A-U base pairs is replaced by a G-C base pair in the somatic 5S rRNA.

Chemical Probes.

The reactivities of purine N7 positions in 3' end labeled RNA were probed with DEPC (A-N7) and DMS (G-N7). Autoradiograms of typical experiments are shown in Figure 3, and the data from several such experiments are summarized in Figure 4. The accessibility of Watson-Crick base pairing positions was tested by reacting the 5S rRNAs with DMS (A-N1, C-N3) or CMCT (G-N1, U-N3). The reactivity of cytidine residues was determined by subsequent hydrazinolysis and aniline cleavage of 3' end labeled 5S rRNA (Figure 3). The reactivity of A, G and U residues was determined by primer extension using reverse transcriptase. Because of the nature of this technique, it was not possible to obtain any information regarding the reactivity of nucleotides 103-121, since this region contains the priming site, and the immediately adjacent sequence in which strong pausing by reverse transcriptase is observed. The nucleotide positions corresponding to 1-9 were not well resolved on gels, and the reactivities of these nucleotides could not be assessed. The results from several experiments have been analyzed, and are summarized on the secondary structure models in Figure 5.

The results obtained with the chemical probes provide a more detailed picture of the conformation of each loop. The reactivities at both N7 and Watson-Crick positions confirm the presence of two bulged A residues at positions 49 and 50, a bulged C at position 63, and a bulged A at position 83. Andersen *et al.* have proposed on the basis of phylogenetic sequence comparison that in oocyte 5S rRNA U_{84} is the bulged nucleotide closed by G_{93}-A_{83} and C_{92}-G_{85} base pairs [18]. The high reactivity of the A_{83}-N7 position to DEPC does not appear to support this

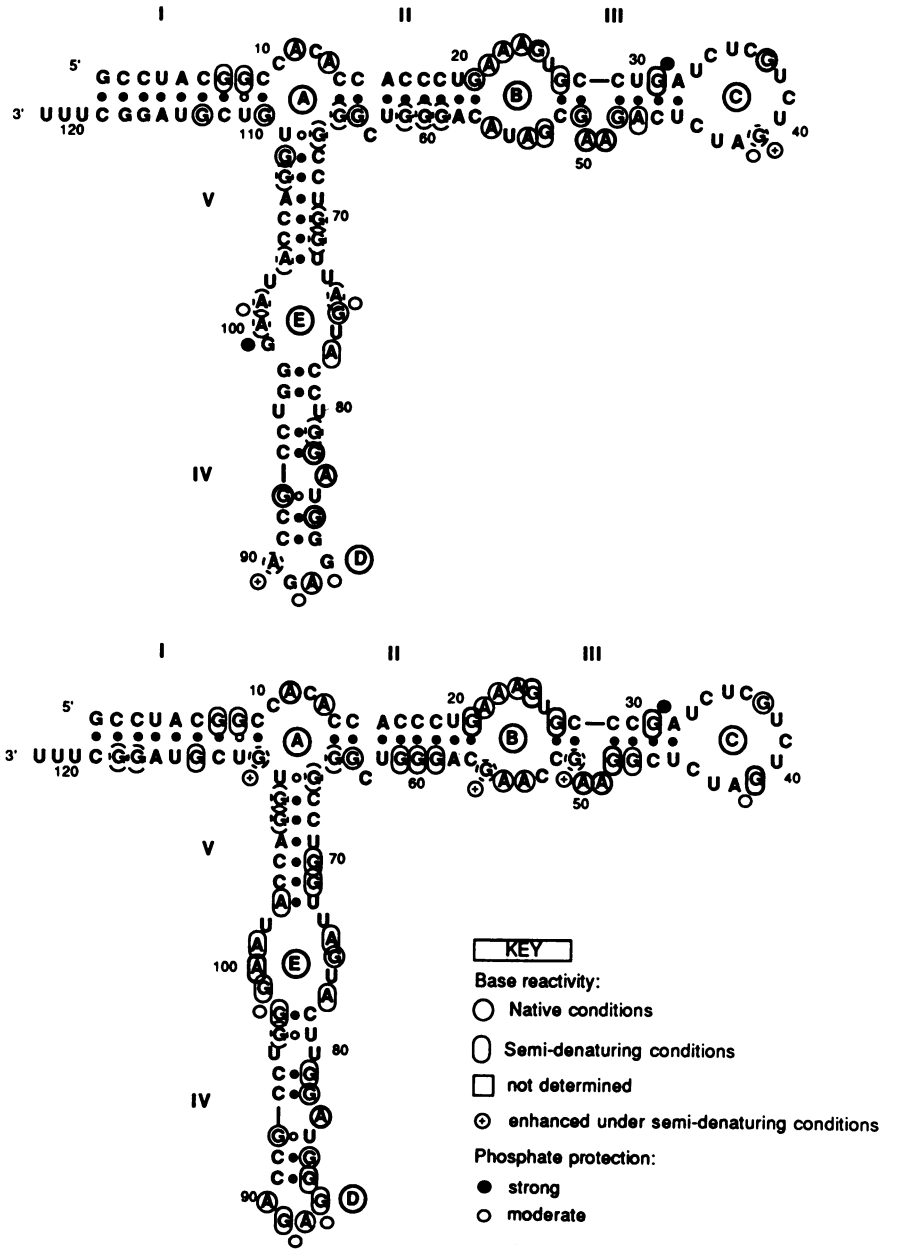

Figure 4. Summary of the chemical modification of purine N-7 positions and phosphates of oocyte (top) and somatic (bottom) 5S rRNAs. The extent of reactivity is indicated by: thick line, strong; thin line, moderate; broken line, weak. Those purines which are not enclosed were reactive only under completely denaturing conditions.

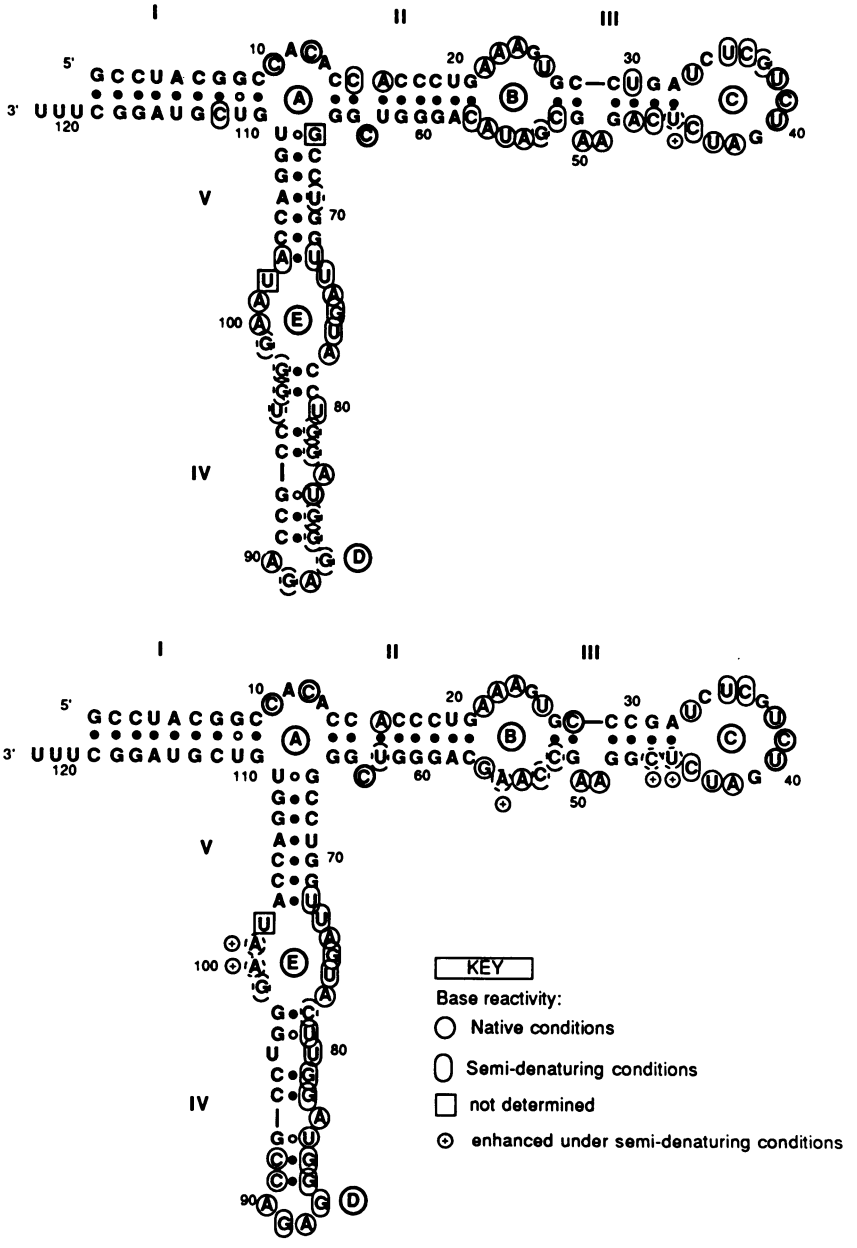

Figure 5. Summary of the chemical modification of A(N-1), G(N-1), C(N-3) and U(N-3) positions of oocyte (top) and somatic (bottom) 5S rRNAs. Symbols are the same as those used in Figure 4.

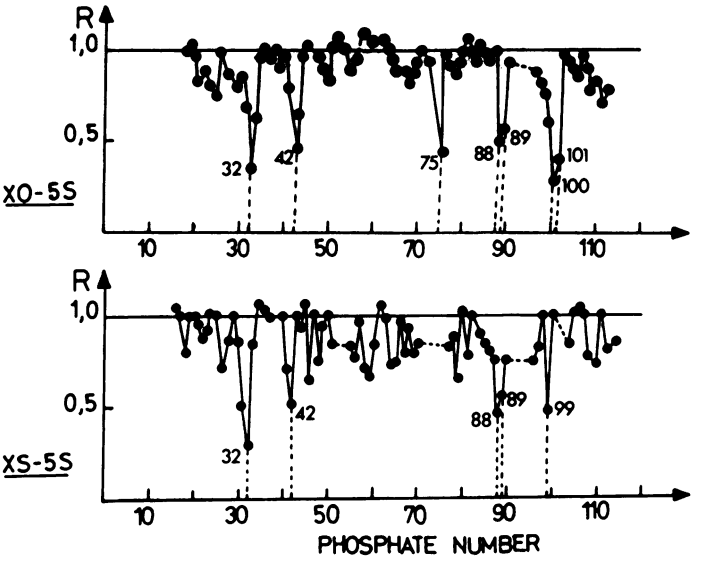

Figure 6. Pattern of phosphate reactivities to ENU under native conditions *vs.* denatured conditions. R values are the ratio between the intensities of the corresponding bands under both conditions, measured as peak heights on the densitograms of the gels. A ratio <1 indicates that alkylation of a given phosphate is lower in the native molecule than it is in the denatured molecule. The reactivity of some phoshpates could not be analysed due to unspecific degradation, and are indicated by a broken line connecting adjoining dots.

model, although the fact that the U_{84}-N3 position is reactive to DMS indicates that the putative G-U pair is not very stable. An equilibrium between the bulged A and bulged U conformations has previously been proposed [18]. It is not unusual for a base pair closing a bulged or internal loop to have a reduced stability, resulting in a local flexibility. These results are the same in both the somatic and oocyte 5S rRNAs.

For loop A, the reactivities of the C-N3 positions and the A-N7 positions under native conditions agree with the nuclease data and indicate that the nucleotides in this loop are essentially single stranded. Similarly, in loop B, seven of the nine nucleotides are reactive to the chemical probes under native conditions. This loop contains three of the somatic/oocyte specific nucleotide substitutions, but the chemical reactivity pattern is the same for both RNAs. The nucleotides at positions 25 and 53 are unreactive, but only the residue at position 53 becomes reactive under semi-denaturing conditions.

The chemical reactivity data of loop C residues is the same for both RNAs. Of the three purine residues, only G_{37} is clearly reactive at the N7 position under native conditions, and the N7 of A_{42} remains unreactive under semi-denaturing conditions. Under native conditions, the sequence UCU_{40} is highly reactive at the Watson-Crick positions, and also is extremely susceptible to cleavage by single-strand specific nucleases. The extension of helix III by the pairing of U_{33}-A_{42} and C_{34}-G_{41} has been proposed in several structural models [18,19,42], but is only partially supported by the chemical reactivities: although C_{34}-N3 and G_{41}-N1 are totally unreactive even under semi-denaturing conditions, the formation of a U_{33}-A_{42} base pair is contradicted by the reactivity of the Watson-Crick

positions of both of these residues under native conditions. The lower reactivity of phosphate 42 towards ENU alkylation (see below) suggests that this phosphate may be involved in a tertiary interaction.

The tetranucleotide sequence $CUCG_{37}$ of loop C is unreactive at the Watson-Crick positions under native conditions, and only partially reactive under semi-denaturing conditions. It has been proposed that the $UCUC_{36}$ sequence in rat 5S rRNA has the potential to form a Watson-Crick base paired tertiary interaction with the $GAGA_{90}$ sequence of loop D [43]. However, the reactivity of the A residues in loop D at both N7 and N1 positions under native conditions argues against such a simple interaction in *Xenopus* 5S rRNA. In contrast to the A residues, the G residues at positions 87 and 89 in loop D are unreactive at N7 and N1 positions under native conditions, and only become partially reactive under semi-denaturing conditions. Furthermore, phosphates 88 and 89 have a decreased reactivity towards ENU under native conditions. A similar result has been reported for the analogous phosphates in *E. coli* 5S rRNA [23]. These results, coupled with the high accessibility of this region to single-strand specific nucleases, suggest that loop D has an unusual conformation. The pattern of reactivity at Watson-Crick and N7 positions in loop D of *Xenopus* 5S rRNA is similar to that found for other four membered loops in 16S rRNA [44]. This similarity suggests that these loops adopt a characteristic conformation that might be stabilized by magnesium coordination, which would contribute to the observed results.

The chemical reactivity data for loop E indicates that a complex conformation exists in this region of the 5S rRNA. Once again, the data are very similar for the somatic and oocyte 5S rRNAs. Only the N7 of G_{75} is reactive in both RNAs under native conditions. In the somatic 5S rRNA, all of the purines are fully reactive at N7 under semi-denaturing conditions, while for the oocyte 5S rRNA A_{74}, A_{100} and A_{101} are partially reactive under these conditions, and G_{99} remains unreactive. This difference between the two RNAs mirrors the degree of accessibility of the loop E region towards nucleases, and suggests that the conformation of loop E is more stable in the oocyte 5S rRNA than it is in the somatic 5S rRNA.

In both RNAs, the AA_{101} sequence was at least partially reactive at the N1 positions under native conditions. Although this sequence was reactive in both somatic and oocyte 5S rRNAs, the degree of reactivity was difficult to assess because of the relatively strong pausing in this region. On the other side of the loop, the two adenosine residues were reactive at the N1 position under native conditions, while the rest of the residues became reactive at their respective Watson-Crick positions only under semi-denaturing conditions. In previous studies on the structure of *Xenopus* oocyte 5S rRNA, the lack of single-strand specific nuclease cleavages in loop E, coupled with the presence of one or two weak RNase V_1 cleavages [18,19] has resulted in the proposal of a base paired conformation for this region consisting of A_{74}-U_{102}, G_{75}-A_{101}, U_{76}-A_{100} and A_{77}-G_{99} [18]. The A_{74}-U_{102} base pair is ruled out by the reactivity of A_{74}-N1 under native conditions. Similarly, formation of the U_{76}-A_{100} base pair seems unlikely given the reactivity of the N1 of A_{100} under native conditions: however, the N3 of U_{76} is only reactive under semi-denaturing conditions.

Using the chemical reactivity data, a model structure for loop E can be proposed based upon non-canonical base pairing between U_{73}-U_{102}, A_{74}-A_{101}, G_{75}-A_{100} and U_{76}-G_{99}. A U (N3-H, O4)-U (O2, N3-H) base pair was found in the crystal structure of yeast $tRNA^{asp}$, which forms a dimer through the GUC anticodon [45], and was proposed for the binding site of ribosomal protein S8 on 16S rRNA [46]. Similarly, A(N6-H, N7-H)-A(N7-H, N6-H) pairs have also been observed in the crystal structure of $tRNA^{Asp}$[45], and A(N7-H, N6-H)-G(N1-H, O6) pairs

have been proposed in the case of ribosomal RNAs [47]. In the structure we propose for *Xenopus* 5S rRNA, the non-reactivity of A_{77}-N7 towards DEPC suggests that this adenine should be stacked. The proposed interactions are in agreement with the chemical reactivities of the loop E nucleotides, with the exception of the U_{73}-U_{102} pairing. This pairing is only partially supported by the data, since it was not possible to determine the reactivity of the U_{102} residue.

The reactivity of the phosphates in both 5S rRNAs towards alkylation was tested under native conditions at 20 °C and 37 °C, and the results are shown in Figure 6. As shown in Figure 4, the phosphates at positions 32, 42, 75, 88, 89, 100 and 101 of oocyte 5S rRNA were protected from reaction with ENU. In the case of somatic 5S rRNA, unreactive phosphates could not be easily identified by inspection, but densitometry revealed that phosphates 32, 42, 88, 89 and 99 were significantly less reactive under native conditions (Figure 6). These include phosphates in loops C, D and E. These phosphates are adjacent to nucleotides which have unreactive N7 or Watson-Crick positions under native conditions, suggesting that either the phosphates are directly involved in the same tertiary interactions as the neighbouring bases, or may form Mg^{2+} binding sites which help to stabilize the tertiary interactions. The phosphate protection pattern observed for the *Xenopus* 5S rRNAs is similar to that observed for three other eukaryotic 5S rRNA molecules [23]. However, in the *Xenopus* 5S rRNAs, additional phosphate protection is observed in loop C which was not observed in yeast, fungal or rat 5S rRNAs [23].

The only residues within helices which are reactive to chemical probes under native conditions are those immediately adjacent to bulged nucleotides. The reactivity of these sites probably results from dynamic breathing of the base pairs closing the bulge. The data are consistent with the formation of five helical stems in the 5S rRNA: reactivity under semi-denaturing conditions suggests that helices III and IV are less stable than the other three helices. In helix IV uridines 80 and 96, which appear to be a non-pairing opposition, are unreactive at their N3 positions towards CMCT in native conditions. We propose that these two uridines form a non-canonical base pair involving two hydrogen bonds formed between U_{80} (N3-H, O4) and U_{96} (O2, N3-H).

Differences in the Conformational Stability of Somatic *vs.* Oocyte 5S rRNA

The somatic and oocyte 5S rRNAs of *Xenopus* can be distinguished by migration on polyacrylamide gels in the presence of 4 M urea [39]. In order to assess the structural basis for this difference in migration, the mobilities of two chimeric 5S rRNAs (Figure 1) under these conditions were compared to the wild type RNAs. As the autoradiogram in Figure 7A shows, the relative resistance of oocyte 5S rRNA (lane 1) to denaturation in 4 M urea results in a faster mobility compared to somatic 5S rRNA (lane 2). The mobility of the chimeric 5S rRNAs is dictated by the sequence in the 30-56 region of the molecule: the chimer carrying the five somatic-specific substitutions in this area has the same mobility as the somatic wild type RNA, while the chimer carrying the five oocyte-specific substitutions in this region has the same mobility as the oocyte wild type RNA. Therefore the greater sensitivity of the somatic 5S rRNA to mild denaturation in urea is the result of one or more of the five somatic-specific nucleotide substitutions found in helix III-loop B.

The renaturation properties of the somatic, oocyte and two chimer 5S rRNAs from *Xenopus* were investigated. After isolation from 7 M urea-12% acrylamide gels, both the somatic and oocyte 5S rRNAs exist as a mixture of two resolved bands on a non-denaturing 10% polyacrylamide gel (Figure 7B and C, lane 1). Renaturation of both RNAs in TMK buffer by heating at 50 °C and cooling either slowly (lane 3) or rapidly (lane 7) results in conversion

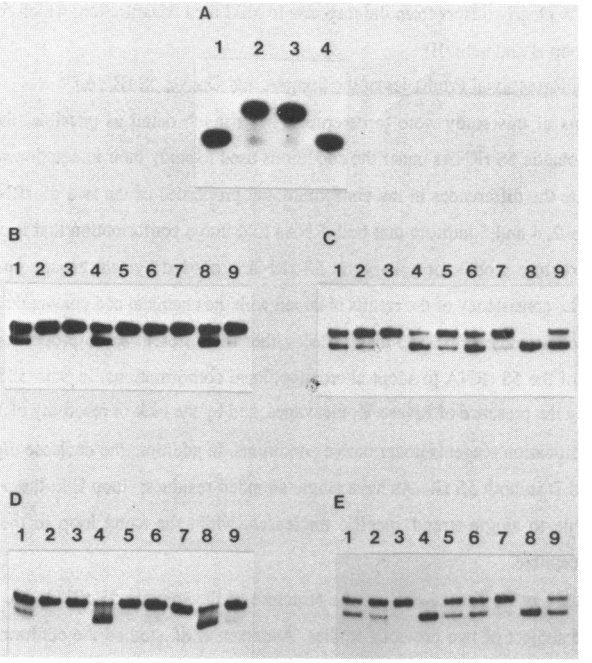

Figure 7. Conformational analysis of oocyte, somatic and chimeric 5S rRNAs. A. Migration under semi-denaturing gel electrophoresis in the presence of 4M urea: 1, Xlo-wt; 2, Xls-wt; 3, 5'-Xls chimer; 4, 5'-Xlo chimer. B-E. Migration on non-denaturing gels after renaturation in: 1, no renaturation; 2 and 6, TK; 3 and 7, TMK; 4 and 8, TMK + EDTA; 5 and 9, TEK; the RNA in lanes 2-5 was slowly cooled after incubation at 55 °C, while the RNA in lanes 6-9 was quickly cooled on ice after incubation. B, Xlo-wt; C, Xls-wt; D, 5'-Xls chimer; E, 5'-Xlo chimer.

to 100% of the slower moving band. The same renaturation process carried out in TK buffer (lanes 2 and 6) also completely resolves the mixture into the slower moving species for the oocyte 5S rRNA, but does not do so for the somatic 5S rRNA. A similar result is observed upon renaturation in TEK buffer (lanes 5 and 9). When the two RNAs are renatured in TMK buffer with two fold molar excess (to Mg^{2+}) of EDTA added (lanes 4 and 8), the somatic 5S rRNA is completely converted to the faster migrating species, while the oocyte 5S rRNA remains a conformational mixture. Under all of the conditions employed, the conformation of the oocyte 5S rRNA represented by the slower migrating species is either more readily formed or more stable than is found for the somatic 5S rRNA. In the case of the somatic 5S rRNA, excess free Mg^{2+} ions are required for the stable formation of the slower migrating species.

The same experiments were conducted on the 5'-Xls chimer (Figure 7D) and the 5'-Xlo chimer (Figure 7E). A comparison of these data reveals that the oocyte conformational properties discussed in the above paragraph can be conferred upon the somatic 5S rRNA simply by substituting a cytidine for uridine at position 79 (compare Fig. 7B and 7D). Conversely, the somatic conformational properties can be transferred to the oocyte 5S rRNA by substituting a uridine for cytidine at position 79 (compare Fig. 7C and 7E). The origin of the sequence specificity of this

conformational property clearly differs from the response to mild urea denaturation, which follows the somatic-specific substitutions in loop B and helix III.

Comparison of the Conformational Properties of the Somatic and Oocyte 5S rRNAs

The primary aims of this study were to determine in as much detail as possible, the solution structure of *Xenopus* oocyte and somatic 5S rRNAs under the conditions used to study their interaction with transcription factor IIIA, and to investigate the differences in the conformational properties of the two 5S rRNA species. The results summarized in Figures 2, 4 and 5 indicate that both RNAs fold into a conformation that is consistent with the generalized secondary structure model for eukaryotic 5S rRNA proposed by two groups on the basis of sequence comparison [11,12]. The consistency of the results obtained with the chemical and enzymatic structure probes is very high, and the few inconsistencies observed may reflect either the different steric properties of these two types of probes, or the ability of the 5S rRNA to adopt alternative, local conformations. In both 5S rRNAs, all five helical stems are confirmed by the presence of RNase V_1 cleavages, and by the lack of reactivity of Watson-Crick positions towards chemical modification reagents under native conditions. In addition, the nuclease digestion studies indicate that loops A, B, C and D in both 5S rRNAs have single stranded residues: loop E in the oocyte 5S rRNA is only very mildly susceptible to single-strand specific nucleases, while the same loop in the somatic 5S rRNA is considerably more susceptible.

Although this study is the first to analyze the structure of the somatic 5S rRNA, the oocyte 5S rRNA from *Xenopus* has been the subject of two previous studies. Andersen *et al.* studied the conformation of the oocyte 5S rRNA in 50 mM Tris pH 7.6, 10 mM $MgCl_2$ and 25 mM KCl at 0 °C using the ribonucleases T_1, T_2, A and V_1 [18]. Our results are in agreement with theirs for the nuclease digestions, although at 0 °C the oocyte 5S rRNA displays fewer nuclease cleavage sites than we observe at 20 °C. One major difference between their results and ours concerns the presence of minor RNase V_1 cleavages at A_{32}, U_{73} and U_{76} which they observed [18], but are not present under our conditions of hydrolysis. When we increase the Mg^{2+} ion concentration to 20 mM, we observe minor RNase V_1 cuts at U_{33}, U_{35} and U_{72}, a result similar to their observation. While this work was in progress, Christiansen *et al.* published a study comparing the accessibility of oocyte 5S rRNA to enzymatic and chemical probes in the presence and absence of TF IIIA [19]. Although their studies were conducted at 0 °C in buffers containing 2.5 mM $MgCl_2$, there is a good correlation between their data and ours. At 0 °C, they also observe RNase V_1 cleavages at U_{33} and U_{35}, suggesting that at low temperatures, a high magnesium concentration is not required to stabilize the stacking in loop C. There are only minor differences between their reported chemical reactivities, and those which we observed.

For the most part, the susceptibility towards chemical and nuclease probes is very similar for the somatic and oocyte 5S rRNAs. However, as the studies on urea and EDTA denaturation show, the oocyte-specific substitutions in loop B/helix III confer upon the RNA a greater stability to partial denaturation in the presence of 4M urea, while the oocyte-specific C_{79} substitution confers a greater stability to denaturation in the presence of EDTA. In comparing the nuclease and chemical probing data for the oocyte and somatic 5S rRNAs, two regions of significant difference appear which can be discussed in the context of the greater conformational stability of the oocyte 5S rRNA towards urea and EDTA denaturation. The 3' half of helix III in the somatic 5S rRNA is cleaved moderately by a number of single-strand specific nucleases, whereas this region of the oocyte 5S rRNA is resistant to cleavage. The

RNase V_1 cleavages in the 5' half of this helix in the somatic RNA, and all of the chemical modification data, are consistent with the formation of this helix. Although alternative conformations for helix III in eukaryotic and prokaryotic 5S rRNAs have been proposed [12], and in the case of a fragment of *E. coli* 5S rRNA have been observed by NMR [48], none are consistent with the *Xenopus* data. Therefore, we conclude from the nuclease data that helix III is less stable in the somatic *vs.* oocyte 5S rRNA, and that it is likely this relative instability that accounts for the urea denaturation properties of the somatic and the 5'-Xls chimeric 5S rRNAs. The nuclease data indicate that loop E is less stable in the somatic *vs.* oocyte 5S rRNA, and the chemical modification data under semi-denaturing conditions (1 mM EDTA) indicate an extensive melting of the 5' half of helix IV up to the tandem cytidine residues past loop D. As the conformational studies with the chimeric 5S rRNAs show, this instability is apparently caused by the single C to U difference between the oocyte and somatic 5S rRNAs at position 79.

The interaction of the somatic and oocyte 5S rRNAs with TF IIIA has been compared, along with the chimeric 5S rRNAs [32]. The somatic 5S rRNA binds to TF IIIA with a 2-3 fold stronger affinity, and it is the somatic-specific substitutions in loop B/helix III that account for this enhanced affinity. The magnitude of this effect is less than the free energy of one hydrogen bond, suggesting that the increase in affinity results from a subtle conformational difference which either strengthens one of the hydrogen bonds in the complex, or allows for the formation of an extra, fortuitous ionic contact in the somatic 5S rRNA-TF IIIA complex. The enhanced affinity of somatic 5S rRNA for the protein would appear to be the result of a subtle conformational difference in helix III.

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