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5S RNA Structure and Interaction with Transcription Factor A. 1. Ribonuclease Probe of the Structure of 5S RNA from *Xenopus laevis* Oocytes†

Janet Andersen, Nicholas Delihas,* Jay S. Hanas, and Cheng-Wen Wu

ABSTRACT: The structure of *Xenopus laevis* oocyte (Xlo) 5S ribosomal RNA has been probed with single-strand-specific ribonucleases T_1, T_2, and A with double-strand-specific ribonuclease V_1 from cobra venom. The digestion of 5′- or 3′-labeled renatured 5S RNA samples followed by gel purification of the digested samples allowed the determination of primary cleavage sites. Results of these ribonuclease digestions provide support for the generalized 5S RNA secondary structural model derived from comparative sequence analysis. However, three putative single-stranded regions of the molecule exhibited unexpected V_1 cuts, found at C_{36}, U_{73}, U_{76}, and U_{102}. These V_1 cuts reflect additional secondary structural features of the RNA including A·G base pairs and support the extended base pairing in the stem containing helices IV and V which was proposed by Stahl et al. [Stahl, D. A., Luehrsen, K. R., Woese, C. R., & Pace, N. R. (1981) *Nucleic Acids Res. 9*, 6129-6137]. A conserved structure for helix V having a common unpaired uracil residue at Xlo position 84 is proposed for all eukaryotic 5S RNAs. Our results are compared with nuclease probes of other 5S RNAs.

The 5S RNA is found complexed with specific proteins in the ribosomes of all organisms and also exists in 7S ribonucleoprotein (RNP) particles of amphibians, teleosts (Picard et al., 1979), and HeLa cells (Gruissem & Seifart, 1982). This RNA has been shown to be phylogenetically conserved not only in overall length (about 120 nucleotides) but also in sequence at certain nucleotide positions and in the internal chain lengths between conserved residues (Delihas & Andersen, 1982). Secondary structural models for both eukaryotic and eubacterial 5S RNAs have been proposed on the basis of comparative sequence analysis (Nishikawa & Takemura, 1974; Fox & Woese, 1975; Luehrsen & Fox, 1981; Studnicka et al., 1981; Böhm et al., 1981). Recently, these models have merged into one generalized secondary structural model for all phylogenetic classes of 5S RNA (Delihas & Andersen, 1982; Böhm et al., 1982; DeWachter et al., 1982; Kuntzel et al., 1983).

As a part of a study of 5S RNA conformation and RNA-protein interactions, we have probed the 5S RNA from *Xenopus laevis* oocytes (Xlo) with ribonucleases. This information also provides a background for the study of the oocyte

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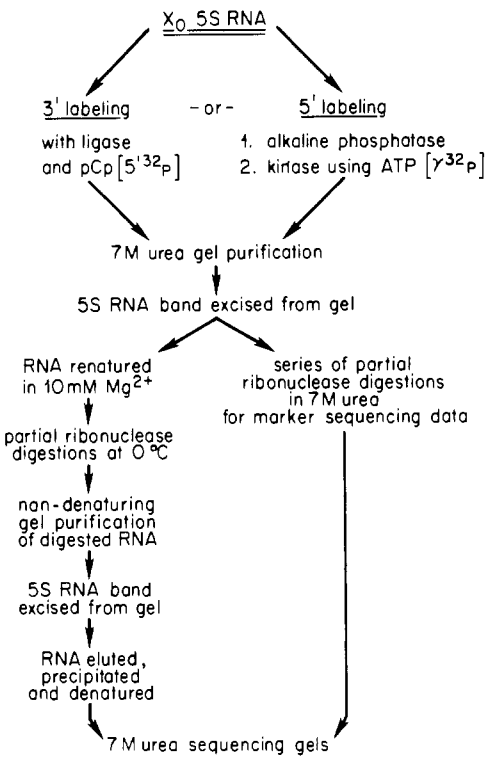

FIGURE 1: Flow chart outlining the sequence of events in the RNase probe experiments involving labeling, renaturation, partial digestions, and gel electrophoresis of Xlo 5S RNA.

5S RNA when complexed to transcription factor protein A (TFIIIA) in the 7S particle. We have used an approach similar to that taken by Douthwaite & Garrett (1981) in their ribonuclease study of eubacterial *Escherichia coli* 5S RNA since these authors have established criteria for distinguishing primary cleavage sites from secondary sites. The similarity of assays allows also for a comparison of a eukaryotic 5S RNA with a eubacterial 5S RNA. We have determined the primary cleavage sites produced by partial ribonuclease digestions of Xlo 5S RNA and have found evidence for the continuation of base pairing between helices IV and V which includes A·G base pairing. Our results are discussed with respect to the generalized secondary structural model derived by phylogenetic comparisons.

Materials and Methods

Preparation and Purification of 5S RNA. The method of Pelham & Brown (1980) was used to prepare 7S particles from immature ovaries of *X. laevis*. The particle was further purified to homogeneity by DEAE-cellulose chromatography as described by Hanas et al. (1983) and was stored at −70 °C until needed. The 5S RNA was prepared from purified 7S particles by phenol extraction or by urea treatment of the particle.

^{32}P End Labeling of 5S RNA. The treatment of 5S RNA is outlined in a flow chart in Figure 1. The 5S RNA was either 3′ labeled with [5′-^{32}P]pCp using T_4 RNA ligase (England & Uhlenbeck, 1978) or 5′ labeled with [γ-^{32}P]ATP using polynucleotide kinase following treatment with alkaline phosphatase of the RNA (Donis-Keller et al., 1977). Labeled 5S RNA was electrophoresed on a preparative 12% polyacrylamide gel containing 7 M urea in 50 mM tris(hydroxymethyl)aminomethane (Tris)–borate (pH 8.3) and 1 mM ethylenediaminetetraacetic acid (EDTA) (TBE). Using an autoradiogram as a template and noting the migration of wheat germ 5S RNA, we excised labeled Xlo 5S RNA from the gel. The 5S RNA was eluted by shaking the gel slice overnight at 37 °C in 1 mL of an elution buffer containing 0.1% sodium dodecyl sulfate (SDS), 0.5 M NH_4OAc, 0.01 M $Mg(OAc)_2$, 0.1 mM EDTA, and 50 μg of *E. coli* tRNA carrier (SDS buffer). The eluant was filtered, and the labeled RNA was subsequently precipitated with ethanol and dried. The purified 5S RNA was resuspended either in deionized water or in a renaturation buffer containing 50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, and 25 mM KCl.

Ribonuclease Digestions and Cleavage Analysis. The 5S RNA resuspended in deionized water was reserved for RNA sequencing in order to obtain position markers on sequencing gels. Sequence marker data and alkaline ladders were obtained by the method of Donis-Keller et al. (1977) and Lockard et al. (1978). Enzymes used in RNA sequencing were from P-L Biochemicals.

The buffered 5S RNA was renatured by being taken up into sterilized, drawn-out glass capillary tubes, which were then sealed and placed in a water bath at 60 °C for 5 min. The RNA was allowed to cool slowly (~1 °C/min) to room temperature before being placed on ice.

The renatured 5S RNA was retrieved from the capillary tubes and was subjected to a series of gentle partial ribonuclease digestions at 0 °C with ribonucleases T_1, T_2, and A (Calbiochem-Behring) and ribonuclease V_1 from cobra venom (a gift from Dr. William Getty of P-L Biochemicals). The concentration ranges used were 1 unit/10 μg of RNA to 1 unit/1000 μg of RNA for RNase T_1, 1 unit/50 μg of RNA to 1 unit/1000 μg of RNA for RNase T_2, 1 μg/500 μg of RNA to 1 μg/10000 μg of RNA for RNase A, and 1 unit/9 μg of RNA to 1 unit/170 μg of RNA for RNase V_1. The incubation times were 30 min for RNases T_1 and T_2, 20 min for RNase V_1, and 5 and 10 min for RNase A. The partial digestions were stopped by adding an RNase inhibitor, RNasin (Promega Biotec) and diluting the samples with gel loading buffer [50 mM Tris–borate (pH 7.5) containing 1 mM $MgCl_2$ and 50% glycerol] and immediately followed by electrophoresis on a preparative nondenaturing 8% of 12% polyacrylamide gel containing 50 mM Tris–borate (pH 7.5) and 1 mM $MgCl_2$ (TBM). The electrophoresis was performed at 500 V for about 20 h at 4 °C. The 5S RNA bands that comigrated with undigested 5S RNA were detected from the autoradiogram and sliced from the gel. The partially digested RNA samples were eluted from the gel slice, precipitated, and dried in the same manner as mentioned above. The dried RNA was subsequently resuspended in 25 mM sodium citrate (pH 5), 1 mM EDTA, and 7 M urea (CUB 5) and denatured by heating at 50 °C for 5 min. Deionized formamide was added to the samples to help maintain the denatured conditions of the fragments and to facilitate layering on the final, thin sequencing gels composed of 8% or 12% polyacrylamide, 7 M urea, and TBE buffer. According to Geiger counter readings, equal amounts of ^{32}P were loaded into each well. Gel electrophoresis was carried out at 1500–1700 V until marker dyes (xylene cyanol FF and bromophenol blue) reached predetermined points on the gel. The gels were then autoradiographed at −70 °C on Cronex X-ray film using an intensifier screen. Each cleavage position was determined by noting its position relative to marker sequence data and alkaline ladders. The determination of the extent of cleavage was made by visual analysis of band intensities on the autoradiogram.

Results

Heterogeneity of ^{32}P End Labeled 5S RNA. Labeled eukaryotic 5S RNAs show size heterogeneity that is due to variable transcription termination (Bogenhagen & Brown, 1981). Figure 2 depicts three major bands representative of

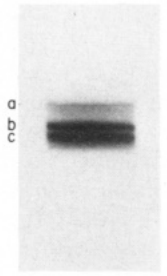

FIGURE 2: Size heterogeneity of 3′-labeled Xlo 5S RNA as determined by electrophoresis in 12% polyacrylamide purification gel containing 7 M urea. Band b is enriched in oocyte 5S RNA. Bands a and c each contain oocyte 5S RNA and a trace amount of somatic 5S RNA as explained in the text.

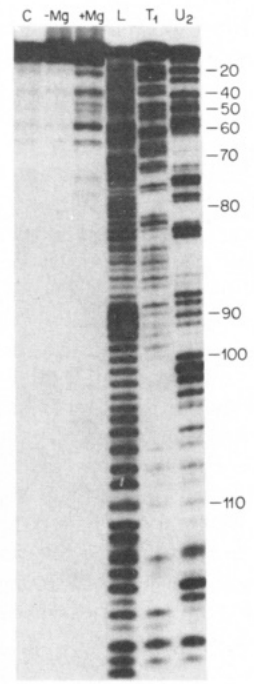

FIGURE 3: Gel patterns on a denaturing 12% polyacrylamide gel showing minor hydrolysis that occurs on 3′-labeled Xlo 5S RNA during renaturation. C is a control showing Xlo 5S RNA without renaturation; −Mg is Xlo 5S RNA heated to 60 °C and slowly cooled but without Mg^{2+}; +Mg is Xlo 5S RNA renatured in the presence of 10 mM Mg^{2+}; L is an alkaline ladder; T^1 and U_2 are partial digestions with RNase T_1 and U_2, respectively, in the presence of 7 M urea.

three different size molecules of labeled Xlo 5S RNA; the difference in size is due to additional U residues at the 3′ end of the molecule. Mobility shift analyses (Pirtle et al., 1980; Silberklang et al., 1977) of 3′-labeled Xlo 5S RNA reveal that there are at least five different sizes of this 5S RNA (data not shown). Although the major band was excised from the purification gels after the RNA was labeled (band b in Figure 2), on occasion, two different size molecules were still present in the final sequencing gels. This phenonemon would cause "doublet" bands to appear on these sequencing gels.

The Xlo 5S RNA used in this study was extracted from oocyte 7S particles (Picard & Wegnez, 1979; Hanas et al., 1983). Sequencing of the end-labeled 5S RNA revealed that a fraction of this RNA had the *X. laevis* somatic 5S RNA sequence, which differs from the oocyte 5S RNA sequence at several positions (Ford & Brown, 1976). We were able to enrich the sample with the oocyte 5S RNA by cutting out a major band and isolating the RNA from the purification gel (see band b in Figure 2). With the oocyte-enriched sample, no change in cleavage patterns was detected due to the presence of the trace amount of somatic 5S RNA.

Table I: Primary Ribonuclease Cleavage Sites in Renatured Xlo 5S RNA

	RNase				
nucleotide	basal[a]	A	T_1	T_2	V_1
$\sim U_4$					(+)
C_{10}	++	+			
A_{11}				+	
C_{12}	++	+			
A_{13}				+	
C_{14}	(+)				
C_{15}	(+)				+
U_{20}					(+)
A_{22}				(+)	
A_{23}				(+)	
A_{24}				(+)	
G_{25}	(+)				
G_{27}			(+)		
U_{30}					(+)
A_{32}					(+)
C_{36}					(+)
G_{37}			++		
U_{38}				+	
C_{39}	++	++		+	
U_{40}	(+)			(+)	
C_{46}	+				+
G_{48}			(+)		
A_{49}				++	
A_{50}				+	
C_{52}	(+)				(+)
G_{53}			(+)		
U_{55}	+				
A_{56}	+				+
C_{57}	+				
C_{63}	(+)				
C_{67}	(+)				
U_{69}					(+)
U_{73}	(+)				+
U_{76}	(+)				+
A_{83}	(+)			+	+
U_{84}					(+)
G_{86}			(+)		
G_{87}			+		
A_{88}				++	
G_{89}			++		
$\sim C_{91}$					+
U_{102}	(+)				+
C_{105}	(+)				+
G_{110}					+
C_{112}	(+)				+

[a] Cleavage sites that occur during renaturation prior to partial ribonuclease digestions. (+) signifies minor cleavage.

Basal Cleavages. Upon renaturation in a buffer containing 10 mM Mg^{2+}, labeled 5S RNA that had been previously gel purified in 7 M urea displayed a small amount of partial hydrolysis at characteristic regions of the molecule. A comparison of the denatured control sample and renatured sample (see Figure 3) demonstrates the extent of such hydrolysis (about 4–7%). To provide a complete picture of the accessible nucleotides in Xlo 5S RNA, we have included these renaturation cleavages in our data, calling them "basal" cleavages (Table I). The major basal cleavages occur at positions C_{10}, C_{12}, and C_{39}, pyrimidine positions in single-stranded regions of the generalized structural model. Since these cleavages are not random but consistently appear at the same positions of the molecule, they probably occur at a time when the molecule is in a folded conformation. Similar basal cleavages have been observed at corresponding positions in the 5S RNAs of renatured rat liver (positions C_{10}, U_{12}, ~40, and ~110) (Toots et al., 1982) and of renatured *Bacillus stearothermophilus* (positions U_{12}, C_{39}, and C_{85}) (Douthwaite & Garrett, 1981). The presence of basal cleavages at similar sites in other renatured 5S RNAs implies a common mechanism of cleavage.

The use of autoclaved buffers and standard sterilized glassware did not alter the nature or extent of the basal cleavages. However, the presence of Mg^{2+} was the factor that markedly augmented basal cleavages during renaturation (see Figure 3). We calculated from ^{32}P cpm values that up to 7% of the molecules were hydrolyzed during renaturation in the presence of Mg^{2+} and 1.3% in the absence of Mg^{2+}—about a 5-fold increase in the hydrolysis in the presence of Mg^{2+}. This minor hydrolysis in the presence of Mg^{2+} may be similar in nature to the hydrolysis of RNA by Pb(II) (Brown et al., 1983). However, we could not totally exclude the possibility that the Mg^{2+} had increased the activity of trace contaminating enzymes like ribonuclease A whose activity can be enhanced by divalent cations (Eichhorn, 1981).

Ribonuclease Cleavages. Both 5′- and 3′-^{32}P-labeled renatured RNA samples were subjected to partial ribonuclease digestions in order to distinguish primary cleavages from secondary cleavages (Douthwaite & Garrett, 1981). An initial ribonuclease cut, or primary cleavage, in an RNA may alter the original conformation of the molecule allowing subsequent cuts, or secondary cleavages. Cleavage sites observed following only one type of labeling are considered secondary (Douthwaite & Garrett, 1981). This conservative criterion was used for distinguishing between primary and secondary cleavages in our assay even though the possibility exists that a minor primary cleavage site may be considered to be secondary due to extensive cleavage of an adjacent major site that is closer to the labeled end of the molecule. Primary cleavages represent the nucleotides that are accessible to hydrolysis in the molecule of original conformation. However, the accessibility of these nucleotides varies so that major and minor cuts have to be taken into consideration when inferring the conformation of the molecule.

The ribonuclease treatment of renatured 5S RNA employed the single-strand-specific ribonucleases T_1, T_2, and A which predominantly cleave at G, A, and pyrimidines, respectively, in partial digestions. In addition, double-strand-specific ribonuclease V_1 from cobra venom was used, which has been successfully applied to probe tRNA conformation and has been shown to be sensitive to tertiary interactions including non-Watson–Crick base pairing such as A·G pairing (Lockard & Kumar, 1981). All digestions were performed in the renaturation buffer and at 0 °C for times varying from 5 to 30 min (see Materials and Methods). The low temperature minimizes the effect of "breathing" of the 5S RNA during the incubation. We endeavored to use the same buffer for all our digestions to minimize variation in the assay. This is one of the reasons we excluded S_1 nuclease from our assay since it requires low-pH buffer. An RNase inhibitor was added uniformly to all the samples at the end of the incubation times, and the RNA was immediately loaded on a nondenaturing purification gel (Figure 4). Full-length molecules of partially digested 5S RNA were isolated from the gel to eliminate the appearance in sequencing gels of cleavage bands from partially digested 5S RNA fragments (Douthwaite & Garrett, 1981). The partially digested 5S RNA was denatured and run on thin sequencing gels along with an alkaline-generated "ladder", and partial RNA sequencing digestions were performed in 7 M urea as markers. Figure 5A shows T_2 and V_1 digestions of renatured 3′-labeled Xlo 5S RNA while Figure 5B shows the same digestions of the 5′-labeled molecule.

Table I lists the major and minor primary cleavage sites from the partial ribonuclease digestions of renatured Xlo 5S RNA. Figure 6 shows the positions of the partial ribonuclease digestions on Xlo 5S RNA as it is drawn in the secondary

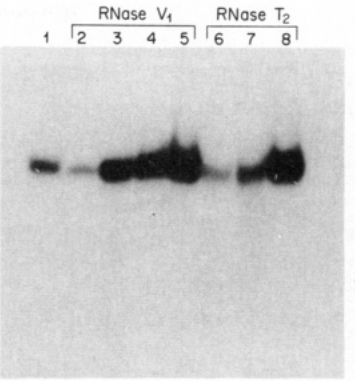

FIGURE 4: Purification of 3′-labeled Xlo 5S RNA by a nondenaturing 12% polyacrylamide gel: lane 1 is an undigested sample; lanes 2–5 are samples digested at 0 °C for 20 min with RNase V_1 at 1:9, 1:17, 1:43, and 1:86 units of V_1 to micrograms of RNA, respectively; lanes 6–8 are samples digested at 0 °C for 30 min with RNase T_2 at 1:50, 1:100, and 1:1000 units of T_2 to micrograms of RNA, respectively.

structural model for 5S RNA. Figure 6A shows the single-strand-specific enzymatic cleavages while Figure 6B shows the double-strand-specific cleavages.

Enhanced Basal Cleavages. Partial ribonuclease digestions enhanced the intensities of certain basal cleavage bands which appeared in the autoradiogram of sequencing gels (e.g., Figure 5). We have inferred from this enhancement that additional ribonuclease-catalyzed hydrolysis has taken place at these positions on intact molecules. In noting whether a single-stranded-specific or a double-strand-specific enzyme catalyzed the additional hydrolysis, we were able to make a qualitative decision about the single- or double-stranded nature of the region of hydrolysis. We have listed these sites both as basal cleavage sites and as enzyme-specific cleavage sites in Table I and have marked the position in Figure 6A,B with a small "e" for enhancement of basal cleavages.

Discussion

Support of the Generalized Secondary Structural Model. Only primary cleavage sites are indicated on the sequence of Xlo 5S RNA which has been drawn in Figure 6 according to the consensus-generalized model (Delihas & Andersen, 1982). On the whole, the data strongly support the generalized secondary structural model for 5S RNA. Single-strand-specific RNases T_1, T_2, and A cleave at single-stranded regions and looped-out positions (i.e., A_{49}) (see Figure 6A) while double-strand-specific RNase V_1 cleaves in double-stranded regions, and at some looped-out positions (i.e., A_{83}) (see Figure 6B). However, we did find some exceptional cleavages. For example, C_{36} exhibited minor hydrolysis by RNase V_1 (Figure 6B). Positions U_{73}, U_{76}, and U_{102} also exhibited V_1 cleavages in both 5′- and 3′-labeled 5S RNA (see Figure 6B). The latter V_1 cleavages represented dramatic enhancement of basal cleavages (see Figure 5B). Interestingly, the putative single-stranded regions opposite C_{36}, U_{73}, and U_{76} (i.e., positions 42–44 and 99–102) did not exhibit any single-strand-specific ribonuclease cleavages in Xlo 5S RNA. These two observations suggest that these nucleotides may be involved in base pairing not shown in the secondary structural model. Whether the V_1 cleavage at C_{36} implies a secondary or a tertiary interaction is uncertain; however, on the basis of the V_1 cleavage sites at U_{73}, U_{76}, and U_{102}, we favor a secondary structural model with extended base pairs between helices IV and V (Stahl et al., 1981). Other putative single-stranded regions that do not show expected cleavage are looped-out C_{63}, G_{66}, and U_{109}. Perhaps these nucleotides are sterically shielded due to the conformation of the molecule, or perhaps they are

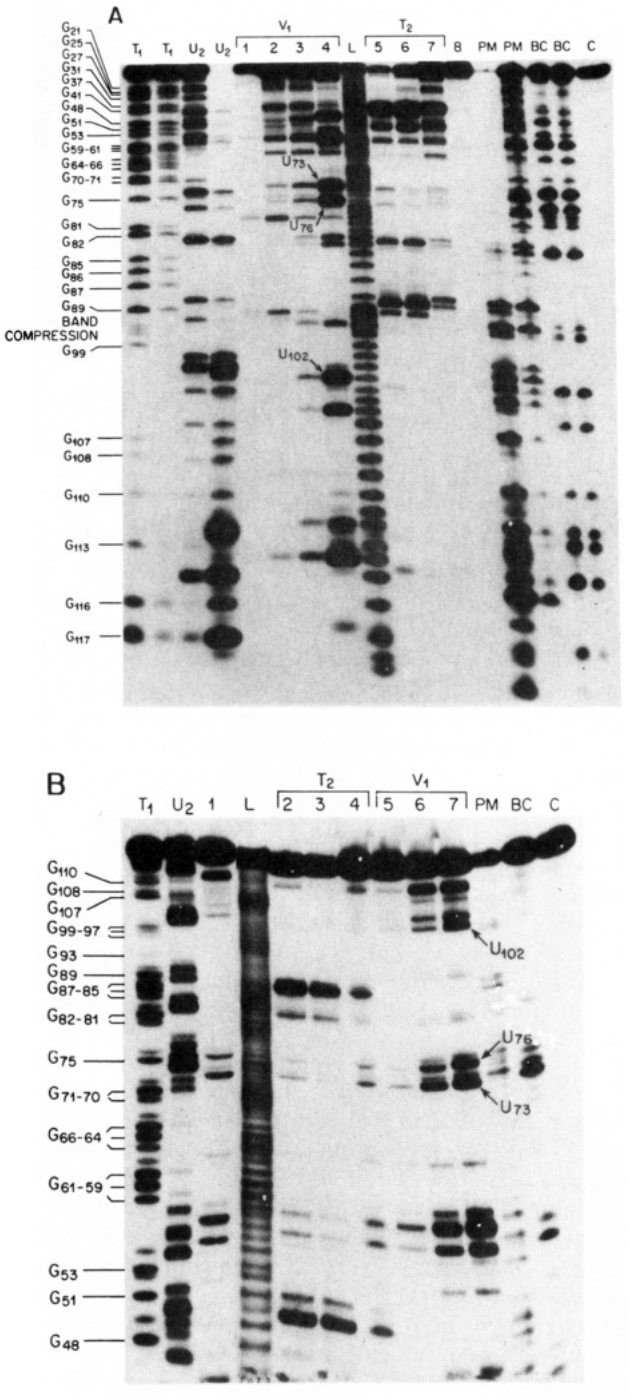

FIGURE 5: Sequencing gels showing the cleavage pattern of Xlo 5S RNA after partial digestion with RNases T_2 and V_1 and nondenaturing gel purification. Arrows have been drawn on the gels to show the V_1-enhanced basal cleavages at positions U_{73}, U_{76}, and U_{102}. (A) 3′-Labeled sample on a 12% polyacrylamide gel: lanes 1–4 are digestions with RNase V_1 at concentrations of 1:9, 1:17, 1:43, and 1:86 units of V_1 to micrograms of RNA, respectively; lanes 5–7 are digestions with RNase T_2 at concentrations of 1:50, 1:100, and 1:1000 units of T_2 to micrograms of RNA, respectively; lane 8 is an undigested, renatured sample (basal cleavages are unusually light in this particular gel). Lanes marked T_1, U_2, PM, and BC are partial digestions with RNases T_1, U_2, Phy M, and BC, respectively, in 7 M urea; the lane marked C is undigested, unrenatured control. (B) 5′-Labeled sample on an 8% polyacrylamide gel: lane 1 is undigested, renatured control; lanes 2–4 are digestions with RNase T_2 at concentrations of 1:50, 1:100, and 1:1000 units of T_2 to micrograms of RNA, respectively; lanes 5–7 are digestions with RNase V_1 at concentrations of 1:43, 1:86, and 1:170 units of RNase V_1 to micrograms of RNA, respectively. Lanes marked T_1, U_2, PM, BC, and C are as described for (A).

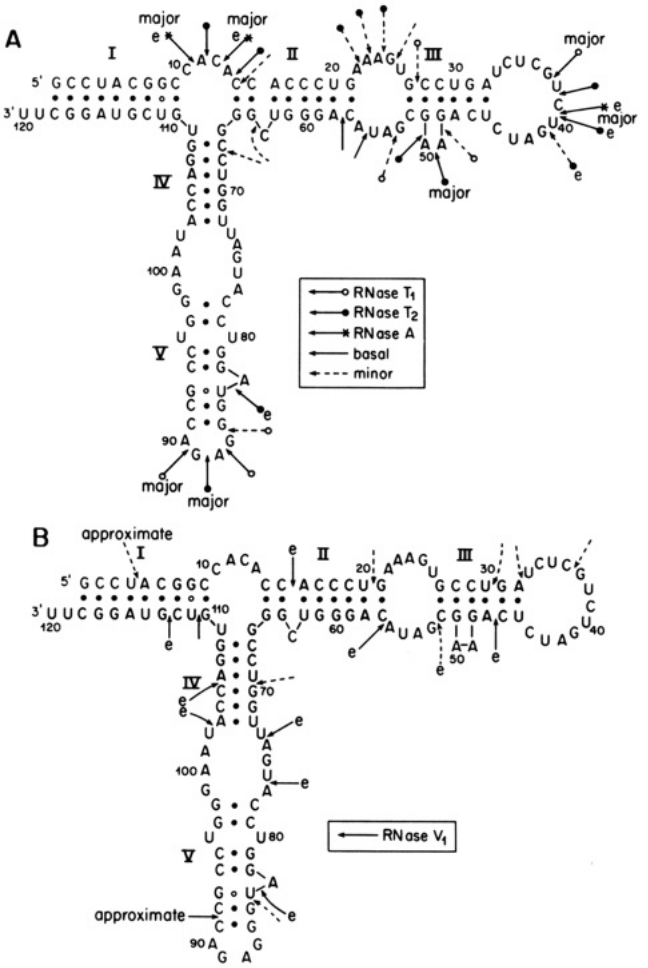

FIGURE 6: Xlo 5S RNA sequence drawn according to the consensus secondary structural model. (A) Arrows mark the primary single-strand-specific ribonuclease cleavages. The insert in the figure explains the symbols used to distinguish between RNases T_1, T_2, and A. Minor cleavages are represented by dashed line arrows; "e" denotes enhancement of basal cleavage (see text). (B) Arrows mark the primary RNase V_1 cleavages . Certain cleavage sites are marked as approximate since their exact positions were obscured by band compression on sequencing gels.

protected from single-stranded enzymatic digestion by being base paired.

In this study, we did not detect any evidence for more than one major conformation of the renatured molecule in solution. If more than one conformation exists, we would have expected to see an area of the molecule that exhibited both single-strand-specific and double-strand-specific cleavages, implying that some molecules in the population of 5S RNA are single stranded in that region while others are double stranded. Such an event only occurs (expectedly) around looped-out positions (i.e., in helices III and V). We did not see any other evidence for an alternate conformation in renatured Xlo 5S RNA.

Comparison to Other Data. Figure 7 shows the comparison of the nuclease cleavage data of various 5S RNAs obtained from other laboratories to data for Xlo 5S RNA (Douthwaite & Garrett, 1981; Pieler et al., 1983; Pieler & Erdmann, 1982; Rabin et al., 1983; Toots et al., 1981, 1982; Troutt et al., 1982; Wildeman & Nazar, 1982; Speek & Lind, 1982). The shaded area of the figure highlights the helical regions of 5S RNA based on the secondary structural model. The nuclease probes of the conformation of other 5S RNAs on the whole support the generalized model for 5S RNA derived by comparative sequence analysis. However, the cleavage patterns of different 5S RNAs do vary (see Figure 7). For example, no cleavage

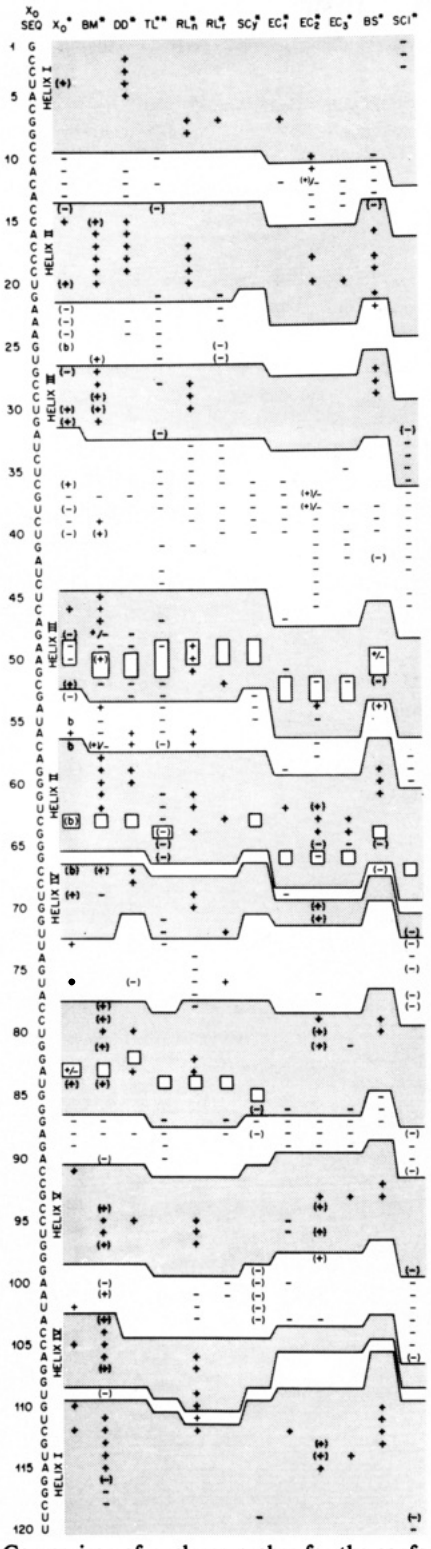

FIGURE 7: Comparison of nuclease probes for the conformation of several 5S RNAs: solid circle, RNase T_1, T_2, and/or A used; asterisk, nuclease S_1 used; parentheses enclosing plus or minus signs, minor cleavage; minus sign, single-specific cleavage; plus sign, double-strand-specific cleavage (RNase V_1) (not all 5S RNAs listed were probed with RNase V_1); b, basal cleavages in Xlo 5S RNA (see text; Xlo, *Xenopus laevis* oocyte 5S RNA; BB, *Bombyx mori* 5S RNA (Troutt et al., 1982); DD, *Dictyostelium discoideum* 5S RNA (Troutt et al., 1982); TL, *Thermomyces lanuginosus* 5S RNA (Wildeman & Nazar, 1982); RL_n, native rat liver 5S RNA (Toots et al., 1981); RL_r, renatured rat liver 5S RNA (Toots et al., 1982); SCy, spinach cytoplasmic 5S RNA (Pieler et al., 1983); EC_1, *E. coli* 5S RNA (Pieler & Erdmann, 1982); EC_2, *E. coli* 5S RNA (Speek & Lind, 1982); EC_3, *E. coli* 5S RNA (Douthwaite & Garrett, 1981); BS, *Bacillus stearothemophilus* 5S RNA (Douthwaite & Garrett, 1981); SCl, spinach chloroplast 5S RNA (Pieler et al., 1983).

(single-strand or double-strand specific) is found in *B. stearothermophilus* 5S RNA in the region of the molecule that is helix IV in the consensus model, while RNase V_1 cuts are found in Xlo and other eukaryotic 5S RNAs. Also, V_1 cleaves on the 3′ side of helix I in Bombyx *mori* 5S RNA but on the 5′ side of helix I in *Dictyostelium discoideum* 5S RNA (Troutt et al., 1982). The diversity in these patterns may reflect actual tertiary conformational differences between 5S RNAs. Diversity may also reflect the sequence specificity of certain enzymes, the differences in salt, buffer, and temperature conditions during digestions, or the differences in methodology in analyzing data (i.e., using only one type of labeled RNA). Variations in salt and Mg^{2+} concentrations and in incubation temperatures have been shown to change the cleavage pattern of the same 5S RNA (Wildeman & Nazar, 1982; Toots et al., 1982; Rabins et al., 1983). Lack of gel purification of partially digested 5S RNA could cause the appearance of cleavage sites that occur on fragments of 5S RNA that break away from the original molecule (Douthwaite & Garrett, 1981). Not all 5S RNA cleavage sites listed in Figure 7 were deduced from a comparison of data from digestions of 5′- and 3′-labeled molecules. Fine coordination of cleavage sites from different 5S RNAs to determine 5S RNA conformation is difficult due to the variables in the assays from different laboratories.

Other 5S RNAs exhibit the unusual V_1 cleavages similar to those found in the putative single-stranded regions encompassing positions C_{36}, U_{73}, U_{76}, and U_{102} of Xlo 5S RNA and lack single-strand-specific enzymatic cleavages in these putative single-stranded regions. There is also a correlation of protected regions in 5S RNAs (see Figure 7). *B. mori* 5S RNA has RNase V_1 cleavages in the two corresponding putative single-stranded regions of the molecule mentioned previously (at C_{39}, C_{40}, and A_{101}) (Troutt et al., 1982). Renatured rat liver 5S RNA has an RNase V_1 cleavage at positions U_{72} and U_{76} in the putative single-stranded region between helices IV and V (Toots et al., 1982). *B. mori* 5S RNA shows no single-strand-specific enzymatic cleavage in the regions opposite the V_1 cuts found in the putative loop regions (positions A_{42}–C_{44} and G_{99}–U_{102} in Xlo 5S RNA). However, renatured rat liver 5S RNA shows two S_1 nuclease cuts at A_{100} and A_{101} (Toots et al., 1982). In general, few 5S RNAs show single-strand-specific cuts in the region between helices IV and V and in the loop bound by helix III at the nucleotides that are closest to the helix even though these regions are putatively single stranded in the generalized secondary structural model (see Figure 7).

The looped-out position of helix II, corresponding to C_{63}, protected in Xlo 5S RNA from single-stranded-specific ribonuclease cuts is also protected from single-strand-specific ribonuclease probes in the *B. mori*, *D. discoideum*, and rat liver 5S RNAs. The looped-out position of helix II shows a V_1 cleavage in rat liver and *D. discoideum* 5S RNAs. The corresponding looped-out position in *E. coli* and *B. stearothermophilus* 5S RNAs is accessible to single-strand-specific ribonucleases.

We believe that the lack of single-strand-specific cuts in the region between helices IV and V most likely reflects base pairing that occurs at these nucleotides to form additional secondary structure. Several investigators have postulated tertiary interactions between the hairpin loop bound by helix III and the 5′ side of the internal loop between helices IV and V (Pieler & Erdmann, 1982). However, the positions involved in such interactions do not appear to be phylogenetically conserved. Alternative secondary structures have been pro-

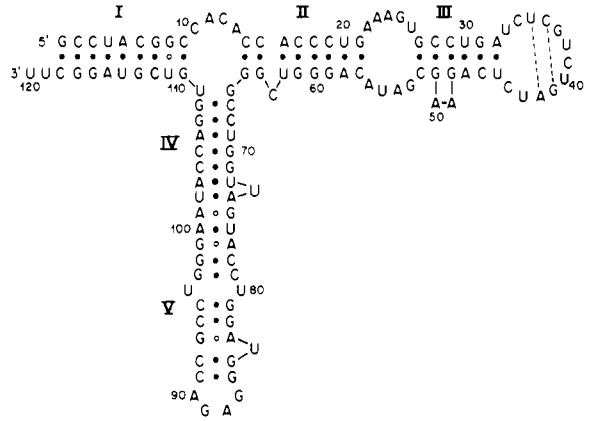

FIGURE 8: Proposed Xlo major oocyte 5S RNA secondary structural model. On the basis of RNase V_1 cleavage data, the Xlo 5S RNA sequence has been redrawn according to the generalized secondary structural model with extended base pairing between helices IV and V. Potential phylogenetically conserved base pairing between C_{36} and G_{41} and between U_{35} and A_{42} is noted even though these appear to be thermodynamically unstable.

posed. Thompson et al. (1981) have postulated continued base pairing across the loop bound by helix III, and Stahl et al., 1981) have postulated continued base pairing in the internal loop between helices IV and V which would contain A·G base pairs. We favor the extension of base pairing between helices IV and V as mentioned above.

Existence of A·G Base Pairs in 5S RNA. There are phylogenetic reasons to include A·G base pairs in the arm of 5S RNA that contains helices IV and V. As pointed out by Stahl et al. (1981), helix V can be extended in most 5S RNAs by the inclusion of conserved A·G bonds. Interestingly, the 5S RNA from the thermoacidophile archaebacterium *Sulfolobus acidocaldarius* contains Watson–Crick base pairs along the whole arm by helices IV and V in the generalized model. Stahl et al., (1981) postulated that the presence of Watson–Crick base pairs in this region of the *Sulfolobus* 5S RNA provided phylogenetic evidence for the extension of base pairing between helices IV and V in all 5S RNA which would include phylogenetically conserved A·G base pairs. Also, Gram-positive and most other eubacterial 5S RNAs have the conserved pair A_{104}·G_{72}. In *Streptococcus cremoris* 5S RNA, helix IV appears to be "rearranged" with an insertion of one base pair, but the A·G pairing is maintained at positions 103 and 73 (Neimark et al., 1983). The significance of this transposition lies in the apparent conservation of the A·G pair as opposed to the conservation of nucleotides A and G at positions 104 and 72, respectively.

Experimental evidence for the presence of A·G base pairs in nucleic acids comes from cobra venon RNase V_1 cleavages at sites having A·G pairs in tRNA (Lockard & Kumar, 1981) and in 16S RNA (Douthwaite et al., 1983), and in the detection of A·G base pairs in oligodeoxynucleotides by proton magnetic resonance spectroscopy (Kan et al., 1983).

Thus, we have redrawn the Xlo 5S RNA sequence in Figure 8 with extended base pairing between helices IV and V which include A·G base pairs. We have noted that the looped-out position in helix V, A_{83}, can be paired with G_{93} if one includes A·G base pairing in this region, too. The putative A·G base pair is phylogenetically conserved in eukaryotic 5S RNAs with the exception of the 5S RNAs from green plants, chicken, and turtle which have Watson–Crick base pairs at this corresponding position (Delihas & Andersen, 1982; Brownlee & Cartwright, 1975; Lazar et al., 1983; Roy, 1977). With the inclusion of the A·G base pair, the looped-out position becomes the common eukaryotic position U_{84} and the structure of helix V becomes conserved for all eukaryotic 5S RNAs. It is possible that this structure is one of two alternating structures for helix V since there is no evidence that an A·G base pair would be preferred to the purine·U pair that it excludes. Also, the covalent bond between A_{83} and U_{84} is cleaved by both single-strand- and double-strand-specific ribonucleases in our study. Thus, helix V in Figure 8 is shown to include this putative A·G base pair. We have also sketched potential base pairs involving U_{35} and C_{36} in the figure even though these do not appear to be thermodynamically stable.

Phylogenetically conserved A·G base pairs in the region of 5S RNA between helices IV and V may have functional significance. This base pairing may readily give way to Watson–Crick base pairing in an interaction of 5S RNA with another RNA. Alternatively, the unconventional bulging of the helix produced by the A·G base pair may position the nucleotides optimally to interact with amino acids in a protein.

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5S RNA Structure and Interaction with Transcription Factor A. 2. Ribonuclease Probe of the 7S Particle from *Xenopus laevis* Immature Oocytes and RNA Exchange Properties of the 7S Particle†

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ABSTRACT: The 5S RNA complexed in the 7S particle of immature *Xenopus laevis* oocytes was ^{32}P labeled at its 3′ end and then subjected in situ to partial digestion using ribonucleases T_1, T_2, A, and V_1 in order to study the conformation of the complexed RNA and its interaction with the transcription factor A (TFIIIA). Digested samples were gel purified to retrieve 5S RNA that was still complexed with the transcription factor protein, and the cleavages in these RNAs were analyzed on sequencing gels. The RNA associated with the 7S particle is very susceptible to ribonuclease activity despite the presence of the protein. Also, the 5S RNA in the 7S particle is in a different conformation from renatured *Xenopus laevis* (Xlo) 5S RNA and appears to have less secondary structure predominantly in the stem that includes helices IV and V. A species of native Xlo 5S RNA which was isolated from 7S particle preparations under nondenaturing conditions revealed a conformation that was more similar to the 5S RNA in the 7S particle than to renatured 5S RNA. Comparison of data from partial ribonuclease digestions performed on renatured 5S RNA, on the native 5S RNA, and on the complexed 5S RNA allowed us to approximate sites of protein-induced structural change in the complexed 5S RNA, which may signal protein interaction domains. These sites include the 5′ side of helices III and V. In another approach to the study of 5S RNA–TFIIIA interactions, we have observed that incubation of ^{32}P-labeled Xlo 5S RNA with 7S particles results in the incorporation of labeled RNA into 7S particles. Heterologous eukaryotic 5S RNA can also be incorporated into 7S particles. These results suggest that a generalized eukaryotic 5S RNA structure can be recognized by TFIIIA.

The 5S ribosomal RNA is found complexed with a 37-kilodalton transcription factor protein (TFIIIA) in a 7S ribonucleoprotein (RNP) particle of the previtellogenic oocytes of *Xenopus laevis* (Xlo) (Picard & Wegnez, 1979; Pelham & Brown, 1980; Honda & Roeder, 1980). TFIIIA also binds to an intragenic transcriptional control region (which includes residues 50–83) in the 5S RNA gene to initiate transcription (Sakonju et al., 1980; 1981; Bogenhagen et al., 1980; Sakonju & Brown, 1982). Transcription of the 5S DNA in vitro is feedback inhibited by Xlo and heterologous 5S RNAs (Pelham & Brown, 1980; Gruissem & Seifart, 1982). Although the binding of TFIIIA to 5S DNA has been studied extensively by using DNase I footprinting techniques (Engelke et al., 1980; Sakonju et al., 1981; Sakonju & Brown, 1982; Hanas et al., 1983), little is known concerning the interaction of the complexed 5S RNA with TFIIIA in the 7S particle.

In this paper, we present the results of two types of experiments which approach the subject of RNA–protein interactions in the 7S particle. One method has been to probe the structure of 5S RNA in the particle with ribonucleases. By using a similar assay, Douthwaite et al. (1982) previously determined the ribonuclease cleavage sites in *Escherichia coli*

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