Secondary structure of *Tetrahymena thermophila* 5S ribosomal RNA as revealed by enzymatic digestion and microdensitometric analysis

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ABSTRACT

The secondary structure of [³²P] end-labeled 5S rRNA from *Tetrahymena thermophila* (strain B) has been investigated using the enzymes S1 nuclease, cobra venom ribonuclease and T2 ribonuclease. The results, analyzed by scanning microdensitometry and illustrated by three-dimensional computer graphics, support the secondary structure model of Curtiss and Vournakis for 5S rRNA (1). Aberrant mobility of certain RNA fragments on sequencing gels was observed as regions of band compression. These regions are postulated to be caused by stable internal base-pairing. The molecule was probed with T2 RNase in neutral (pH 7.5) and acidic (pH 4.5) buffers and only minor structural differences were revealed. One of the helices was found to be susceptible to enzymatic attack by both the single-strand and double-strand specific enzymes. These observations are evidence for the existence of dynamic structural equilibria in 5S rRNA.

INTRODUCTION

Knowledge of the secondary and tertiary structure of 5S ribosomal RNA is important in understanding the structure and function of the ribosome. Enzymes with secondary structure specificity can be used to identify susceptible phosphodiester bonds in large RNA molecules (1). *Tetrahymena thermophila* 5S rRNA although only 120 nucleotides in length, has not been subjected to structure analysis by either Nuclear Magnetic Resonance or X-ray crystallography. Other physical techniques do not generate the same detailed information as that obtainable with the enzymatic structure-mapping method (2). The sequences of all known 5S rRNAs can be folded to conform to the unified secondary structure model of De Wachter et al., proposed on phylogenetic and thermodynamic grounds (3). The De Wachter et al. base pairing scheme has been slightly modified by Curtiss and Vournakis (1,4) since in the case of the 34 eucaryotic 5S rRNAs sequences that they examined it is possible to elongate helix C by allowing a two-base bulge. The present study uses three secondary structure-specific enzyme probes to experimentally test the modified model. The probes employed were the double-strand specific cobra venom ribonuclease (CV RNase), and the single-strand specific enzymes S1 nuclease and T2 ribonuclease. CV RNase

requires a pH 7.5 Mg^{++} containing buffer for activity. S1 nuclease requires an acidic (pH 4.5) buffer and Zn^{++} as a cofactor. T2 RNase, known to possess single-strand specificity (5), has no divalent cation requirement. Although maximally active at pH 4.5, T2 RNase still retains considerable activity at pH 7.5. It is conceivable that the secondary structure of 5S rRNA differs in the two buffer conditions required for this set of enzymes; S1 nuclease buffer and CV RNase buffer vary by 3 pH units and in the divalent cation present. Cleavage of the same phosphodiester bond by CV RNase and S1 nuclease in their respective buffers could be attributed to changes in base pairing in the two buffers and invalidate the combining of digestion data for structure determination. T2 RNase digestions were, therefore, carried out in both buffers in order to compare the cleavage patterns obtained.

The enzymatic cleavage results were quantitated by scanning microdensitometric analysis of autoradiographs. A three-dimensional computer graphics system was then employed to display the quantitative results using a surface plot format.

MATERIAL AND METHODS

Materials

Tetrahymena thermophila 5S rRNA (strain B) was prepared as described by Kumasaki et al (6). T4 RNA ligase and T4 polynucleotide kinase were purchased from Bethesda Research labs. Cobra Venom RNase and S1 nuclease were purified as previously described (3). T2 RNase (grade VI) was purchased from Sigma Chemical Co. [^{32}P]-3'ATP (8000 Ci/mmol) and 5'-[^{32}P]-3' cytidine bisphosphate (2000 Ci/mmol) were purchased from Amersham-Searle.

End Labeling

Purified 5S rRNA was [^{32}P] end-labeled at either the 3' or 5' terminus, as reported previously (2), and digested exhaustively with P1 nuclease and T2 RNase respectively to identify the labeled nucleotide (7).

Digestion Condition

Digestions with the sequence-specific enzymes T1, U2, CL3 and Bacillus cereus RNases, and with alkali to yield sequence information were performed as previously described (8,9). Digestions with the secondary structure-specific enzymes S1 nuclease, Cobra Venom RNase and T2 RNase were employed to identify double-stranded and single-stranded regions of the RNA molecule (10). Each reaction contained 10,000 to 50,000 Cerenkov cpm of [^{32}P] RNA and 2.5 μ g of unfractionated yeast tRNA carrier in a total volume of 7 μ l. For structure specific reactions the sample was equilibrated by preincubation for 3 minutes

at 37°C before addition of an appropriate enzyme dilution. The reaction was then incubated at 37°C for 1 to 10 minutes and quenched by the addition of an equal volume of a solution of 16 M urea, 0.05% (w/v) brophenol blue, 0.05% (w/v) xylene cyanol, 1mM EDTA, 2µg/µl tRNA, and immediately chilled on dry ice. Cobra Venom RNase reactions were carried out in one of two buffers; CV buffer A: 200mM MaCl, 5mM MgCl₂, 10mM tris pH 7.5; CV buffer B: 100mM NaCl, 2mM MgCl₂, 50mM tris pH 7.5. S1 nuclease reactions were carried out in S1 buffer: 200mM NaCl, 10mM ZnSO₄, 40mM NaOAc pH 4.5. T2 RNase reactions were carried out in CV buffers A and B, and S1 buffer.

Gel Electrophoresis

The samples were heated at 90°C for 20 seconds before loading onto a 10% to 20% acrylamide, 8.3M urea gel (40 x 33 x 0.04 cm), and electrophoresed at 1,000 V, constant voltage (2).

Autoradiography

Autoradiographs of the gels were made using Kodak X-Omat RF X-ray film. The gels were exposed at -20°C and exposure times were such that bands scanned had an optical density of no greater than 1.0 measured at a wavelength of 500 nm, to ensure a linear relationship between optical density and cpm.

Densitometric Analysis of Autoradiographs

Autoradiographs were scanned using a Jarrel-Ash model 23-100 scanning microdensitometer. The peak areas for each band were calculated, and have been shown to be proportional to cpm and concentration (unpublished results). Control peak areas were subtracted from digestion lanes to give corrected band densities.

Representation of Data

Three-dimensional plots of the data were generated using the statistical analysis system (SAS) GMAP surface program run on Syracuse University's IBM (4341) computer.

RESULTS

Homogeneity of end-labeled 5S rRNA

End analysis and sequencing gels of end-labeled RNA showed homogeneously labeled termini, and full length 5S rRNA. Typical autoradiographs covering the entire length of the 5S rRNA molecule are shown in figures 1 and 2. The 3' terminus of 5S rRNA was found to label with higher efficiency compared to the 5' terminus, as would be expected from the secondary structure model (1).

Sequence Heterogeneity

The sequence, and sequence heterogeneity, determined in this study for

FIGURE 1

An autoradiograph of a 10% polyacrylamide, 8.3M urea gel, showing products of 3'-[³²P] end-labeled *Tetrahymena thermophila* 5S rRNA digested with S1 nuclease, Cobra Venom RNase and T2 RNase at 37°C. The structure-specific reactions were preincubated at 37°C for 3 min. before addition of enzyme. The gel was prerun at 1,000V for 60 min. The samples in lanes 1 - 8 were loaded and the gel electrophoresed at 1,000V for 130 min. or until the xylene cyanol marker dye had migrated 40 cm. Samples in lanes 9 - 16 were then loaded and the gel electrophoresed at 1,000V for a further 180 min. The reactions loaded in lanes 1 - 3 and 9 - 11 were performed in S1 buffer. The reactions loaded in lanes 6 - 8 and 14 - 16 were performed in Cobra Venom buffer. Lanes 4 and 12 show a ladder resulting from alkali digestion. Lanes 5 and 13 contain digests performed with U2 RNase. Figure 1A shows the digestion patterns from nucleotides 5 - 65, and figure 1B the digestion patterns from nucleotides 51 - 120, numbered from the 5' terminus.

FIGURE 2

An autoradiograph of 3'-[^{32}P] end-labeled 5S rRNA showing secondary structure results with S1 nuclease and Cobra Venom RNase for nucleotides 83 - 36. Lanes 1 - 4 contain a control digest, a T1 RNase digest, an alkali ladder and a U2 RNase digest, respectively. Lanes 5 - 8 contain S1 nuclease reactions incubated at 37°C for 1,2,5, and 10 min. Lanes 9 - 12 contain Cobra Venom RNase reactions incubated at 37°C for 1,3,5 and 10 min respectively in Cobra Venom buffer B. Both S1 nuclease and Cobra Venom RNase structure reactions were preincubated 37°C for 3 min. before addition of the enzyme.

Tetrahymena thermophila (strain B) 5S rRNA agrees with previously published results (11). Sequence heterogeneity was found at nucleotide positions 2 (C/U), 4 (A/G), 115 (U/C) and 117 (G/A). The sequence heterogeneity is such that the possibility of basepairing in helix A is maintained (see figure 4).

Regions of Compression

A problem often encountered when sequencing RNA is band compression. Band

FIGURE 3
Regions of compression in digestion fragments of 3'- or 5'-[32P] end-labeled 5S rRNA. An autoradiograph of each compression region is shown alongside the postulated fragment secondary structure and band assignment. Brackets indicate comigration of fragments. **Region 1.** The autoradiograph includes a section of a 10% acrylamide, 8.3M urea sequencing gel. 3'-[32P] end-labelled 5S rRNA was digested with the sequence-specific RNases T1, U2 and *Bacillus cereus*. Bands #17 and #18 comigrate. **Region 2.** The autoradiograph shows a section of a 5'-[32P] end-labeled 5S rRNA sequencing gel. Bands #66 and #67 comigrate. With 3'-[32P] end-labeled RNA, compression is seen in bands #57 and #58, at the other side of the hairpin loop (Data not shown). **Region 3.** The autoradiograph shows a 3'-[32P] end-labeled RNA sequencing gel. Bands #96 and #97 comigrate.

compression in gel autoradiographs is often due to residual secondary structure in digestion fragments causing the fragments to migrate faster than expected. This phenomenon can lead to misnumbering due to band inversion. Most compression problems can be overcome by heating the sample before loading, and running 8.3M urea gels at about 40°C. With 3' end-labeled substrate band compression is found on the 5' side of the hairpin loop, and vice versa with 5' end-labeled RNA. Despite running the gels under the denaturing conditions described, three regions of persistent band compression were observed as shown in figure 3. The left panels show examples of band compression in autoradiographs, while the postulated explanation for this phenomenon is shown on the right.

Compression in region 1 was noted using 3' end-labeled RNA. Sequence-specific enzymes cleave on the 3' side of the recognized nucleotide generating labeled fragments lacking that nucleotide. Sequencing lanes T1 RNase, U2 RNase, *Bacillus cereus* RNase, and an alkaline cleavage ladder show the sequence of this region. The postulated explanation and band assignment is shown on the right of figure 3. Bands #17 and #18 comigrate. Band #17 probably contains a fragment which can form the hairpin loop G18 - G21 basepaired to C29 - C26. Due to secondary structure this fragment will migrate faster than a completely denatured fragment of the same length. Band #18, corresponding to a fragment one nucleotide shorter whose 5' nucleotide is G19, is postulated not to form a hairpin loop and migrates normally resulting in comigration with band #17. An alternative explanation should be that bands #17 and #19 comigrate due to base-pairing of nucleotides G19 - G21 with C28 - C26. Since nucleotides 18 and 19 are both guanosines, it is not possible to distinguish between these alternatives, but we feel that the first is most likely on energetic grounds. Compression in region 2 is shown in figure 3, second panel. Both sides of this hairpin loop were observed as regions of band compressions.

In digestions of 5' end-labeled RNA, bands #66 and #67 were found to comigrate. With 3' end-labeled RNA bands #57 and #58 comigrated. These observations are consistent with the postulated secondary structure shown for region 2. Compression in region 3 is shown in the bottom panel of figure 3. Comigration of bands #96 and #97 led us to postulate the existence of the hairpin loop shown, ie. basepairing between U95 - G99 and C104 - G108.

The position of proposed hairpin loops for each compression region observed is shown in the secondary structure model of 5S RNA in figure 4. The compression regions 1, 2, and 3 shown in figure 3 are labeled A, B, and C, respectively, in this figure. The secondary structure model is that of Curtiss

FIGURE 4

Hairpin loops postulated to form in compression regions 1,2, and 3 shown in figure 3 are shown in the context of the uncleaved 5S rRNA molecule and labelled regions A,B and C respectively. The molecule is folded according to the secondary structure model of Curtiss and Vournakis (1). The 5 helices are labeled A-A', B-B', C-C', D-D' and E-E' in order of occurrence beginning at the 5' terminus. The regions I1' and I2' are similarly located opposite I1 and I2. The single-stranded regions are labeled M for multibranch loop, and I1, I2 for internal loops. The nomenclature is according to De Wachter et al. (3,11). For easy comparison, the model is shown in the same projection as the three-dimensional representations in Figure 6 below.

and Vournakis (1). This is the same as the model of De Wachter et al. (3,11) with a minor modification involving the basepairing of U33 - C34 with A42 - G41 which extends helix C by two basepairs and creates a two-base bulge at nucleotide positions 43 - 44.

Secondary Structure Results

Figures 1 and 2 are autoradiographs of 3' end-labeled 5S rRNA digested with the enzymatic structure probes S1 nuclease, Cobra Venom RNase and T2 RNase. Figure 1 shows data covering almost the whole length of the 5S rRNA molecule, from nucleotide 5 - 120. Figure 2 includes nucleotides 37 - 83 and shows the reproducibility of the structure reactions. In most cases the cleavages by Cobra Venom RNase and the single-stranded probes S1 and T2 are distinct, identifying helical and looped regions of the molecule, respectively. Positions where cleavage occurs with both double and single-strand specific probes may indicate tertiary interactions, or unstable helices.

A. COBRA VENOM RNase

B. S1 NUCLEASE

C. T2 RNase IN CV BUFFER

FIGURE 5

Comparison of results obtained by visual and densitometric analysis of structure reactions of 3'-[^{32}P] end-labeled 5S RNA. The unfolded 5S rRNA molecule, 120 nucleotides in length, is represented along the X axis. The position of every tenth nucleotide is indicated by a vertical dash. The 5' terminus is on the left of the figure. The secondary structure of the molecule is shown schematically, the heavy horizontal lines indicating basepaired regions. The Y axis shows the amount of cleavage by structure-specific enzymes. The upper portion of each panel shows the mean average of 13 experiments in which the autoradiographs were analysed by visual examination. The data covers nucleotides 5 - 120. The lower portion of each panel shows the densitometric results obtained by scanning the autoradiograph in Figure 1. The data covers nucleotides 10 - 120. Fig. 5A shows Cobra Venom RNase results, 5B S1 nuclease results and 5C combined T2 RNase results in CV buffers A and B.

FIGURE 6

Three-dimensional representations of *Tetrahymena thermophila* 5S rRNA, folded according to the secondary structure model of Curtiss and Vournakis, illustrate the susceptibility of the molecule to cleavage by enzymatic probes. The secondary structure model is shown in the XY plane in the same projection as in Figure 4. The XY plane represents the phosphodiester bonds between nucleotides where cleavages occur. The numbers refer to the base positions numbered from the 5' terminus. The height of the peak (the Z value) is a measure of the susceptibility of the bond to enzymatic cleavage. Figure 6A shows Cobra Venom RNase results. The mean average from 13 experiments, analyzed visually is plotted as the Z value. The data covers nucleotides 5 - 120. Figure 6B shows S1 nuclease results. The data are plotted from the densitometric analysis of figure 1, lanes 2 and 10, covering nucleotides 10 - 120. Figure 6C shows T2 RNase results in S1 buffer. Densitometric data are plotted from figure 1, lanes 3 and 11, covering nucleotides 10 - 120. Figure 6D shows T2 RNase results in CV buffer A. Densitometric data are plotted from Figure 1, lanes 6 and 14, covering nucleotides 1 - 120.

Figure 5 shows, in histogram form, the results obtained by two methods of data analysis, visual and densitometric. We were unable to obtain data on the first four nucleotides by visual analysis, and the first nine nucleotides by densitometric analysis, at the 5' end of the molecule, because these bands were not resolvable on autoradiographs. The visual analysis was performed by scoring the intensity of bands, subtracting the control bands, and calculating the mean average. The relative strength of the cleavages, which occur 3' to the numbered nucleotide, were compared using an arbitrary numerical scale. Since the extent of digestion and film exposure time vary with each experiment the results were not identical. However, the relative densities of bands were consistent between experiments. The densitometric data comes from scanning the lanes of the autoradiogram shown in figure 1. Values were corrected by subtracting control cleavages. The densitometric analysis gave similar results to those obtained by visual examination. Differences in the data obtained by these two methods are due to several factors. The densitometer is more sensitive than the eye to low band densities. The resolution of the densitometer decreases near the top of autoradiograms where the bands lie closer together at the 5' end of the molecule. The visual analysis is the mean of 13 experiments which together cover virtually the whole length of the molecule, while the densitometric data come from a single experiment.

Data obtained from the visual and densitometric analyses of autoradiographs were used to create the computer-generated three-dimensional representations shown in figure 6. The XY plane shows the secondary structure model of the 5S molecule in the same projection as in figure 4. The phosphodiester bonds in the RNA are represented by squares of equal area. Peak heights are shown in the third (Z) dimension and represent relative enzyme cleavage intensities at the phosphodiester bonds obtained either by visual analysis (figure 6A) or by densitometric analysis (figures 6B, 6C, and 6D).

Cobra Venom RNase, which cleaves in double-stranded regions, may also recognize tertiary interactions involving non-Watson-Crick basepairing (12). CV RNase reactions were carried out in buffers A and B. Few differences were observed in the digestion patterns in the two buffers. Figure 6A shows the results of the mean values of CV RNase digestions obtained by visual analysis. It is apparent that every helix is recognized by the enzyme. Regions of intense cleavage occur on both strands, usually opposite one another in the secondary structure model.

Figure 6B, 6C and 6D show the results of densitometric analysis of digestions with S1 nuclease, and T2 RNase in Cobra Venom and S1 buffers. The

data were obtained by scanning the lanes of the autoradiograph shown in figure 1. As shown in figure 6B, S1 cleaves at every single-stranded region in the secondary structure model apart from the H2 and I2' regions. S1 nuclease also cleaves at the 2 two-base bulges in helix C, and the single-base bulge in helix B. The cleavages in helices B, D and E are likely to be due to instability causing the helices to "breathe", with helix E being particularly susceptible.

T2 RNase digestions were carried out in both S1 and Cobra Venom buffers, and the results were compared as shown in figures 6C and 6D. Despite the three unit pH difference between the buffers, the T2 cleavage patterns in the two buffers are similar. Although cleavage intensities vary according to the buffer, strand scission occurs in both buffers in all single-stranded loops, and supports the secondary structure model.

DISCUSSION

The results of the secondary structure analysis on Tetrahymena thermophila 5S rRNA reported above support the 5S secondary structure model of Curtiss and Vournakis (1). Cobra Venom RNase cleaves at the bulged bases in the C' region of 5S rRNA. Phosphodiester bonds at positions 48, 49 and 50, which compose the two-base bulge at the 3' end of helix C, are cleaved, indicating that this region is helical. Nucleotide 43, in the 5' bulge of helix C, is also cleaved by CVR. In the model of De Wachter et al. (3) the C' helical region extends from the lower half of helix E, shows a low proportion of G-C base pairs (0.25 G-C, 0.50 A-U, 0.25 G-U). In contrast, the upper half of helix E, which is virtually resistant to S1 nuclease and T2 RNase cleavage, contains a high proportion of G-C base pairs (0.75 G-C, 0.25 G-U). Melting experiments are presently underway to test the prediction that the lower half of helix E is thermally unstable.

Three regions in helix E cleaved by both single-strand and double-strand specific probes, may be an example of helix instability. Due to the existence of ambiguous basepairing opportunities in regions I1-C and I2-E of eucaryotic 5S rRNAs it is possible that several different secondary structures can form. These alternative basepairing schemes were found in a large collection of 5S rRNAs and are postulated to exist in dynamic equilibrium with each other. Changes in secondary structure could serve as articulations and allow the RNA to exist as a flexible and dynamic molecule (15). Similarly, it is possible to construct different basepairing schemes for the I2-E region of the 5S rRNA from Tetrahymena thermophila. Such a dynamic equilibrium between different structures could contribute to the observed susceptibility of helix E to S1

nuclease and T2 RNase. "Breathing" of helices may be important in RNA binding by proteins. The sequence of the bases 73 - 77 in the I2 loop are highly conserved among eucaryotic 5S rRNAs (1). The positive transcription factor TFIIIA from Xenopus laevis when complexed to its 5S rRNA induces conformational changes in the C and E helical regions (16). These changes may be related to the location of binding sites for the factor. Exchange reactions using 5S rRNA from other species showed that the binding of TFIIIA was relatively non-specific. The protein may recognize common elements of 5S rRNA primary and/or secondary structure (16). It is interesting to note that the potential protein interaction domain at E is within the unstable E helical region.

Three persistent regions of band compression were found in the experiments described above. The secondary structures postulated to account for the compression are short-range hairpin loops. Since compression is found in the strongly denaturing conditions used, the structures formed must be very stable. In each case, the postulated hairpins consist of a helix containing three G-C base pairs and a four-base loop closed by a G-C base pair. Physical studies on oligoribonucleotides indicate that the smallest observed loop size is four bases (17,18). For hairpin loops with three or more G-C base pairs, such as those proposed above, the most stable loop size is unknown.

Adenine residues predominate in the postulated hairpin loops of T. thermophila 5S rRNA digestion fragments as proposed above (see figure 3). Examination of the secondary structure model for E. coli 16S rRNA (18) shows adenine residues to be disproportionally represented in the single-stranded regions (73% A) compared to the whole molecule (24% A). Purine-purine base stacking is known to be a major contributor to the overall stability of RNA molecules (20). Infrared measurements of 5S rRNA from Thermus thermophilus have recently shown the unpaired adenines, to be basestacked (21). These residues are mostly conserved (1) indicating the importance of basestacked adenines in stabilizing the structure of 5S rRNA. This could explain the high proportion of adenine residues in single-stranded regions in E. coli 16S rRNA and in the T. thermophila 5S rRNA molecule studied here.

In summary, we find that the data shown above support the secondary structure model of Curtiss and Vournakis (1), and that this structure is maintained in both neutral and acidic buffers.

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