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Enzymatic and Chemical Structure Mapping of Mouse 28S Ribosomal Ribonucleic Acid Contacts in 5.8S Ribosomal Ribonucleic Acid†

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ABSTRACT: Secondary structure mapping experiments using S1 nuclease, RNase T_1, and diethyl pyrocarbonate as conformational probes have identified those regions in mouse 5.8S rRNA containing major sites of interaction with 28S rRNA. One site encompasses the 3′-terminal 20 nucleotides and corresponds to the region identified previously as a component of an RNase-resistant 5.8S/28S rRNA junction complex. A second site, located at the 5′ terminus, has not been defined precisely but is believed to involve approximately 20–30 nucleotides. The existence of these sites of interaction is supported by comparing sequences of eukaryotic 5.8S and 28S rRNA with those of the prokaryotic 23S rRNA. Evidence for the occurrence of at least three helical regions in the central portion of the mouse 5.8S rRNA molecule is also presented.

Intermolecular RNA–RNA interactions mediate reactions that occur during protein synthesis, and probably other cellular functions. Such interactions have been difficult to explore because they generally are very tenuous. An RNA–RNA interaction of unknown role occurs in the large subunit of eukaryotic ribosomes, where 5.8S rRNA exists in the form of a specific complex with 28S rRNA (Pene et al., 1968). The noncovalent association presumably is maintained by hydrogen bonding and base stacking, and the complex is readily isolated by the usual extraction procedures. The stability of the 5.8S/28S rRNA complex affords an opportunity to investigate the details of an intermolecular RNA–RNA interaction.

We have demonstrated previously that the 5.8S/28S rRNA complex of mouse ribosomes can be dissociated by heating and reconstituted into a structure which exhibits the same thermal denaturation properties as those of its native counterpart (Pace et al., 1977). Limited ribonuclease digestion of the reconstituted complex between radioactive 5.8S rRNA and unlabeled 28S rRNA, and subsequent fractionation of the hydrolysis products, led to the identification of a specific "junction fragment" that contained part of the interacting regions of 5.8S and 28S rRNAs. The 5.8S moiety of the RNase-resistant fragment consisted of 42 nucleotides derived from the 3′ terminus of 5.8S rRNA. It was proposed that the 3′-terminal 20 nucleotides of the 5.8S molecule engage with 28S rRNA. The remaining 22 residues form an intramolecular GC-rich helix which, if allowed to engage in coaxial helix stacking, could in principle enhance the stability of the bimolecular interaction.

The isolated junction fragment was considerably less stable to thermal denaturation than the intact complex, however (Pace et al., 1977). This suggested that it is not the only region of interaction between 5.8S and 28S rRNAs. In an effort to define additional points of interaction and to assess conformational changes in 5.8S rRNA induced by annealing to 28S rRNA, we have exploited the structure mapping techniques described by Wurst et al. (1978) and Peattie & Gilbert (1980). In these procedures the sensitivity of a 5′- or 3′-end-labeled RNA molecule to nuclease or structure-specific reagent attack is analyzed by a modification of the rapid RNA gel sequencing

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techniques. As conformational probes we have used several single-strand-specific endonucleases, including nuclease S1, the ribonucleases T_1, A, and U_2, and the A-specific reagent diethyl pyrocarbonate. When the nuclease and chemical cleavage patterns for 28S-annealed and unannealed 5.8S rRNAs were compared, two probable points of contact between these two rRNA molecules have been delineated.

Experimental Procedures

Materials. T4 polynucleotide kinase and T4 RNA ligase were prepared by the methods of Cameron & Uhlenbeck (1977) and Walker et al. (1975), respectively. Bacterial alkaline phosphatase was obtained from Sigma Chemical Co., ribonucleases T_1 and U_2 were from Sankyo, and P1 nuclease was from P-L Biochemicals, Inc. S1 nuclease was purified from crude α-amylase powder (Sigma) by the procedure of Rushizky et al. (1975) and was kindly and generously supplied by Drs. R. Wurst and J. Vournakis. [γ-^{32}P]ATP and [5′-^{32}P]pCp[1] were synthesized by the procedures of Maxam & Gilbert (1977) and Stahl et al. (1979), respectively. Acrylamide and *N,N′*-methylenebis(acrylamide) were purchased from Eastman Chemicals and urea was from Fisher Scientific Co.

Cell Growth and RNA Purification. Mouse fibroblasts (L-929 strain) were grown in suspension in Eagle's minimal essential medium supplemented with 0.05% methylcellulose and 5% (v/v) calf serum (Pace et al., 1977). The labeling and extraction of total cellular RNA and the purification of 28S and 5.8S rRNAs by sucrose gradient centrifugation and gel electrophoresis have been described (Pace et al., 1977; Walker & Pace, 1977).

End Labeling of Mouse 5.8S rRNA. Twenty micrograms (0.4 nmol) of mouse 5.8S rRNA was dephosphorylated with bacterial alkaline phosphatase as described by Donis-Keller et al. (1977) with the exception that the reaction was incubated at 60 °C. After incubation, the alkaline phosphatase was removed by two phenol extractions, and the dephosphorylated 5.8S rRNA was recovered from the final aqueous phase by ethanol precipitation. Ten micrograms (0.2 nmol) of dephosphorylated mouse 5.8S rRNA was phosphorylated at the 5′ terminus with [γ-^{32}P]ATP and T4 polynucleotide kinase and then purified by gel electrophoresis as described (Donis-Keller et al., 1977). Dephosphorylated 5.8S rRNA was labeled at the 3′ terminus under conditions similar to those described by England & Uhlenbeck (1978). A 100-μL reaction mixture containing 50 mM Hepes[1] (pH 8.3), 10 mM $MgCl_2$, 5 mM DTT,[1] 8 μM ATP, 10% dimethyl sulfoxide, 0.2 nmol of dephosphorylated 5.8S rRNA, 0.2 nmol of [5′-^{32}P]-pCp,[1] and 25 units of T4 RNA ligase was incubated for 24 h at 5 °C. We find that with 5.8S rRNA it is essential to carry out the ligase reaction at low temperature; very little pCp addition is observed at 25–37 °C. Presumably the 3′ end of the 5.8S rRNA is sufficiently ordered in structure that it is not accessible to the enzyme. The reaction was terminated by ethanol precipitation, and the product was purified by gel electrophoresis. Following electrophoresis, the 5.8S rRNA bands were located in the gels by UV shadowing (Hassur & Whitlock, 1974) and autoradiography, excised, and eluted by shaking at 4 °C in buffer containing 0.1 M NaCl, 1 mM EDTA,[1] 0.1% $NaDodSO_4$,[1] and 10 mM Tris-HCl[1] (pH 7.4). Eluates were filtered through glass wool, and the RNA was recovered by ethanol precipitation.

Annealing of 5.8S to 28S rRNA. Complexes between 5.8S and 28S rRNAs were generated by incubating unlabeled 28S rRNA and either 3′- or 5′-end-labeled 5.8S rRNA in 10 mM Tris-HCl (pH 8.0), 2 mM EDTA, and 0.4 M NaCl for 60 min at 58 °C. The optimal RNA concentration for annealing was about 1 mg/mL at a molar ratio of 28S to 5.8S rRNA of 1.5. After incubation, the reaction was chilled rapidly to 0 °C and diluted with an equal volume of 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA. Annealed complexes were then resolved from unannealed 5.8S rRNA by centrifugation in a 5–20% (w/v) sucrose gradient in 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 0.1 M NaCl as described previously (Walker & Pace, 1977). Fractions of 0.3 mL were collected and assayed for radioactivity. Those fractions constituting the 28S region of the gradient were pooled, and the RNA was precipitated with ethanol. After centrifugation of the RNA and removal of residual ethanol by drying under vacuum, the 5.8S/28S rRNA complex was dissolved in 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 0.1 M NaCl and stored at −20 °C.

A control reaction containing 5.8S rRNA, but no 28S rRNA, was incubated under identical conditions and processed as described above with the sucrose gradient centrifugation step omitted.

Analysis of Termini. P1 nuclease digestion of end-labeled 5.8S rRNA and chromatography on PEI[1] thin-layer plates in 1.0 M LiCl have been described (Wurst et al., 1978).

Partial Digestions with RNases T_1 and U_2 under Denaturing Conditions. The procedure used to produce partial digests of end-labeled 5.8S rRNA in the presence of 7 M urea has been described (Donis-Keller et al., 1977). Ribonucleases T_1 and U_2 were both used at the level of 5×10^{-3} unit/μg of RNA.

Limited Alkaline Hydrolysis. The conditions used for the partial hydrolysis of end-labeled 5.8S rRNA with base were those described by Wurst et al. (1978) with the exception that the reaction was incubated for 20 min.

Partial Digestion of End-Labeled 5.8S rRNA under Nondenaturing Conditions. Unannealed and 28S-annealed 5.8S rRNAs were digested with S1 nuclease in a 10-μL reaction mixture containing 10 mM $ZnSO_4$, 0.2 M NaCl, 40 mM sodium acetate (pH 5.5), 10 μg of carrier RNA, and 0.6 unit of enzyme (Wurst et al., 1978). Reactions were incubated at 37 °C for different lengths of time and then terminated by adding 40 μL (20 μg) of carrier RNA in 2.5 mM EDTA and 5 μL of 1% diethyl pyrocarbonate in ethanol. The reactions were immediately frozen at −70 °C and lyophilized. Partial digestions of unannealed and 28S-annealed 5.8S rRNAs with RNases T_1, U_2, and A generally were performed in 10 μL of 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 0.2 M NaCl containing 10 μg of carrier RNA and 2.5×10^{-2} unit of enzyme. After incubating at 0 °C for the appropriate time, the reactions were terminated as described above. Partial digestions also were carried out in 1 mM $MgCl_2$ instead of EDTA, but no significant differences in the digestion patterns were observed.

Diethyl Pyrocarbonate Cleavage. Unannealed and 28S-annealed 5.8S rRNAs were treated with diethyl pyrocarbonate under "native" (presence of 10 mM $MgCl_2$), "semidenaturing" (presence of 1 mM EDTA), and "denaturing" (90 °C) conditions, as described by Peattie & Gilbert (1980), except that the kinetics of modification were examined. At time intervals following addition of the diethyl pyrocarbonate, samples were withdrawn and frozen for subsequent lyophilization and aniline cleavage as described. The chemical modification and cleavage

[1] Abbreviations: Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; ^{32}pCp, cytidine [5′-^{32}P]bisphosphate; Hepes, *N*-(2-hydroxyethyl)piperazine-*N′*-2-ethanesulfonic acid; DTT, dithiothreitol; PEI, poly(ethylenimine); DEP, diethyl pyrocarbonate.

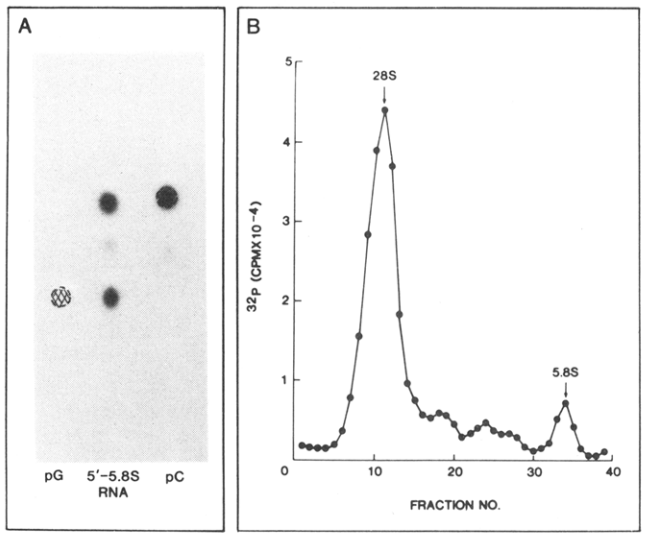

FIGURE 1: (A) End-group analysis of 5′-^{32}P-labeled 5.8S rRNA. Mouse 5.8S rRNA was dephosphorylated and then labeled at the 5′ terminus with [γ-^{32}P]ATP and T4 polynucleotide kinase. A small aliquot of the labeled RNA was digested to completion with P1 nuclease, and the resulting mononucleotides were chromatographed on thin layers of PEI-cellulose in 1 M LiCl. After autoradiography, the spots were excised from the plate and their radioactive contents determined. Unlabeled pG, visualized under UV light, and ^{32}P-labeled pC were used as markers. Their positions are indicated by hatched areas. (B) Purification of 28S-annealed 5.8S rRNA. Mouse 5.8S rRNA, labeled at either the 5′ or 3′ terminus, was incubated with unlabeled 28S rRNA at 58 °C in 0.4 M NaCl. The complexes were resolved from unannealed 5.8S rRNA by centrifugation through preformed 5–20% (w/v) sucrose density gradients in 0.1 M NaCl/10 mM Tris-HCl, pH 7.4/1 mM EDTA. The gradients were centrifuged in a Spinco SW41 rotor at 20000 rpm for 12–13 h at 4 °C. Fractions were collected and monitored for radioactivity.

protocols require several ethanol precipitations, so equivalent recoveries in compared samples were of concern. Therefore, prior to resolution of cleavage products on sequencing gels, samples were monitored for radioactivity, and the same amounts of label were applied to all gel slots to be compared.

Polyacrylamide Gel Electrophoresis. Prior to electrophoresis, the lyophilized samples were dissolved in 10 M urea, 0.05% xylene cyanol, and 0.05% bromophenol blue and heated for 30 s at 50 °C. Aliquots of each reaction were analyzed in adjacent lanes of either 20% or 15% polyacrylamide slab gels (1.5 × 200 × 400 mm) containing 7 M urea as described elsewhere (Maxam & Gilbert, 1977; Donis-Keller et al., 1977).

Results

5′- and 3′-End-Labeled 5.8S rRNA. End-labeled substrates for the structure mapping procedure were prepared as detailed under Experimental Procedures. Direct sequence analysis of mouse 5.8S rRNA had previously revealed the existence of heterogeneity at the 5′ terminus (Nazar et al., 1976), which would have significant consequences on the interpretation of the sequencing gels. It was necessary, therefore, to document the extent of such heterogeneity in our 5.8S rRNA preparations. 5′-End-labeled 5.8S rRNA was digested to completion with P1 nuclease, and the resulting mononucleotides were resolved by chromatography on thin sheets of PEI-cellulose using 1 M LiCl (Figure 1A). Liquid scintillation counting indicated that 60% of the 5′ ends were pG and the remainder pC. Thus, when "reading" the sequencing gels on 5′-end-labeled RNA, it must be recognized that two autoradiographic bands will result for each cleavage event.

Preparation of 28S-Annealed and Unannealed 5.8S rRNA. We have demonstrated previously (Pace et al., 1977) that annealing of 5.8S rRNA to 28S rRNA occurs at 58 °C in the presence of 0.4 M NaCl. Figure 1B illustrates the preparative separation of 28S-annealed and unannealed 5.8S rRNA by sucrose gradient centrifugation and that approximately 70% of the end-labeled 5.8S rRNA enters into complex formation with 28S rRNA. The annealed complex was collected from the 28S region of the sucrose gradient and used in the structure mapping experiments described below.

Higher Order RNA Structure Mapping. It was anticipated that 5.8S rRNA would not possess the same secondary conformation in the presence of 28S rRNA as it does in its absence. Secondary structural differences should, therefore, manifest themselves as differences in the susceptibilities of particular nucleotides to single-strand-specific nucleases or modifying reagents. Hence, when enzymatic digests or chemical modification patterns of 28S-annealed and unannealed 5.8S rRNA under native conditions are compared, it is possible to delineate those nucleotides in 5.8S rRNA that interact with 28S rRNA or are conformationally altered by the interaction. This approach has been used successfully by Wurst et al. (1978), with S1 nuclease, and Peattie & Gilbert (1980), with chemical agents, to examine the higher order structure of tRNA in solution. The structure mapping procedures are extensions of the rapid gel sequencing procedures described by Donis-Keller et al. (1977) and Peattie (1979). Briefly, end-labeled RNA is cleaved with structure- and base-specific nucleases or reagents under native conditions such that, on the average, less than one nick per molecule is introduced. The resulting partial digests of the RNA substrate are resolved by gel electrophoresis on the basis of chain length. A comparison of the mobility of the resulting fragments to fragments of known length delineates the attacked residues, which are considered to be located in single-stranded regions of the RNA substrate. Using nucleases S1 (little base specificity), T_1 (G specific), A (pyrimidine specific), and U_2 (A specific) and diethyl pyrocarbonate (A specific), we have compared the conformations of unannealed and 28S-annealed 5.8S rRNA. Reactions were incubated for different lengths of time to observe the kinetics of band appearance. This was necessary to discriminate between those nucleotides that were accessible to the probes at the beginning of the reaction and those whose accessibility might result from a progressive change in the conformation of the substrate as the reaction proceeds. Vournakis and his colleagues (Wurst et al., 1978) have discussed the importance of this kinetic approach to structure mapping and the interpretation of the gel bands.

Structure Mapping with Nucleases. Figure 2 presents autoradiograms of some of the many digestion patterns examined, in this case nuclease S1. Each gel also was loaded with end-labeled 5.8S rRNA digested partially with RNases T_1 and U_2 under denaturing conditions (presence of urea) or with base, to align the native cleavages with the nucleotide sequence. The derived alignment is consistent with the nucleotide sequence of rat 5.8S rRNA determined by Nazar et al. (1976) with the exception that we can account for only 156 instead of 158 nucleotides. In particular, residues G_{51}–C_{52} in the sequence derived for rat 5.8S rRNA are not revealed by the gel analysis of the mouse L-cell 5.8S rRNA or its gene.[2] It is possible that species differences between rat and mouse 5.8S rRNA occur in this region, although we think it unlikely.

The results shown in Figure 2 and many other experiments are imposed on the structure shown in Figure 3. Arrows indicate prominent cleavage sites; the nuclease-susceptible

[2] T. A. Walker et al., unpublished results.

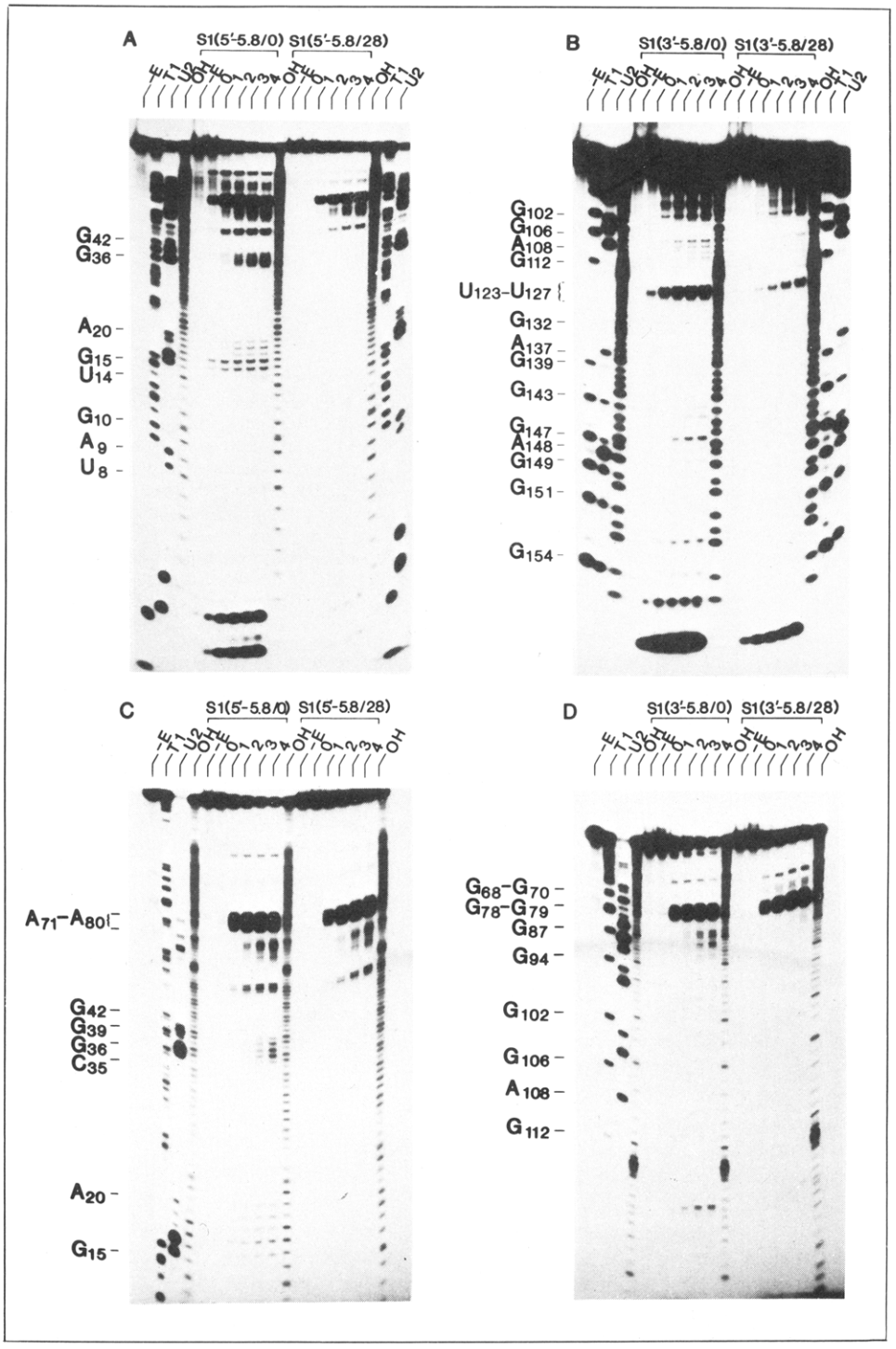

FIGURE 2: S1 nuclease structure mapping of 28S-annealed and unannealed 5.8S rRNA. 5′- (panels A and C) or 3′- (panels B and D) end-labeled 5.8S rRNA, either alone (5.8/0) or annealed to unlabeled 28S rRNA (5.8/28), was partially digested with S1 nuclease under nondenaturing conditions as described under Experimental Procedures. Portions of each digest were electrophoresed on either 20% (panels A and B) or 15% (panels C and D) polyacrylamide–7 M urea slab gels to resolve oligomers of different lengths. Numbers and letters to the side of the gels represent the location and identity of nucleotides relative to the 5′ end of 5.8S rRNA, as determined by comparing the mobility of the S1-generated oligomer with fragments of known length produced in the alkaline hydrolysates (OH^-). T_1 and U_2 denote RNase T_1 and RNase U_2 hydrolysates of end-labeled 5.8S rRNA produced under denaturing (50 °C, 7 M urea) conditions to locate in the sequence the positions of G and A residues, respectively. Samples were also incubated in the absence of enzyme (–E) to monitor extraneous degradation of the end-labeled substrates. The numbers 0–4 refer to S1 nuclease reactions incubated for increasing lengths of time to observe the kinetics of band appearance. For a more thorough discussion of the principle underlying the structure mapping technique, consult the text and Wurst et al. (1978).

regions of 5.8S rRNA in the 5′- and 3′-terminal regions, which clearly are protected when in complex with 28S rRNA, are shaded. In the simplest interpretation, these regions represent contact sites between 5.8S and 28S rRNA. Residues 36–42 also are susceptible in the free 5.8S rRNA, but not in the 5.8S/28S complex. However, this region remains susceptible to diethyl pyrocarbonate in the complex (see below), so we do not consider it to be a contact site. Presumably the 28S rRNA sterically shields this region from the bulky enzymes, whereas little barrier is offered to the diethyl pyrocarbonate.

Three helical domains, labeled arms II–IV in Figure 3, are indicated by the occurrence of adjacent complementary sequences and the partial nuclease digestion patterns. It is evident in the digestion patterns that there are only a few (see

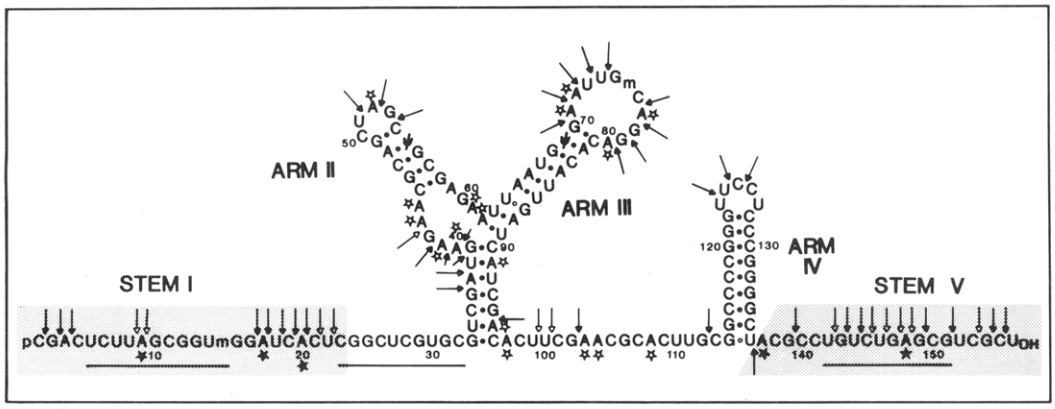

FIGURE 3: Summary of the structure mapping experiments using S1 nuclease, RNase T_1, and a chemical agent, diethyl pyrocarbonate, as conformational probes. The sequence of the molecule is presented with the central region, encompassing nucleotides 33–136, arranged in one of the possible secondary structures consistent with the available data. The 5′- and 3′-terminal regions possess as yet indeterminate secondary structures. Closed arrows indicate phosphodiester bonds cleaved rather efficiently while open arrows denote those bonds cleaved to a lesser extent. In all cases, residues which are susceptible to S1 nuclease cleavage were also cleaved by all other nucleases with the appropriate base specificity. Adenine residues susceptible to modification by diethyl pyrocarbonate are indicated with open stars, and those susceptible in unannealed, but not in the annealed complex, are depicted with solid stars. Shaded areas represent nuclease-susceptible regions in 5.8S rRNA which are protected when 5.8S rRNA is in complex with 28S rRNA; these regions represent, therefore, putative contact sites between the two rRNA molecules. The three sequence tracts which are underlined represent potential contact sites whose occurrence in the 5.8S rRNA would be predicted from comparative sequence analyses of 5.8S and 28S rRNAs with *Escherichia coli* 23S rRNA.

below) susceptibility differences between free and 28S-associated 5.8S rRNA within nucleotides ca. 45–136, which includes arms II–IV. The most significant of the 5.8S contacts with the 28S rRNA is located in the 5′-terminal ca. 20–30 nucleotides and the 3′-terminal ca. 20 nucleotides, both regions of which, even in the free 5.8S rRNA, seem substantially structured.

The structure at the termini could arise in two ways: (1) local (e.g., hairpin) independent folding of the terminal sequences or (2) more long-range pairing of the 5′- and 3′-terminal sequences. In fact most of the proposed foldings for 5.8S rRNA, which generally ignore the 28S rRNA, include a pairing of the 5′ and 3′ termini (Nazar et al., 1975; Luoma & Marshall, 1978). Such theorized pairings are always unsatisfactory in their expected stabilities because of bulges and mispairings required to align the few base complements in the 5′- and 3′-terminal 5.8S rRNA sequences. Moreover, the little complementarity that does exist is not consistent in positioning when phylogenetically diverse 5.8S rRNA sequences are compared (Olsen & Sogin, 1982). We resolved the question of whether structure within the 5′-terminal ca. 40 residues of free 5.8S rRNA is due to local vs. long-range folding by examining the nuclease S1 digestion pattern of the 5′ half of the 5.8S rRNA in the absence of the 3′ half; a sequencing gel is shown in Figure 4A. The cleavage pattern within residues 1 to ~30 is equivalent to that of the intact, free 5.8S rRNA. It is the pattern expected for a hairpin, i.e., a sensitive loop flanked by resistant stretches. In fact, the sequence within residues 1 to ~35 can be folded into a hairpin which is rather more satisfying than the 3′–5′-end pairing. We do not attribute any functional significance to this hairpin, however, since it does not stand the test of phylogenetic comparison (Olsen & Sogin, 1982). Probably it unfolds upon association with 28S rRNA (see below). We do not know the structure of the 3′-terminal ca. 20 residues in the free 5.8S rRNA but consider it moot, since it undoubtedly rearranges in contact with 28S rRNA (see below).

Arms II, III, and IV shown in Figure 3 almost certainly exist, for several reasons: (1) they are composed of adjacent Watson–Crick complements of good theoretical stabilities (Tinoco et al., 1973), (2) the duplex stem components of the arms are resistant to digestion, but the apex loops are highly sensitive, and (3) the analogous local structures are predictable from the nucleotide sequences of 5.8S rRNA from other eukaryotes (Olsen & Sogin, 1982).

All of the partial digestion data are consistent with the occurrence of arm II. Additionally, the pattern of band compression in the relevant regions of the alkaline hydrolysis lanes of the sequencing gels suggests the presence of a hairpin composed of adjacent complements. For example, 5′-end-labeled fragments in Figure 2C, which include nucleotides only through the apex of arm II (up to residues ca. 53), are evenly spaced on the gels, indicating no peculiar conformational effects on electrophoretic mobility. Higher in the gel of Figure 2C, 5′-labeled fragments which include sufficient amounts of nucleotides 54 to ~60 to form arm II (presumably with at least the complementary residues ca. 45–49) exhibit compression of band spacing, indicative of stable secondary structure.

Arm III is probably a real structure, at least in the 5.8S rRNAs of the higher eukaryotes. The stem of arm III (pairing between residues 62–70 and 81–89) is quite stable to digestion by RNases T_1 and U_2 (data not shown), as required by the proposed structure, but, following extensive digestion by nuclease S1, becomes labile (Figure 2C). We feel that this observation does not detract from the proposed pairing, however. The differing susceptibilities of stem III to the various nucleases are understandable in terms of the specificities of the enzymes and the fact that the apex loop of arm III is the most accessible region in the entire molecule to enzymatic attack. Nuclease S1, which is relatively nonspecific as to the cleaved residue, rapidly and completely trims any unpaired residues in the loop of arm III, thereby removing residues probably important for stabilizing, by stacking interactions, the adjacent duplex. In contrast, because they are specific for G or A, RNases T_1 and U_2 introduce only a few cuts in the loop. The residual dangling ends would continue to provide stacking stability to the stem, minimizing secondary digestions. Moreover, the stem is inaccessible to diethyl pyrocarbonate (see below). The stem of arm III certainly is not very stable, however, and the apparent stability of the pairing in the stem deteriorates in the lower eukaryotes (Olsen & Sogin, 1982).

Arm IV certainly exists. The high GC content of the adjacent complements, the extreme resistance of the stem to

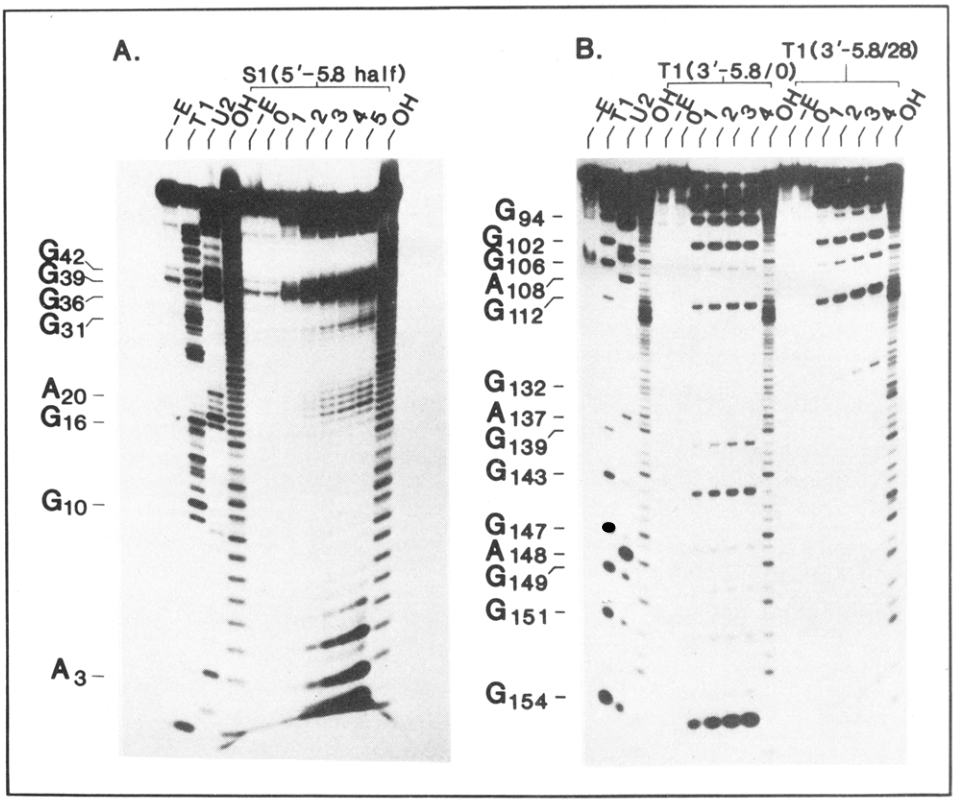

FIGURE 4: (A) S1 nuclease structure mapping of the end-labeled 5′-terminal "half" of 5.8S rRNA (residues 1–73). The labeled substrate was prepared as described (Peters et al., 1982), and analyzed as detailed in Figure 2 and under Experimental Procedures. (B) RNase T_1 structure mapping of 3′-end-labeled, 28S-annealed (5.8/28) and unannealed (5.8/0) 5.8S rRNA. The reaction products were resolved on 20% polyacrylamide–7 M urea slab gels and autoradiographed. Labeling of the autoradiographs is the same as in the legend to Figure 2.

nuclease digestions, and severe band compression in this region of the sequencing gels are all compatible with it. Moreover, arm IV is readily isolated from nuclease digests of 5.8S rRNA (Lightfoot, 1978; T. A. Walker, unpublished results). Thermodynamic data on the melting of the isolated arm IV from yeast have been gathered and are consistent with its proposed structure (Lightfoot, 1978).

Structure within residues ca. 35–45 and ca. 90–110 remains vague. Although pairing is shown between residues 33–39 and 90–96, this conjecture is based mostly on the existence of the paired complements, which deteriorates in the lower eukaryotes. The structure mapping data generally are consistent with the existence of a weak pairing, however. The data suggest that this entire region of the molecule is highly ordered, but we cannot interpret how. The structure tightens and/or reorders somewhat upon association with 28S rRNA; the G_{39}–A_{44} segment evidently is buried in the 28S rRNA superstructure (see below). The relative resistance of these regions to the various agents generally is increased, although the susceptibility of a few residues is enhanced. It is clear, however, that there are no outstanding differences in the nuclease sensitivities of this region of the 5.8S rRNA upon association with 28S rRNA, so we conclude that it is not involved in immediate contacts.

There are no major differences in the relative nuclease sensitivity patterns of the free and 28S-bound 5.8S rRNA in arms II and III. Arm IV of the 5.8S rRNA, however, is perturbed in a peculiar way by association with 28S rRNA. The apex of arm IV is less susceptible to nuclease S1 (Figure 2B) or RNase A (not shown) when the 5.8S rRNA is in complex with 28S rRNA, but not sufficiently so that direct contact with the loop is indicated. Surprisingly G_{132}, which is in the middle of the stem of arm IV, is rendered accessible to digestion by RNase T_1 (Figure 4B) and (less so) to nuclease S1 (Figure 2B) upon complex formation. Additionally, a C residue at approximately position 118 becomes susceptible to RNase A (data not shown). Precise location of this susceptible C residue is impossible because of the severe band compression due to secondary structure in this portion of the gel. The susceptibility of these residues must mean that the 28S rRNA exerts a rather profound influence on this highly stable duplex, perhaps kinking it, for example, by intercalation. We found this result surprising because, as pointed out, G residues in that hairpin are extremely inaccessible to RNase T_1 digestion, even under denaturing conditions. It is interesting to note that other 5.8S rRNAs have non-Watson–Crick pairings at about the same position as in the mouse 5.8S rRNA arm IV we observe to be perturbed by the 28S rRNA. This points to functional significance for the local instability in arm IV. The stem of arm IV probably is very important to the 5.8S/28S complex. It is part of the nuclease resistant junction complex isolated previously (Pace et al., 1977). Possibly it contributes substantially to the interaction by coaxial helical stacking (Grosjean et al., 1976) on stem V (see below) which results from pairing the 3′ end of the 5.8S rRNA with the 28S rRNA.

Residues within the regions 1 to ~30 and 137 to 156 are rendered resistant to the nucleases upon binding 28S rRNA (Figure 2A,B); they seem to be the primary contacts that maintain the complex. We denote these putative pairings as stems I and V in Figure 3. The 3′ boundary of the 5′-binding region is rather arbitrary. Definition of the actual residues involved is obscured by the presumably aberrant hairpin discussed above. If the pairings suggested below are correct, this hairpin must unfold upon binding to the 28S rRNA. The 5′- and 3′-terminal half-molecules are capable of independently binding to 28S rRNA with about the same binding energy (Peters et al., 1982), so we presume that the sequence extents of the pairings are about the same, ca. 15 residues, based on

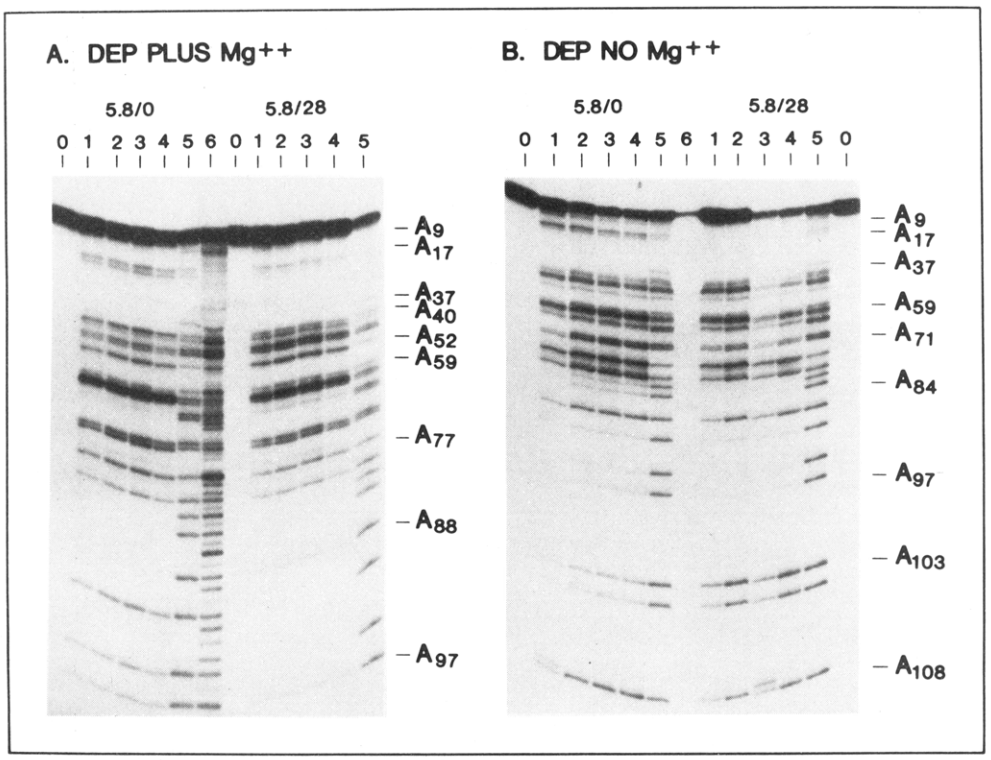

FIGURE 5: Structure mapping with diethyl pyrocarbonate. 5.8S rRNA, labeled at its 3′-end with [5′-^{32}P]pCp, alone or following annealing to 28S rRNA as described under Experimental Procedures, was treated with diethyl pyrocarbonate as detailed by Peattie & Gilbert (1980). 20 μL of diethyl pyrocarbonate was added to 200-μL reactions in 50 mM sodium cacodylate, pH 7.0/10 mM $MgCl_2$ (panel A) or 50 mM sodium cacodylate, pH 7.0/1 mM EDTA (panel B). Following incubation at 37 °C, aliquots were withdrawn at 30 (lane 1), 60 (lanes 2, parts A and B), 90 (lanes 3, parts A and B), or 120 min (lanes 4, parts A and B), then precipitated and washed with ethanol, and subjected to cleavage by aniline as detailed by Peattie & Gilbert (1980). Samples applied to lanes 0 (parts A and B) received no diethyl pyrocarbonate but were subjected to aniline treatment. Samples applied to lane 5 (parts A and B) were treated with diethyl pyrocarbonate in 50 mM sodium cacodylate, pH 7.0/1 mM EDTA at 90 °C for 15 min and then subjected to aniline cleavage to display all A residues. Lanes 6 (parts A and B) contained [5′-^{32}P]pCp-appended 5.8S rRNA which had been partially digested by nuclease S1, to delineate all residues. Following all the modification and cleavage steps, all the dried samples were dissolved in electrophoresis buffer, their ^{32}P contents were monitored by Cerenkov radiation, and equal amounts of radioactivity were applied to lanes 0–5 for each compared RNA species.

the 3′ pairings. The binding site(s) for the 5′ end remain(s) conjectural, but the 3′ end likely associates with the 5′ end of the 28S rRNA (see below).

Structure Mapping with Diethyl Pyrocarbonate. The nucleases are bulky probes for structure, so we wished to use a more freely diffusing agent, as well. Diethyl pyrocarbonate, specific for nonstructured A residues and one of the more reliable chemical probes, was used, as detailed under Experimental Procedures. Because the aniline cleavage generates heterogeneous chemistry at the 3′ end of the cleaved phosphodiester bond (Peattie, 1979), only 3′-end-labeled molecules could be examined. We inspected the sensitivity of free and 28S-associated 5.8S rRNA to the diethyl pyrocarbonate, both in the presence and absence of Mg^{2+}. Figure 5 shows one comparative analysis of the free and bound 5.8S rRNA. As a somewhat simplified distillation of the data, residues susceptible to the agent are indicated with stars in Figure 3. Those attacked in the free 5.8S rRNA, but not in the complex, are indicated by filled stars. The data generally are consistent with the enzymatic digestion analyses. An important exception is that nucleotides G_{39}–A_{44}, which are insensitive to nucleases when bound to 28S rRNA, remain sensitive to the diethyl pyrocarbonate (Figure 5). We suggest, therefore, that this region is not a contact between the 5.8S and 28S rRNAs. Rather, the 28S rRNA superstructure probably sterically limits the access of the bulky nucleases. Some reordering of this region by the 28S rRNA is indicated, however. For example, A_{40} is considerably more susceptible to the agent than its 3′ neighbor, A_{41}, but when bound to the 28S rRNA, both are freely available. Residues in the 5′- (Figure 5) and 3′- (not shown) terminal contact regions are resistant to the agent in the complex, in the absence of Mg^{2+}, although in its presence A_{17} is somewhat susceptible. This suggests that pairing between the 5′ end of the 5.8S and the 28S rRNA is not a perfect RNA–RNA duplex, but in the absence of information on the 28S rRNA binding sequence, we cannot address this structure further.

It was pointed out above that the stem of arm III is quite weak, and the evident pairing degenerates in the 5.8S rRNAs of the lower eukaryotes. The instability of this stem also is reflected in the diethyl pyrocarbonate data. Residues A_{65} and A_{88}, which might be expected to be resistant to the reagent, require Mg^{2+} for stability; they are attacked in its absence. In contrast, residues A_{95} and A_{97} are susceptible in the presence of Mg^{2+}, but not so much so in its absence, so are indicated as sensitive in Figure 3. We cannot interpret the available data in terms of structural transitions; however, it seems that the stem connecting arms II and III is not a straightforward, short helix.

Discussion

Taken together, the enzymatic and chemical cleavage data provide a reasonable estimate for the folding of the mouse 5.8S rRNA, free and in association with the 28S rRNA, as shown in Figure 3. The following are important considerations:

(1) The existence of arms II, III, and IV, which are predictable from the folding of adjacent complements in the sequence, are supported by the partial cleavage data. Considerable additional structure is evident in the regions surrounding these arms, but we cannot interpret the details with

confidence. Except for the perturbation of arm IV (see above) and a general tightening of the structure, the association with 28S rRNA causes little reordering within residues ca. 30–135. Residues 36–42 are rendered inaccessible to nucleases in association with 28S rRNA, but remain sensitive to diethyl pyrocarbonate, so probably are not contact sites for the 28S rRNA.

(2) The 5′- and 3′-terminal ca. 20 residues of the 5.8S rRNA evidently are not associated with one another, in contrast to most proposed foldings for the molecule (Nazar et al., 1975; Luoma & Marshall, 1978). Significant structure is evident in the termini of even the free 5.8S rRNA, but these presumably reorder in contact with the 28S rRNA. The nuclease S1 digestion data show that structure within the 5′ end of the free 5.8S rRNA probably is due mainly to local, loose hairpin formation, rather than more long range pairing with the 3′ end of the molecule. Nevertheless, it remains possible that some sort of 5′–3′ interaction occurs in the free 5.8S rRNA. This suggestion derives from the finding that 5′ and 3′ half-molecules of 5.8S rRNA associate with 28S rRNA at substantially lower annealing temperatures than the intact 5.8S rRNA (Peters et al., 1982). One interpretation of this observation is that association of the 5.8S rRNA termini, in the intact molecule, precludes binding to the 28S rRNA and that the aberrant pairing must be dissociated in order for annealing to occur.

(3) One primary contact between the 5.8S and 28S rRNA is within the 5′-terminal ca. 20–30 residues. Upon complex formation, this region is very resistant to all the agents. The 5′ half of 5.8S rRNA is capable of independently associating with the 28S rRNA (Peters et al., 1982), probably through this interaction. The contact between the 5′ segment of the 5.8S and the 28S rRNA, which we denote as stem I in Figure 3, almost certainly is not by a continuous Watson–Crick pairing, however. Residue A_{17} and to a lesser extent residue A_{20} remain in the complex somewhat susceptible to diethyl pyrocarbonate in the presence of Mg^{2+}. In the absence of information on the 28S rRNA contact sequence, we make no attempt to interpret this in any detail. However, sequence homologies between the 5.8S and 28S rRNAs (Olsen & Sogin, 1982), and the prokaryotic 23S rRNA (Nazar, 1980; Jacq, 1981; Noller et al., 1981), suggest the nature and the locale in the 28S rRNA for this contact (see below).

(4) It is clear that the 3′-terminal ca. 20 residues of the 5.8S rRNA indicated as stem V in Figure 3 provide part of the contact with the 28S rRNA. In complex, this region is rendered inaccessible to all of the nucleases tested, and it is part of the previously isolated, nuclease-resistant junction complex. As with the 5′ half, the 3′ half of 5.8S rRNA is capable of independently binding to the 28S rRNA (Peters et al., 1982).

In attempting to identify the binding sites for the 5.8S on the 28S rRNA, we sought complementarities between the available sequences. That of the mammalian 28S rRNA is not known, but limited information from yeast (Bayev et al., 1981) and *Xenopus* (Hall & Maden, 1980) is in the literature. Nazar (1980) and Jacq (1981) have pointed out that the eukaryotic 5.8S rRNA is homologous to the 5′ end of the prokaryotic 23S rRNA, so it is possible to extend the phylogenetic comparison into the prokaryotes. A detailed analysis of sequence and secondary structural homologies in these various RNA species are presented by Olsen & Sogin (1982).

Figure 6 summarizes the relationships between the 5.8S/28S rRNA complex and the homologous region of the bacterial 23S rRNA. The 5.8S and 28S rRNA genes are not contiguous in their transcriptional unit (Hall & Maden, 1980; Figure 6A),

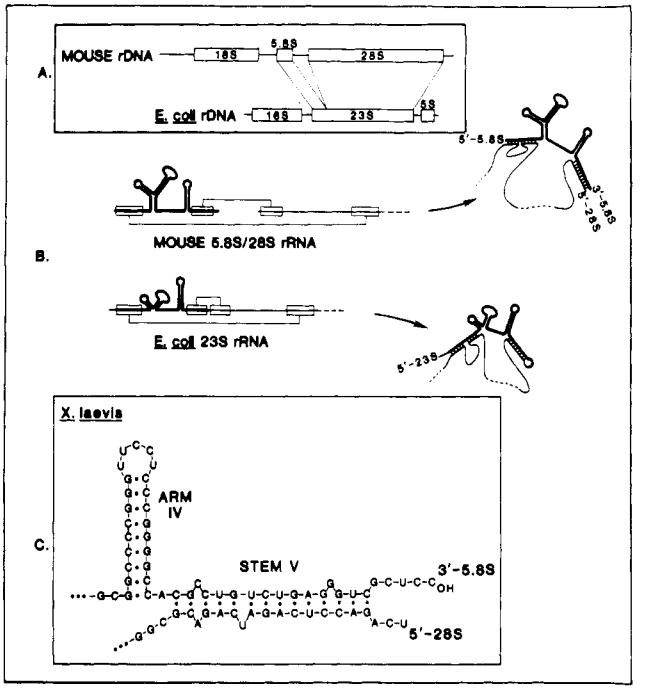

FIGURE 6: (A) Comparison of the transcriptional organization of the rRNA genes in eukaryotic and prokaryotic rDNA. The figure emphasizes the structural homology between the *E. coli* 23 rRNA gene and the eukaryotic 5.8S and 28S rRNA genes. (B, upper figure) Schematic representation of the topology of the contact sites between 5.8S and 28S rRNAs. The designated pairings are supported by comparative sequence analyses and the secondary structure model for *E. coli* 23S rRNA (Noller et al., 1981). The bold line represents the 5.8S rRNA sequence and the thin line that of the 28S rRNA. In the mouse 5.8S/28S rRNA interaction, the discontinuity between the 5.8S and 28S rRNA sequences in the initial rRNA transcript is indicated. (B, lower figure) The bold line (with appropriate secondary structure indicated) represents the 5′-terminal region of *E.coli* 23S rRNA which is structurally homologous to the eukaryotic 5.8S rRNA. The thin line denotes the remainder of the 23S sequence. Regions in the sequence which are proposed to base pair are indicated with boxes linked by thin lines. The pairings proposed for the *E. coli* 23S rRNA (Noller et al., 1981) are residues 16–25 with residues 515–524 and residues 34–43 with residues 437–447. (C) Proposed model for the base pairing between the 3′ terminus of 5.8S rRNA and the 5′-terminus of 28S rRNA. The sequences shown are those for *Xenopus laevis* (Ford & Mathieson, 1978; Hall & Maden, 1980).

but the homologous sequences are adjacent in the bacterial 23S rRNA (Brosius et al., 1980; Figure 6A). From comparisons of several prokaryotic 23S rRNAs, Glotz et al. (1981) and Noller et al. (1981) conclude that the 23S rRNA sequence homologous to the 5′-terminal 5.8S rRNA segment, which contacts 28S rRNA, is paired at two sites within a region ca. 500 residues from the 5′ end of the 23S rRNA. The sequences in the 5.8S rRNA which are homologous to the paired, prokaryotic 23S rRNA sequences are underlined in Figure 3. This region of 28S rRNA is not yet sequenced, so we cannot address in detail any pairing which may occur between the 5′ end of 5.8S rRNA and the interior of 28S rRNA. We suggest, however, on the basis of the phylogenetic comparisons, that the analogous pairing occurs between 5.8S and 28S rRNAs, as diagrammed in Figure 6B. Such pairing requires that structure within the 5′-terminal segment of the free 5.8S rRNA, interpreted above as possibly an imperfect hairpin, would unfold upon association with the 28S rRNA.

The bacterial 23S rRNA segment homologous to the 5.8S rRNA 3′-terminal contact site forms a local hairpin with the region homologous in sequence to the 5′ end of 28S rRNA. Inspection of the available 5.8S and 28S rRNA sequences revealed similar complementarity; that of *Xenopus* is shown

in Figure 6C. Analogous complementarity is evident in the 5.8S and 28S rRNAs of other eukaryotes (Olsen & Sogin, 1982).

In summary, then, we propose that the 5.8S/28S rRNA complex involves two main contacts. One of these consists of pairing between the 3′-terminal ca. 20 residues of the 5.8S rRNA and the 5′ end of the 28S rRNA. A second contact is suggested to occur between the 5′-terminal region of the 5.8S rRNA and two segments of the 28S rRNA several hundred nucleotides (at least 300–400, on the basis of the prokaryotic homologue) from the 5′ end of the 28S rRNA. The termini of the 5.8S rRNA thus are seen to span and organize several hundred nucleotides of the 28S rRNA sequence into a structured domain, as schematically indicated in Figure 6B.

The association of the 3′ end of the 5.8S rRNA and the 5′ end of the 28S rRNA seems reasonably solid, based on phylogenetically conserved complementarities in the eukaryotic rRNAs (Olsen & Sogin, 1982) and their prokaryotic 23S rRNA homologues (Noller et al., 1981). There is, however, a conceivable alternative to the proposed association of the 5′ end of the 5.8S rRNA with the 28S rRNA. In bacterial 23S rRNA, there exists good complementarity between the 5′ and 3′ termini (Glotz et al., 1981; Kelly & Cox, 1981), and it has been suggested (Kelly & Cox, 1981) that the 5′ end of the 5.8S rRNA has the same relationship in interacting with the extreme 3′ end of the 28S rRNA. Such pairing of the ends of the 5.8S rRNA with the ends of the 28S rRNA would effectively circularize the latter. Although this may be an attractive pairing, the best sequence alignment of the 5′ terminus of the 5.8S rRNA on the bacterial 23S rRNA does not overlap the region which pairs with its 3′ end. Rather, the alignment begins about 12 residues into the 23S rRNA, excluding the sequence which pairs with the 23S rRNA 3′ end. The 5.8S rRNA 5′ contact with the 28S rRNA coincides with the region of the bacterial 23S rRNA which interacts internally, as discussed above. Moreover, the limited complementarity which exists between the 5.8S rRNA 5′ end and the 28S rRNA 3′ end is not phylogenetically conserved (Olsen & Sogin, 1982). Thus, although a model whereby 5.8S rRNA would span the termini of the 28S rRNA is attractive, strict interpretation of the prokaryotic and eukaryotic sequence homologies argues against it. Further analysis of the 28S rRNA contacts for the 5.8S rRNA will be required to resolve this point.

The proposed model for the 5.8S/28S rRNA complex also has implications for the posttranscriptional processing of these molecules. rRNAs of 5.8S and 28S are derived in the nucleolus from the cleavage of a 32S precursor (Maden & Robertson, 1974). Several hundred precursor-specific residues linking the mature 5.8S and 28S rRNA sequences are removed, evidently in simultaneous (or nearly so) cleavage events, since intermediate precursors have not been detected. The removal of the intervening precursor segment requires cleavage at the 3′ end of the 5.8S sequence and at the 5′ end of the 28S rRNA, so it is satisfying that the proposed pairing draws these processed sites into proximity. This is a common theme in the maturation of the prokaryotic rRNAs.

Finally, the homology of the eukaryotic 5.8S rRNA with the 5′ end of the prokaryotic 23S rRNA raises the question of the utility of the evolutionary fragmentation of rRNA. The 5.8S/28S rRNA complex is not the only example of this. The ribosomes of flowering plant chloroplasts have a 4.5S rRNA, which corresponds to the 3′ end of the bacterial 23S rRNA (MacKay, 1981). Some bacteria have fragmented 23S rRNA (Lessie, 1965; Marrs & Kaplan, 1970; Robinson & Sykes, 1971); insects have further fragmented 5.8S rRNA, which is clipped at the apex of arm IV (Pavlakis et al., 1979). The diversity of the cleavage patterns suggests that the advantage conferred to the organism is subtle. Perhaps constructing the rRNA from fragments enhances its conformational mobility.

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Independent Binding Sites in Mouse 5.8S Ribosomal Ribonucleic Acid for 28S Ribosomal Ribonucleic Acid†

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ABSTRACT: Limited digestion of mouse 5.8S ribosomal RNA (rRNA) with RNase T_2 generates 5′- and 3′-terminal "half-molecules". These fragments are capable of independently and specifically binding to 28S rRNA, so there exist at least two contacts in the 5.8S rRNA for the 28S rRNA. The dissociation constants for the 5.8S/28S, 5′ 5.8S fragment/28S, and 3′ 5.8S fragment/28S complexes are 9×10^{-8} M, 6×10^{-8} M, and 13×10^{-8} M, respectively. Thus, each of the fragment binding sites contributes about equally to the overall binding energy of the 5.8S/28S rRNA complex, and the binding sites act independently, rather than cooperatively. The dissociation constants suggest that the 5.8S rRNA termini form short, irregular helices with 28S rRNA. Thermal denaturation data on complexes containing 28S rRNA and each of the half-molecules of 5.8S rRNA indicate that the 5′-terminal binding site(s) exist(s) in a single conformation while the 3′-terminal site exhibits two conformational alternatives. The functional significance of the different conformational states is presently indeterminate, but the possibility they may represent alternative forms of a conformational switch operative during ribosome function is discussed.

The 5.8S/28S rRNA complex from the large subunit of eukaryotic ribosomes is a convenient model for exploring some features of intermolecular RNA–RNA interactions. The 5.8S rRNA of mouse is shown in Figure 1, folded as suggested by the existence of complementary sequences, partial nuclease digestions, and chemical modifications (Walker et al., 1982). The shaded sequences at the 5′- and 3′-terminal ca. 20 residues are protected from nucleases and reagents upon the association with 28S rRNA, implying that they are the major contacts. We suggest elsewhere (Walker et al., 1982), on the basis of these data and sequence comparisons with the bacterial 23S rRNA, that the 5′ and 3′ termini of the 5.8S rRNA bind to sites on the 28S rRNA which are several hundred nucleotides distant from each other. The 5.8S rRNA thus is viewed to bridge a rather long stretch of the 28S rRNA, pinching off a conformational domain, as sketched in the inset to Figure 1.

If the described notion for the 5.8S moiety of the complex is correct, the 5′ and 3′ ends of the 5.8S rRNA should be capable of independently associating with the 28S rRNA. This would not be expected if the 5′ and 3′ regions of the 5.8S rRNA were associated with each other, as suggested by many workers (Nazar et al., 1975; Ford & Mathieson, 1978; Luoma & Marshall, 1978; Khan & Maden, 1976), and then interact with the 28S rRNA through some complex tertiary structure. In the present study we show that the 5′- and 3′-terminal 5.8S contacts with the 28S rRNA in fact can occur independently and that they contribute about equally to the overall 5.8S/28S binding energy.

Experimental Procedures

Purification of RNA. Uniformly ^{32}P-labeled and nonradioactive 5.8S and 28S rRNAs and the native 5.8S/28S complex were purified from mouse L-929 cells as detailed previously (Pace et al., 1977; Walker & Pace, 1977). RNAs were stored in aliquots, as ethanol precipitates at −80 °C. Just before use, samples were centrifuged, decanted, and dissolved in 0.1 M NaCl/0.05 M Tris-HCl[1] (pH 7.4)/0.001 M EDTA[1] (STE). Nonradioactive 5.8S rRNA sometimes was prepared from Balb/C mouse livers. Six grams of livers stored at −70 °C was thawed in 50 mL of ice-cold STE. After the tissue was minced, an equal volume of STE-saturated phenol was added and the mixture homogenized in the cold. After cen-

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[1] Abbreviations: Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

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