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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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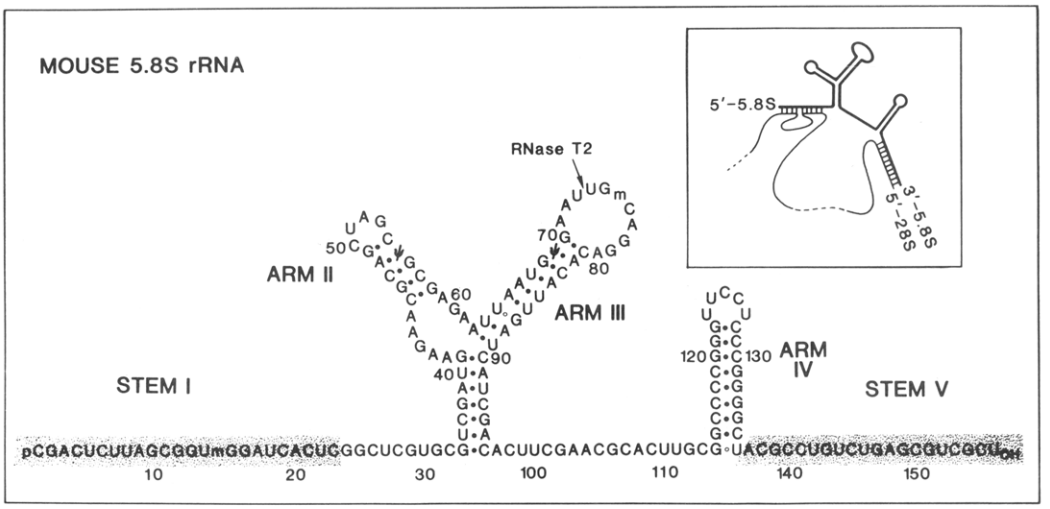

FIGURE 1: Mouse 5.8S rRNA. The nucleotide sequence of the mouse 5.8S rRNA (Nazar et al., 1975) is folded as suggested by the occurrence of vacinal complements, partial nuclease digestion, and chemical modification experiments (Walker et al., 1982). The shaded regions are proposed to interact with the 28S rRNA at different sites, defining a conformational domain as illustrated in the inset. The point of cleavage by RNase T_2, as discussed in the text, is indicated.

trifugation, and a second extraction, 2 volumes of ethanol was added to the aqueous phase, and it was stored at −20 °C. After centrifugation the precipitated RNA was made 1 M in NaCl and incubated overnight to precipitate high molecular weight RNA, including the 5.8S/28S rRNA. The high molecular weight RNA precipitate was taken up in STE, ethanol precipitated, and redissolved in water. After heating at 70 °C for 2 min, followed by rapid cooling to dissociate 5.8S rRNA from 28S rRNA, the solution was adjusted to 0.6 M NaCl/0.5 M sodium acetate, pH 5.5. This was loaded onto a 0.9 × 16 cm methylated albumin silicic acid column (Stern & Littauer, 1971) equilibrated with 0.6 M NaCl/0.05 M sodium acetate, pH 5.5. Absorbancy at 254 nm was monitored as fractions were collected across a 0.6–1.2 M NaCl gradient. The peak of 5.8S rRNA, identified by 8% polyacrylamide gel electrophoresis, was pooled and precipitated with ethanol. rRNA (5.8 S) was further purified by preparative gel electrophoresis as described previously (Donis-Keller et al., 1977). This procedure yielded 400 μg of pure 5.8S rRNA.

Generating Half-Molecules of 5.8S rRNA. rRNA (5.8 S) was subjected to a limited RNase T_2 digestion to cleave 5.8S rRNA into 5′ and 3′ halves. ^{32}P-Labeled 5.8S rRNA at less than 0.08 mg/mL was incubated in 15 mM Tris-HCl, pH 7.3/3 mM EDTA/10 mM NaCl at 70 °C for 5 min and then slow cooled over 4 h to 35 °C. After chilling to 0 °C, the solution was made 0.5 mg/mL in carrier tRNA, 0.2 M in NaCl, and 13 units/mL in RNase T_2 (Sankyo) and incubated for 20 min at 11 °C. The sample was frozen, dried in vacuo, and taken up in 10 M urea/3 mM EDTA, and the fragments were resolved on an 8% polyacrylamide gel containing 8 M urea. RNA was recovered from the gel as described previously (Walker et al., 1982). In preparing the 5′ and 3′ half-molecules from nonradioactive 5.8S rRNA, the same procedure was followed except that no carrier tRNA was added and the RNase T_2 concentration was 2 units/mL. The half-molecules were visualized on a gel by UV shadowing (Hassur & Whitlock, 1974) and recovered as described previously (Walker et al., 1982). Nonradioactive RNAs were labeled at their 5′ ends by using [γ-^{32}P]ATP and polynucleotide kinase as described previously (Maxam & Gilbert, 1977).

Results

Purification and Characterization of 5′ and 3′ Halves of 5.8S rRNA. The approach in these experiments was to determine whether fragments of 5.8S rRNA containing only one of the two putative binding sites can independently bind to the 28S rRNA. We first treated uniformly ^{32}P-labeled 5.8S rRNA with various nucleases and examined the products on denaturing polyacrylamide gels. RNase T_2 proved to be the most useful; it generated primarily two large fragments in good yield (Figure 2A). The fragments were extracted from the gel and complete RNase T_1 digests were characterized by the Sanger two-dimensional fingerprinting technique (Sanger et al., 1965). Autoradiograms of the fingerprints of intact 5.8S and the fragments are shown in Figure 2B–D; the molar yields of the oligonucleotides are given in Table I. The approximate sequences of the RNase T_2 fragments were evident from the molar yields of oligonucleotides, in comparison to the intact 5.8S rRNA. The polynucleotide referred to as the 5′ half-molecule of 5.8S rRNA (5′ 5.8 S) extends from the 5′ terminus of 5.8S rRNA to at least nucleotide 71 (Figure 1). The 3′ terminus of the 5′ 5.8S fragment was not determined. However, the absence of RNase T_1 oligonucleotide number 20 (Figure 2C) indicates that the 5′ half-molecule does not extend to nucleotide 78. Therefore, the T_2 RNase cleavage site(s) generating the 5′ half-molecule lies somewhere between nucleotides 71 and 80, probably at U_{73} (below).

The fragment referred to as the 3′ half-molecule of 5.8S rRNA (3′ 5.8 S) extends from the 3′ terminus of 5.8S rRNA to at least nucleotide 79. However, it, too, is missing the RNase T_1 oligonucleotide number 20 (Figure 2D), so the 3′ half does not extend to nucleotide 71. In separate experiments, the nonradioactive 3′ 5.8S fragment was labeled at its 5′ terminus by using [γ-^{32}P]ATP and polynucleotide kinase (Experimental Procedures) and subjected to RNA sequencing as described by Donis-Keller et al. (1977). The data (not shown) indicated that, although there was some heterogeneity, more than 70% of the 5′ termini were U_{74}.

Association of the 5′ and 3′ Halves of 5.8S rRNA with Each Other. We tested the affinities of the 5.8S rRNA half-molecules for each other. The nonradioactive 5′ half (5′ 5.8 S) was incubated with an equimolar amount of ^{32}P-labeled 3′ half-molecule (3′ 5.8 S) at different temperatures under standard annealing conditions (0.4 M NaCl/3 mM EDTA, pH 7.3). As shown in Figure 3A, a 5′ 5.8S/3′ 5.8S complex, having the same electrophoretic mobility as 5.8S rRNA, forms readily at low temperatures (0–35 °C); there are no significant conformational barriers to the association. This is in contrast

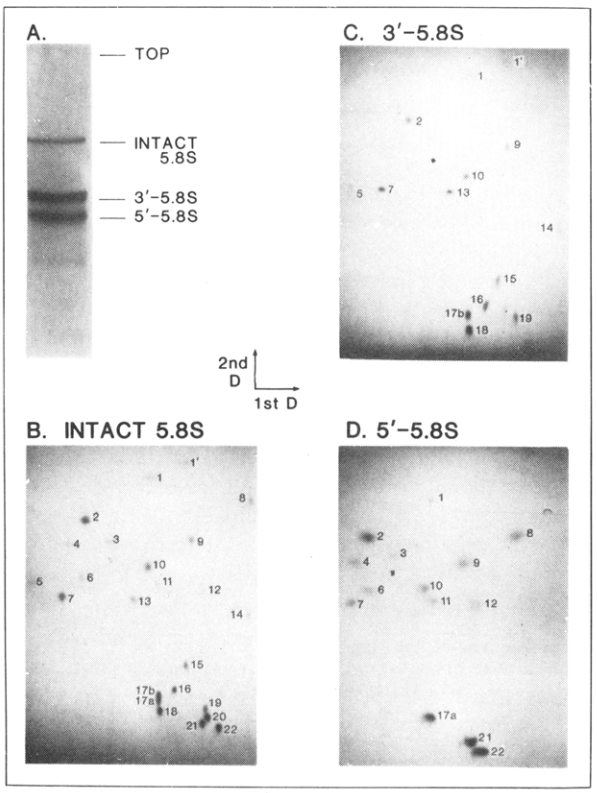

FIGURE 2: Characterization of the 5.8S rRNA half-molecule. (A) 22 μg of uniformly ^{32}P-labeled 5.8S rRNA (1×10^6 cpm/μg) was subjected to annealing, RNase T_2 digestion, and resolution of products on an 8% polyacrylamide gel in 8 M urea, all as detailed under Experimental Procedures. (B–D) The indicated RNase T_2 fragments or intact 5.8S rRNA was digested to completion with RNase T_1, and the resulting oligonucleotides were resolved by two-dimensional electrophoresis, as detailed by Sanger et al. (1965), except that the first dimension of electrophoresis was carried out on cellulose acetate strips saturated with 0.3 M ammonium formate, pH 3.5/7 M urea/1 mM EDTA. The electrode buffer was 0.4 M ammonium formate, pH 3.5/1 mM EDTA. The second dimension of electrophoresis, on DEAE paper, was 0.1 M pyridine/5% formic acid (pH 2.3). The oligonucleotides are numbered according to Table I.

Table I: RNase T_1 Digestion Products of 5′ 5.8S and 3′ 5.8S Fragments

oligo-nucleotide[a]	sequences[b]	5′ 5.8S fragment yield obsd	5′ 5.8S fragment yield ex-pected[d]	3′ 5.8S fragment yield obsd	3′ 5.8S fragment yield ex-pected[d]
1	G	3.0	3	6.1	7
2	CG	3.0	3	2.9	2
3	AG	1.1	1	1.1	1
4	CAG	0.6	1	–[c]	0
5	CCCCG	–	0	0.6	1
6	AAG	0.9	1	–	0
7	AACG	0.8	1	0.8	1
8	UG, ΨG, U_mG	2.3	3	–	0
9	UCG, CΨG	1.8	2	1.0	1
10	CUCG	1.1	1	0.9	1
11	CUAG	0.8	1	–	0
12	AUG	0.8	1		0
13	CUACG	–	0	1.2	1
14	UCUG	–	0	0.9	1
15	CACUUG	0.03	0	1.0	1
16	AUCAUCG	0.01	0	1.0	1
17a	AUCACUCG	1.34	1	–	0
17b	ACACUUCG	–	0	1.0	1
18	ACACAUUG	0.1	0	1.1	1
19	UUCCUCCCG	–	0	0.5	1
20	AAUUG$_m$CAG		0	–	0
21	ACUCUUAG	1.2	1		0
22	AAUUAAUG	1.4	1		0

[a] The numbers refer to oligonucleotides in Figure 2. [b] The sequence is based on the sequence of 5.8S rRNA (Nazar et al., 1975). RNase T_1 generated oligonucleotides from the half-molecules were identified by comparing autoradiograms of their fingerprints with those of 5.8S RNA. No further characterization was pursued. [c] (–) indicates no spot was observed in the autoradiogram. [d] Expected yields are based on the assumption that the half-molecules extend from either the 5′ or 3′ end of 5.8S to a T_2 RNase cleavage site within residues 71–79 (see text).

to 5.8S rRNA, which does not anneal readily to 28S rRNA at low temperatures, but does anneal at high temperatures. Contacts between the 5.8S halves are tenuous, however. When the preformed complex was incubated in 0.1 M NaCl at various temperatures and then resolved on an 8% acrylamide gel, the fragments dissociated in a sharp thermal transition, with a T_m of 38 °C (Figure 3B). Under the same conditions, the 5.8S/28S rRNA complex has a T_m of 52 °C (Pace et al., 1977), so the intramolecular interactions of the 5.8S rRNA are less stable than the intermolecular interactions between 5.8S rRNA and 28S rRNA. We do not know exactly the contacts between the 5′ and 3′ 5.8S halves. The pairings indicated in Figure 1 are reasonable possibilities, but substantial tertiary structure undoubtedly is involved.

Association of the 5.8S Halves with 28S rRNA. We examined the annealing of the ^{32}P-labeled 5′ and 3′ halves, independently, to 28S rRNA at various temperatures, under the standard annealing conditions. The results are presented in Figure 4A, in comparison with the annealing of the intact 5.8S to 28S rRNA. Consistent with the fact that the major contacts with the 28S rRNA seem to be at the termini of the 5.8S rRNA, both halves proved capable of independent association. Although the 5′ 5.8S/28S rRNA complex formation displays a more defined temperature optimum than formation of the 3′ 5.8S/28S rRNA complex, neither is as sharp as that observed for the intact 5.8S/28S rRNA complex (Pace et al., 1977). The measurable formation of the 3′ 5.8S/28S and 5′ 5.8S/28S rRNA complexes at relatively low temperatures (i.e., less than 50 °C) as compared to the negligible formation of 5.8S/28S rRNA complex at similar temperatures (Pace et al., 1977) suggests that the half-molecules offer fewer structural barriers to complex formation than does 5.8S rRNA. This presumably means that the 5.8S rRNA undergoes a temperature-dependent conformational change in order to associate with 28S rRNA, involving, at least in part, disruption of interactions between the 5′ and 3′ halves of 5.8S rRNA.

The thermal stabilities of the 5′ 5.8S/28S rRNA and 3′ 5.8S/28S rRNA complexes were compared to that of the 5.8S/28S rRNA complex. Aliquots of nonradioactive 28S rRNA, complexed with either ^{32}P-labeled 5′ 5.8S or 3′ 5.8S half-molecule or ^{32}P-labeled 5.8S rRNA in 0.1 M NaCl, were incubated at various temperatures and the products analyzed by gel electrophoresis as above. As shown in Figure 4B, the 5′ 5.8S/28S rRNA complex shows a simple, monophasic denaturation profile, superimposable on the initial portion of the 5.8S/28S rRNA thermal denaturation profile. The 3′ 5.8S/28S rRNA complex, however, displays a biphasic denaturation profile. This suggests two different stability populations of 3′ 5.8S/28S rRNA complexes, similar to the phenomenon observed for both the native 5.8S/28S rRNA complex from mouse L cells (Pace et al., 1977) and for the reconstituted 5.8S/28S rRNA complex. The biphasic nature of the thermal denaturation profile of the 3′ 5.8S/28S rRNA complex probably cannot be explained as two different binding sites on 28S rRNA for the 3′ half-molecule. Since the complex was formed in the presence of a more than 3-fold excess of 28S rRNA over the fragment, if there were two sites on the

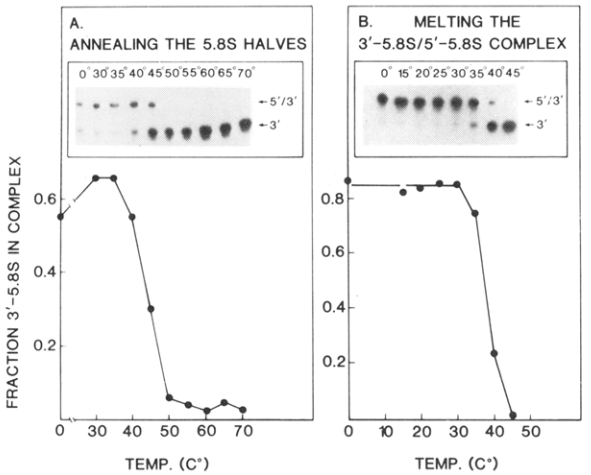

FIGURE 3: Reassociation and dissociation of the 5.8S rRNA halves. (A) Thermal dependence for annealing the 5.8S rRNA halves. Identical aliquots of 5′-^{32}P-labeled 3′ 5.8S fragment (9 ng) and nonradioactive 5′ 5.8S fragment (9 ng) were incubated for 2 min at 80 °C, chilled on ice, and adjusted to 0.4 M NaCl in a final volume of 10 μL. The samples were sealed into capillary tubes, held at the indicated temperatures for 30 min, and then quenched with 20 μL of ice-cold 5% sucrose containing 45 ng of the nonradioactive 3′ 5.8S fragment. Products were resolved by electrophoresis at 4 °C through an 8% polyacrylamide slab gel. The inset is an autoradiogram of the dried gel, from which bands were excised and monitored for ^{32}P content, as plotted. (B) Thermal dissociation of the 3′ 5.8S/5′ 5.8S rRNA complex. An aqueous solution of the 5′ ^{32}P-labeled 3′ 5.8S fragment (81 ng) and nonradioactive 5′ 5.8S fragment (108 ng) was incubated for 2 min at 80 °C, chilled in an ice bath, and made 0.4 M in NaCl. The complex of the halves was formed by incubating this 45-μL reaction at 30 °C for 1 h, chilling in an ice bath, and then diluting to a final NaCl concentration of 0.1 M. This was then divided into eight 25-μL aliquots. Following 2-min incubations at the indicated temperatures, 10 μL of ice-cold 15% sucrose containing 45 ng of the nonradioactive 3′ 5.8S fragment was added to each. Products were resolved and characterized as described in (A).

28S rRNA with different affinities for the 3′ 5.8S half, most of the fragment should bind to the site with higher affinity. This is not the case. We suggest (Discussion) that the biphasic dissociation curves for the native complex and the 3′ 5.8S/28S complex are due to two alternate conformational states involving the contacts between the 3′ half of the 5.8S and the 28S rRNAs.

We (Pace et al., 1977) and others (Sitz et al., 1978) have noted that 5.8S rRNA, under the appropriate annealing conditions, readily forms dimers. Figure 4 shows that the isolated 5′ 5.8S half also forms dimers, so the contacts resulting in the dimerization evidently do not involve the 3′ end of the molecule, as suggested by Sitz et al. (1978). We do not know the nature of the pairings which result in the dimerization; several imperfect, dimeric helices are theoretically possible with the nucleotide sequence of the 5′ 5.8S half. We do not attribute any functional significance to these dimers, since they are observed at higher temperatures than required to effect the 5.8S/28S reassociations and only one 5.8S rRNA molecule is present in each ribosome.

Dissociation Constants for the 5′ 5.8S/28S rRNA, 3′ 5.8S/28S rRNA, and 5.8S/28S rRNA Complexes. The relative affinities of 5.8S rRNA and the two half-molecules for 28S rRNA were quantitated. The dissociation constants for a bimolecular interaction such as 5.8S binding to 28S rRNA normally would be evaluated from saturation binding experiments, with a constant amount of 28S rRNA. Such data yield not only the dissociation constant for the 5.8S/28S rRNA complex but also the number of sites on the 28S rRNA that bind 5.8S rRNA. This approach was not useful in measuring the dissociation constants for the 5.8S/28S rRNA complex and the 5′ 5.8S/28S rRNA complex because at the concentrations of 5.8S rRNA or the 5′ half required to saturate the 28S rRNA, both form appreciable amounts of dimers and higher order aggregates. The dissociation constants for the complexes therefore were determined in a converse manner, by saturating constant amounts of the labeled 5.8S, 5′ 5.8S half, or 3′ 5.8S half with 28S rRNA. Following the annealings as detailed in the legend to Figure 5, the products were resolved by gel electrophoresis and quantitated as above. The data are presented as Scatchard plots (Figure 5); the intercepts indicate the number of binding sites and the dissociation constants. The data for the 5.8S/28S rRNA complex indicate that the 5.8S rRNA will accommodate 1.1 molecules of 28S rRNA, and the dissociation constant for the complex is about 9×10^{-8} M. There is 0.9 binding site on the 5′ half-molecule for 28S rRNA, and the dissociation constant for the complex is ca. 6×10^{-8} M. There is 0.9 binding site on the 3′ half for 28S rRNA, and the dissociation constant for the complex is ca. 13×10^{-8} M.

Since the 3′ half-molecules do not aggregate under standard annealing conditions, a corroborating experiment was carried out, incubating a constant amount of 28S rRNA with various amounts of the ^{32}P-labeled 3′ half of 5.8S rRNA. These results are included in Figure 5A; the analysis indicates one tight binding site for the 3′ half-molecule on 28S rRNA, with a K_d of about the same value as obtained from the experiments designed to saturate the 3′ half-molecule with 28S rRNA. One tight binding site per 28S rRNA molecule indicates that the preparation of 28S rRNA is completely active, at least with respect to binding the 3′ half-molecule.

Competition for 28S rRNA between Intact 5.8S rRNA and the Fragments. The affinities of both the 5′ and the 3′ half-molecules for 28S rRNA are fairly strong, and each is comparable to the affinity of 5.8S RNA for 28S RNA. This does not, however, indicate that the binding of the half-molecules is specific, i.e, that they each bind 28S rRNA at the same site as 5.8S rRNA. Given the size of 28S rRNA and the fact that we do not know the exact nature of the complexes, it is conceivable that the half-molecules could bind to the 28S rRNA at sites that have nothing to do with the specific interaction of 5.8S rRNA with 28S rRNA. We therefore sought to evaluate the specificity of binding in a more direct way. The labeled half-molecules were incubated under standard annealing conditions with either nonradioactive 28S rRNA or nonradioactive 5.8S/28S rRNA native complex, with or without prior denaturation. Aliquots were removed at various times and the products resolved and quantitated as above. If the half-molecules bind 28S rRNA at the same sites as 5.8S rRNA, then 28S rRNA with a resident 5.8S rRNA should show a decreased rate of fragment binding relative to 28S rRNA free of 5.8S rRNA, and the reassociation of the fragments should be retarded in the presence of unbound, intact 5.8S rRNA. The data are shown in Figure 6.

The results for the binding of the 3′ 5.8S fragment were as anticipated (Figure 6A); the fragment bound well to 28S rRNA and poorly to the 5.8S/28S rRNA complex, and binding was diminished by the presence of intact, unassociated 5.8S rRNA. Moreover, the concentration of the 3′ 5.8S/28S complex formed with 28S rRNA alone, in comparison to that formed in the presence of 5.8S rRNA, was about that expected, based on the affinities of the 3′ 5.8S half and the 5.8S rRNA for 28S rRNA and the concentrations of the RNAs. From these results we conclude that the 3′ half-molecule binds 28S rRNA at the same site that 5.8S rRNA binds.

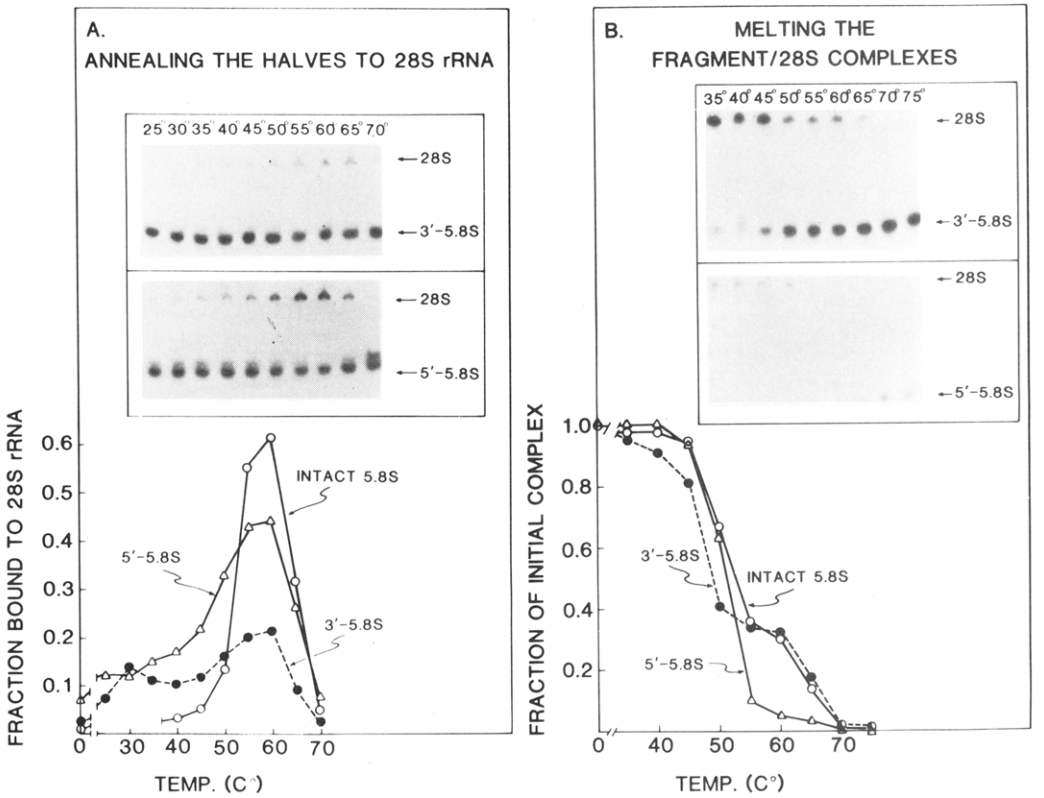

FIGURE 4: Association and dissociation of the 5.8S fragment/28S rRNA complexes. (A) Thermal dependence for formation of the complexes. 12 pmol of uniformly ^{32}P-labeled 5′ 5.8S, 3′ 5.8S, or intact 5.8S rRNA in 63 μL of H_2O was held at 80 °C for 5 min, chilled, and adjusted to 0.4 M NaCl and 0.2 μM 28S rRNA (38 μg) in 115 μL. 10-μL aliquots sealed in capillaries were held at the indicated temperatures for 15 min and then rinsed into 30 μL of 5% sucrose/0.6 mM EDTA. The products were resolved by electrophoresis through a 3% polyacrylamide/0.25% agarose slab gel and monitored as described in the legend to Figure 3. (B) Thermal denaturation of the complexes. Complexes were formed by incubating uniformly ^{32}P-labeled fragments or intact 5.8S rRNA with 28S rRNA in 0.4 M NaCl at 50 °C for 1 h in sealed capillaries. The amounts of materials used were 0.35 μg of 3′ 5.8S rRNA and 86 μg of 28S rRNA per 55 μL, 0.12 μg of 5′ 5.8S rRNA and 32 μg of 28S rRNA per 30 μL, and 0.18 μg of intact 5.8S rRNA and 29 μg of 28S rRNA per 30 μL. Following annealing, aliquots were made 0.1 M in NaCl, incubated in sealed capillaries for 2 min at the indicated temperature, and chilled on ice, and the products were analyzed by gel electrophoresis as above.

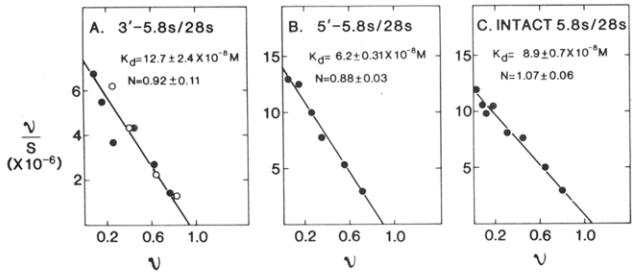

FIGURE 5: Dissociation constants for the fragment and intact complexes. (A) Identical aliquots of the uniformly ^{32}P-labeled 3′ 5.8S fragment (0.032 μg) were incubated 2 min at 80 °C and immediately chilled in an ice bath. NaCl and different amounts of 28S rRNA were added to each aliquot to make 10-mL reactions that were 0.4 M in NaCl. These reactions were heated to 55 °C for 2 h in sealed capillaries and then chilled in an ice bath, made 5% in sucrose, and analyzed by gel electrophoresis as described in the legend to Figure 3. These data are presented as a Scatchard plot with ν representing the fraction of 3′ 5.8S rRNA in complex with 28S rRNA and S designating the concentration of uncomplexed 28S rRNA (filled circles). The line represents a least-squares fit of the data. In a similar experiment, identical aliquots of 2 μg each of 28S rRNA were incubated with different amounts of the ^{32}P-labeled 3′ half-molecule. In these data ν is the fraction of 28S in complex with the 3′ half-molecule, and S is the concentration of the uncomplexed 3′ half-molecule (open circles). (B and C) Identical aliquots of the ^{32}P-labeled 5′ 5.8S fragment (0.048 μg) or 5.8S rRNA (0.046 μg) were treated as outlined above in 20-μL reactions. ν is the fracton of 5′ 5.8S (part B) or 5.8S rRNA (part C) in complex with 28S rRNA, and S is the concentration of uncomplexed 28S rRNA. The lines represent least-squares fits of the data. For all figures, N is the number of binding sites on the fragments or intact 5.8S rRNA for 28S rRNA, and K_d is the dissociation constant for the appropriate complex.

The outcome of experiments with the 5′ 5.8S fragment was not so straightforward (Figure 6B). As with the 3′ half, the 5′ half of the 5.8S rRNA annealed rapidly to 28S rRNA, but it exchanged only slowly with the 5.8S/28S complex. This is consistent with the notion that the bound 5.8S rRNA in the native complex blocks the site that binds the 5′ 5.8S fragment. However, the binding of the 5′ 5.8S half was not competitively inhibited by free, intact 5.8S rRNA present in the denatured 5.8S/28S rRNA complex. This would seem inconsistent with the occupation by the intact 5.8S and 5′ half of the same site on the 28S rRNA, but other interpretations are equally pertinent. For example, some of the 5′ 5.8S fragment may form dimers (above) with other 5′ fragments or the intact 5.8S rRNA and be carried into complex with the 28S rRNA by that presumably artifactitious association. We therefore feel that the more relevant observation is that the native 5.8S/28S rRNA complex binds the 5′ 5.8S fragment only slowly, presumably through exchange with resident, intact 5.8S rRNA, rather than rapidly, by occupying a distinct site.

Discussion

On the basis of several observations, we have suggested previously that ca. 20 residues at both the 5′ and 3′ ends of the 5.8S rRNA are associated with the 28S rRNA (Walker et al., 1982; Pace et al., 1977). These observations are (1) the 5′ and 3′ ends of the 5.8S rRNA are rendered resistant to single-strand-specific nucleases and chemical modifying agents upon binding 28S rRNA, (2) the 3′-terminal segment of 5.8S rRNA is isolatable as a nuclease-resistant complex with an unidentified segment of 28S rRNA (Pace et al., 1977), and

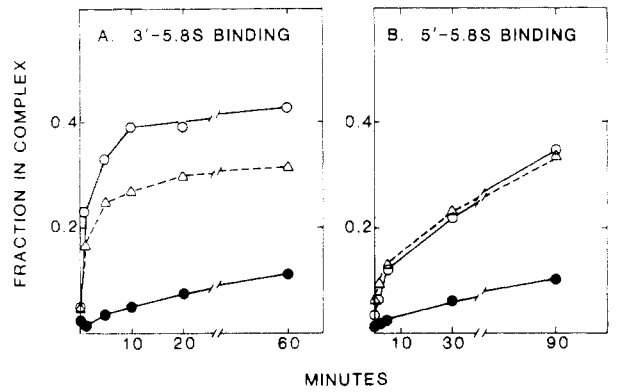

FIGURE 6: Binding of fragments to intact and denatured 5.8S/28S rRNA complex. (A) Three annealing reactions were prepared, each 0.4 M NaCl and 0.01 μg/μL in 5'-^{32}P-labeled 3' 5.8S fragment. One contained 0.67 μg/μL 5.8S/28S rRNA native complex (closed circles), a second contained 0.67 μg/μL 5.8S/28S complex which had been heated to 80 °C for 2 min prior to addition to the annealing reaction (open triangles), and the third reaction contained 0.66 μg/μL 28S rRNA (free of 5.8S rRNA) (open circles). The molar ratios of the 3' 5.8S fragment:native or denatured complex or 28S rRNA was 1:1. Each reaction was split into six aliquots and incubated at 55 °C in sealed capillaries for the indicated period of time. They then were chilled, made 5% in sucrose, and resolved by gel electrophoresis as above. (B) Three annealing reactions were prepared and treated as described above by using 0.006 μg/μL 5'-^{32}P-labeled 5' 5.8S fragment. Two of the reactions contained 0.4 μg/μL 5.8S/28S rRNA (closed circles), one of which was heated (open triangles), and the third reaction contained 0.36 μg/μL 28S rRNA (open circles).

(3) nucleotide sequences in the bacterial 23S rRNA which are homologous to the 5' and 3' termini of 5.8S rRNA are complementary with 23S sequences homologous to the 28S rRNA (Noller et al., 1981). The experiments reported here are consistent with an expectation from this model that the 5' and 3' halves of 5.8S rRNA should be capable of independently associating with the 28S rRNA. The results do not address the exact nature and extent of the contacts. The binding constants of the 5' and 3' fragments are about the same as that of the intact 5.8S rRNA for 28S rRNA, so the binding sites act independently rather than cooperatively under the annealing conditions. Quantitatively, the measured K_d values for the halves are consistent with the postulate (Walker et al., 1982) that the 5.8S rRNA termini form short, irregular helices with the 28S rRNA, with binding energies of ca. −40 to −50 kJ/mol under the annealing conditions. We do not yet have the appropriate sequences of the mouse 28S rRNA, but the theoretical stability (Salser, 1977) of the suggested pairing (Walker et al., 1982) between the 3' end of *Xenopus* 5.8S rRNA and the 5' end of the 28S rRNA would be substantially higher than that (ca. −70 kJ/mol) under the annealing conditions. However, because of the extrapolations required for these comparisons, the fact that the annealings are carried out under near-denaturing conditions, and our ignorance of the detailed structure of the complex, we do not consider this discrepancy serious.

Although the temperature optima for binding the fragments and the intact 5.8S rRNA to the 28S rRNA are similar (ca. 55–60 °C), it is interesting that the fragments are capable of the association at relatively low temperatures (less than 50 °C). This suggests that neither fragment must undergo substantial conformational change to accommodate the 28S rRNA. This contrasts with the intact 5.8S rRNA, which strictly requires the rather high temperatures (55–60 °C) for binding. Presumably, then, competing intramolecular interactions involving the 5' and 3' halves of the 5.8S rRNA preclude their ready association with the 28S rRNA. Possibly the competing folding involves irregular pairing between the 5'- and 3'-terminal segments that normally bind the 28S rRNA. Although the complementarity is not good, such pairings have been proposed (Nazar et al., 1975; Ford & Mathieson, 1978; Luoma & Marshall, 1978; Khan & Maden, 1976). This offers an interesting problem in ribosome biosynthesis, since during transcription, the 5.8S rRNA sequence is completed several hundred residues before the RNA polymerase enters the 28S rRNA gene. Thus, there may exist some mechanism to potentiate the 5.8S/28S rRNA association, perhaps by preventing the 5.8S rRNA from adopting the incorrect conformation. A ribosomal protein, for example, may do this. Or, a precursor-specific RNA sequence in the primary rDNA transcript might interact with part of the 5.8S rRNA, to preclude the abnormal folding. We have documented such a possibility previously, with the finding that a precursor-specific sequence may substantially alter the mature domain of a bacterial 5S rRNA precursor, unless prevented from doing so by another precursor-specific sequence (Stahl et al., 1979).

Comparison of the dissociation curves of the intact 5.8S and the half-molecules from the 28S rRNA hint that the 3' half of the 5.8S rRNA, when bound to the 28S rRNA, may be involved in two metastable conformations. The thermal denaturation of native or reassociated 5.8S/28S rRNA complexes is biphasic; about 65% of the population dissociates at ca. 50–53 °C and the remainder at ca. 65 °C. Since release of the 5.8S rRNA is measured, this must mean that the 5.8S rRNA is associated in two different conformational states, of different stabilities. We do not know whether these different stability forms have functional significance. They may be artifacts of extraction or handling, but conceivably they are the alternative forms of a conformational switch involving the 5.8S and 28S rRNAs. The reasons for the biphasic character of the 5.8S/28S rRNA dissociation probably reside in contacts in the 3' half of the 5.8S rRNA. In the thermal dissociations of the fragment 28S rRNA complexes, the 5' 5.8S/28S T_m was found to be monophasic and the same as that of the less stable native complex component, but the 3' 5.8S/28S dissociation is biphasic, with the higher T_m the same as that of the more stable population of native complexes. The fraction of less stable 3' 5.8S/28S complexes is the same as the fraction of less stable native complexes, and the fragment complex dissociates with a lower T_m than the native complex. In the simplest interpretation, then, the T_m of the low stability native complexes would be defined by the 5' 5.8S contacts, with the 3' 5.8S contacts occupying their less stable conformational state. The native complexes with the more stable 3' 5.8S contacts would have the higher T_m. If, during function, the 3' 5.8S contacts alternate between the low and higher stability states, the switching mechanism likely does not involve the 5' half of the 5.8S rRNA, since the isolated 3' half is capable of entering the two types of complexes with the 28S rRNA.

The two stability states of the 5.8S rRNA in complex with the 28S rRNA very possibly involve arm IV (Figure 1). The 28S rRNA certainly interacts with arm IV (Figure 1); residues in the center of that very stable duplex stalk are sensitive to nuclease digestions in the complex, but not in the free 5.8S rRNA (Walker et al., 1982). The two alternate stability states might be explained, for example, by the alignment, in contrast to the displacement, of arm IV from coaxiality with the postulated imperfect duplex between residues 137–156 and the 28S rRNA. In coaxial alignment, arm IV would stabilize, by stacking forces, the adjacent weak helix. The displacement of arm IV from coaxiality would weaken the 3' 5.8S contact with the 28S rRNA. Again, there is no evidence that such

a conformational switch has any role in ribosome function. In this regard, however, it would be interesting to inspect the dissociation properties of the 5.8S fragment/28S rRNA complexes from other organisms, with diverse sequences.

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Nucleotide Sequence of *Dictyostelium discoideum* 5.8S Ribosomal Ribonucleic Acid: Evolutionary and Secondary Structural Implications†

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ABSTRACT: We have determined the nucleotide sequence of the *Dictyostelium discoideum* 5.8S ribosomal RNA (rRNA). The sequence has relatively low homology with other 5.8S rRNAs and is further distinguished by its lack of modified nucleotides. The homology data indicate that *D. discoideum* diverged from the mainstream of eukaryotic descent at the earliest branch yet characterized by molecular phylogeny. Taking advantage of the considerable divergence of the *D. discoideum* 5.8S rRNA sequence from those of other eukaryotes, we have concluded that there is very little phylogenetically conserved, intramolecular secondary structure. This conclusion is discussed in the light of the variety of evidence for a highly ordered structure of 5.8S rRNA in vitro. We also offer comparative evidence in support of a specific model for the base pairing between the 3′ end of 5.8S rRNA and the 5′ end of eukaryotic 28S rRNA.

The large subunit of most eukaryotic ribosomes contains the 28S, 5S, and 5.8S rRNAs. When isolated under nondenaturing conditions, the 5.8S rRNA is found associated with the 28S rRNA (Pene et al., 1968). Chemical modification and enzymatic susceptibility measurements have been used with limited success to probe the secondary structure of the 5.8S rRNA (Walker et al., 1982; Nazar et al., 1975; Pace et al., 1977; Kelly & Maden, 1980; Wildeman & Nazar, 1981) and its association with the 28S rRNA (Walker et al., 1982; Pace et al., 1977). A complementary method of secondary structure analysis involves comparison of the potential base pairings of homologous RNAs from a variety of organisms. This approach has been successful in establishing universal secondary structures for tRNAs (e.g., Rich & RajBhandary, 1976), 5S rRNAs (Fox & Woese, 1975), and eubacterial 16S (Woese et al., 1980) and 23S rRNAs (Glotz et al., 1981; Noller et al., 1981). However, a lack of sequence diversity has limited the effectiveness of a similar comparative analysis of 5.8S rRNAs.

Molecular phylogeny (Zuckerkandl & Pauling, 1965) indicates that the cellular slime mold *Dictyostelium discoideum* is the deepest known branch within the eukaryotic kingdom. This is based upon the sequences of the 5S rRNA (Hori et

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