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Structure of the 5.8S RNA Component of the 5.8S–28S Ribosomal RNA Junction Complex†

Norman R. Pace,* Thomas A. Walker, and Ellen Schroeder

ABSTRACT: The 5.8S ribosomal RNA of mouse L-cells is dissociable from 28S rRNA by treatment with agents which disrupt hydrogen bonding. The 5.8S-28S rRNA association is restored by appropriate annealing procedures, to yield a complex with thermal denaturation properties identical with those of the native 5.8S-28S rRNA complex. Ribonuclease digestion of the artificial 5.8S-28S rRNA complex, radioactive only in the 5.8S rRNA moiety, yields a fragment not released from uncomplexed 5.8S rRNA. This 5.8S-derived fragment has the properties expected of the 5.8S-28S rRNA junction complex, including ribonuclease resistance, a sharply temperature-dependent reduction in apparent size, and the ability of denatured fragments to reanneal with 28S but not 18S rRNA. The 5.8S-28S rRNA junction complex is shown to include the 3′-terminal 42-43 nucleotides of the 5.8S rRNA molecule; however, it is proposed that only the 3′-terminal 20-21 nucleotides are associated by complementary base interaction with the 28S rRNA. The residuum (21-22 nucleotides) of the 5.8S rRNA component of the junction complex is envisaged to form an intramolecular hairpin which stacks upon and hence stabilizes the RNA double helix formed by the 5.8S-28S association.

It is becoming evident that specific RNA-RNA interactions are of paramount importance to the machinations of the protein-synthesizing apparatus. For example, in prokaryotes 16S ribosomal RNA (rRNA) appears to assist in the binding of mRNA to the ribosome (Shine and Dalgarno, 1971; Steitz and Jakes, 1975); 5S rRNA may be involved in the placement and/or displacement of the tRNA molecules from their sites on the ribosome surface (Erdmann et al., 1973); and structural transitions in the tRNA-mRNA complex have been proposed to provide the essence of the translocation process (Woese, 1970). An example of an RNA-RNA interaction which as yet has no known role is the 5.8S-28S rRNA complex which exists in the ribosomes of eukaryotes (Pene et al., 1968). The 5.8S rRNA, which is about 160 nucleotides in length (Nazar et al., 1975), is rather stably associated along a portion of its length with the 28S rRNA, apparently through hydrogen bonding. The 5.8S-28S rRNA complex is dissociated by heat or urea (Pene et al., 1968), and may be reformed from its constituent

† From the National Jewish Hospital and Research Center, and the Department of Biophysics and Genetics, University of Colorado Medical Center, Denver, Colorado 80206. *Received June 1, 1977.* This work was supported by National Institute of General Medical Sciences Grants GM20147 and GM22367, and Career Development Award No. 1-K04-GM00189 to N.R.P.

RNA molecules under appropriate annealing conditions (see below). As one component of a study of the structure and function of the 5.8S–28S rRNA complex, we here define that portion of the mouse 5.8S rRNA nucleotide sequence which is associated with 28S rRNA.

Materials and Methods

(1) *Growth and Labeling of Cells.* Mouse embryo fibroblasts (L-929 strain) were grown in suspension in Eagle's minimal essential medium (MEM)[1] supplemented with 5% calf serum and 0.05% methylcellulose. To isotopically label total cellular RNA, cells were suspended at a density of 5×10^5/mL in phosphate-free MEM containing 0.15% $NaHCO_3$, 0.05% methylcellulose, 0.02 M Hepes, and 5% dialyzed fetal calf serum. Carrier-free $H_3{}^{32}PO_4$, which had been purified by chromatography on a Dowex-1 column immediately before use, was added to a concentration of 200 μCi/mL. After 48 h of incubation at 37 °C, the cells were collected by centrifugation, washed twice in a buffer containing 0.01 M Tris-HCl (pH 7.4), 0.15 M NH_4Cl, 0.002 M $MgCl_2$, and 0.002 M $CaCl_2$, and suspended in 0.05 M Tris-HCl (pH 7.4), 0.1 M NaCl, and 0.001 M EDTA (STE).

(2) *Extraction and Purification of RNA.* RNA was extracted from cells by shaking twice with STE-saturated phenol and then precipitated from 75% ethanol. Total RNA was fractionated by sucrose gradient sedimentation as described previously (Walker and Pace, 1977). The 5.8S rRNA was released from purified 28S rRNA by heating for 2 min at 65 °C in 0.01 M Tris-HCl (pH 7.4) and 0.001 M EDTA, followed by rapid cooling to 0 °C. The solution was once again submitted to sucrose gradient sedimentation. The low molecular weight material containing 5.8S rRNA was collected from the top of the sucrose gradient and purified further by electrophoresis through, and elution from, an 8% polyacrylamide gel (Walker et al., 1974).

(3) *Melting and Reannealing of RNA–RNA Complexes.* Samples of native or artificial 5.8S rRNA complex to be denatured prior to analysis were precipitated from ethanol, dried in vacuo, dissolved in a small volume of 0.003 M EDTA (pH 7.3), and then held in an 80 °C water bath for 1 min. Following rapid chilling in an ice bath, the sample was minipulated as desired. In some experiments it was useful to monitor the dependence of melting upon temperature. In these cases, ethanol precipitates were dissolved in 0.1 M NaCl, 0.003 M EDTA (pH 7.3), and aliquots were sealed into capillary pipettes. The sealed samples were held at indicated temperatures in a Haake circulating water bath for 2 min, chilled in an ice bath, and then subjected to appropriate polyacrylamide gel electrophoresis for separation of products. The products of 5.8S–28S complex denaturation were resolved by electrophoresis through a 3.5% polyacrylamide gel (acrylamide-bis(acrylamide), 20:1 in buffer E (Loening, 1967)); the products of fragment I complex denaturation were resolved in a 15% gel (acrylamide-bis(acrylamide), 100:1 in buffer E) with a short stacking gel of 6% polyacrylamide (acrylamide-bis(acrylamide), 100:1) in one-half concentrated buffer E. After electrophoresis at approximately 8 V/cm for the appropriate time, analytical gels were dried in vacuo in a boiling water bath.

The optimum temperature for annealing 5.8S rRNA with 28S rRNA, which had been stripped of resident 5.8S rRNA, was determined to be about 58 °C in 0.4 M NaCl, 0.003 M EDTA (pH 7.3) (see Results section); these conditions therefore generally were employed. In the case of annealing denatured fragment I to 28S rRNA, incubation was at 50 °C instead of 58 °C. This temperature was chosen because the fragment I T_m is 6–7 °C less than that of the native 5.8S–28S rRNA complex. Annealing mixtures generally were incubated for 1 h, although, at the RNA concentrations employed, the reactions were essentially complete within 10 min. Any samples to be analyzed by gel electrophoresis subsequent to annealing were diluted fourfold with 0.003 M EDTA (pH 7.3) prior to application to polyacrylamide gels.

(4) *Preparation of the 5.8S–28S rRNA "Junction Complex".* Purified ^{32}P-labeled 5.8S rRNA (5–10 μg) of 1×10^6 to 4×10^6 cpm per μg of RNA and 500 μg of nonradioactive 28S rRNA (stripped of resident 5.8S as described above) were mixed, precipitated from ethanol, and dissolved in 108 μL of 0.003 M EDTA (pH 7.3). After thorough mixing to ensure that all RNA was in solution, 12 μL of 4 M NaCl was added and the mixture was sealed in a capillary and held at 58 °C for 1 h. Following incubation the sample was cooled in an ice bath and then added to a polystyrene tube containing 13 μL of 4 M NaCl (i.e., to a final NaCl concentration of about 0.8 M). RNase T1 (15 μL) at 200 μg/mL or a mixture of RNase T1 and RNase A at 100 μg each per mL was added; then the sample was held at 0 °C for 30 min. Following this digestion, the sample was diluted threefold with 0.2% $NaDodSO_4$ and immediately layered over a 15% polyacrylamide (acrylamide-bis(acrylamide), 20:1) slab gel containing buffer E with a 0.5-cm 6% polyacrylamide (acrylamide-bis(acrylamide), 100:1) stacking gel containing one-half concentrated buffer E. Electrophoresis was carried out for 24 h in the cold at 4 V/cm; bands were located by radioautography and excised from the gel. The gel segment containing fragment I was pulverized by grinding with a glass rod and then eluted twice by overnight shaking in the cold in 1 M sodium acetate, 0.003 M EDTA, 0.05 M Tris-HCl (pH 7.3). Gel fragments were removed by filtration through tightly packed glass wool. If the isolated fragment I was to be manipulated in denatured form, $MgCl_2$ to 0.01 M and ethanol to 75% were added and the precipitated RNA was recovered by centrifugation following chilling to −20 °C for several hours. We find that addition of Mg^{2+} is required for good recovery of the fragment from ethanol. However, ethanol precipitation also denatures substantial amounts of the complex population; ethanol is known to destabilize base stacking interaction (Levine et al., 1963). Therefore, if the intact complex was desired, the eluted fragment I was exchanged into the appropriate buffer by passage through a 0.7 × 20 cm Sephadex G-50 column equilibrated with that buffer.

(5) *Two-Dimensional Fingerprint Analysis.* Complete digests of RNA by RNase T1 were analyzed by two-dimensional electrophoresis according to Sanger and Brownlee (1967) with the following modifications in buffers. The first-dimension electrophoresis was carried out on cellulose acetate strips (Schleicher and Schuell) saturated with 0.3 M ammonium formate (pH 3.5), 7 M urea, 1 mM EDTA. The electrode buffer for the first dimension was 0.4 M ammonium formate, 1 mM EDTA and electrophoresis was carried out at 80 V/cm for about 70 min. In the second dimension the buffer consisted of either 7% formic acid or 0.1 M pyridine, 5% formic acid (pH 2.3). This latter buffer is superior to 7% formic acid in that resolution within graticules is improved and, because of the higher salt concentration in the pyridine-formate system, large

[1] Abbreviations used are: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid, disodium salt; $NaDodSO_4$, sodium dodecyl sulfate; DEAE-cellulose, diethylaminoethylcellulose; Tris, tris(hydroxymethyl)aminomethane; STE, 0.1 M NaCl, 0.001 M EDTA, 0.05 M Tris-HCl (pH 7.4); buffer E, 0.02 M sodium acetate, 0.001 M EDTA, 0.04 M Tris-acetate (pH 7.2); fragment I, the ribonuclease-resistant component of the 5.8S–28S rRNA junction complex; MEM, Eagle's minimal essential medium.

oligomers migrate more rapidly in the DEAE paper. Following electrophoresis, oligonucleotides were detected by radioautography and then excised and monitored for radioactivity. Secondary characterizations of selected oligonucleotides were carried out by completely digesting the oligonucleotides on paper with RNase A (50 μg/mL) for 3 h at 37 °C and resolving the products by electrophoresis on DEAE paper in 0.5% pyridine, 5% acetic acid (pH 3.5).

Results

Reconstitution and Characterization of Artificial 5.8S–28S rRNA Complex. If 5.8S rRNA is associated with 28S rRNA via hydrogen bonds along a portion of its length, it is to be anticipated that the bound segment would be resistant to digestion by ribonuclease. At the outset of this investigation we attempted to recover from native, ^{32}P-labeled 5.8S–28S rRNA complex a ribonuclease-resistant segment not present in ^{32}P-labeled 28S rRNA which had been stripped of 5.8S rRNA by heat denaturation. This approach proved unsuccessful, however; the plethora of fragments released by RNase from ^{32}P-labeled 28S rRNA, which is 30-fold larger than the resident ^{32}P-labeled 5.8S rRNA, obscured the region of polyacrylamide gels which in retrospect contained the complex of interest. Recourse, therefore, was had to reconstituting the 5.8S–28S rRNA complex with ^{32}P-labeled 5.8S and nonradioactive 28S rRNA, and then submitting this artificial complex to RNase digestion; any radioactivity released by the RNase must derive from the 5.8S molecule.

The reassociation of denatured 5.8S–28S rRNA complex has previously been reported (Sy and McCarty, 1971), although the fidelity of the process was somewhat suspect; the 5.8S rRNA was observed to anneal extensively to 18S as well as 28S rRNA. During our characterizations of the reannealing of mouse 5.8S and 28S rRNA, we noted essentially no annealing of 5.8S rRNA with carefully purified 18S rRNA under the reconstitution conditions employed. It, therefore, seems likely that the previously observed association of 5.8S with 18S rRNA was due to significant contamination of the 18S rRNA with fragmented 28S rRNA.

The temperature dependence of re-formation of the 5.8S–28S rRNA complex was evaluated by holding mixtures of ^{32}P-labeled 5.8S rRNA and nonradioactive 28S rRNA (previously stripped of resident 5.8S rRNA as detailed in Materials and Methods) in 0.4 M NaCl, 0.003 M EDTA (pH 7.3), at various temperatures for 1 h. Reaction products then were resolved by electrophoresis through a 2.8% polyacrylamide slab gel, all as described in the legend to Figure 1, and Materials and Methods. Following drying of the gel slab and radioautography (presented in the inset to Figure 1), radioactive bands were excised and their ^{32}P content was determined; the fraction of radioactivity associated with the 28S band as a function of incubation temperature is depicted in Figure 1. Noteworthy are the quite sharp temperature dependence of the annealing process, and the rather high temperature required for complex formation. These observations both suggest that the 5.8S association with 28S rRNA is a specific one, involving a relatively extensive region of hydrogen bonding. Under the same conditions, no association of 5.8S with 18S rRNA was seen (data not shown, but see below). We also observe that at annealing temperatures of 65 °C and above, that 5.8S rRNA enters a structure having a substantially lower electrophoretic mobility than 5.8S rRNA not exposed to such conditions (see inset to Figure 1). This novel structure has approximately the electrophoretic mobility expected for a dimer of 5.8S rRNA, but as well could reflect the

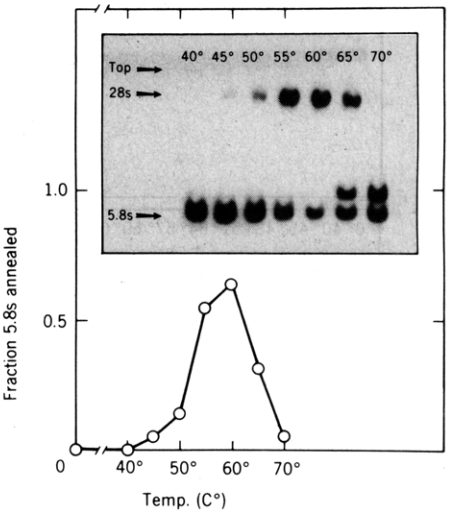

FIGURE 1: Temperature dependence of 5.8S rRNA reassociation with denatured 28S rRNA. Nonradioactive, denatured 28S rRNA at 250 μg/mL and ^{32}P-labeled 5.8S rRNA at 0.75 μg/mL, prepared as detailed in Materials and Methods, were held in sealed capillaries in 0.4 M NaCl, 0.003 M EDTA (pH 7.3), at the indicated temperature for 1 h. Following incubation, the samples were chilled immediately in an ice bath and added to an equal volume of cold E buffer + 0.1% $NaDodSO_4$ containing sucrose crystals. Products were resolved by electrophoresis through a 2.8% polyacrylamide, 0.25% agarose slab gel in E buffer. The inset to the figure is the radioautogram of the dried gel from which bands of radioactivity were excised and monitored for ^{32}P content. The fraction of the total ^{32}P radioactivity located in the 28S band of the gel was determined for each temperature.

assumption by the molecule of a secondary structure with a very much more expanded radius of gyration and hence a lower rate of migration in the polyacrylamide gel. Formation of the structure does not require the presence of 28S rRNA in the annealing mixtures and it is dissipated by heating to 80 °C at low (0.003 M EDTA) but not high (0.4 M NaCl, 0.003 M EDTA) ionic strength. Examination of this structure might yield information bearing on intramolecular secondary and tertiary interactions in the native 5.8S molecule.

It was important in this investigation to seek assurance that the 5.8S–28S rRNA complex which we artificially reassemble is equivalent to that derived from ribosomes. The most significant observation bearing on the identity of the native and artificial 5.8S–28S rRNA complexes is that both exhibit the same temperature dependence of denaturation. This was evaluated, as detailed in the legend to Figure 2, by holding native ^{32}P-labeled 5.8S–28S or artificial ^{32}P-labeled 5.8S–nonradioactive 28S rRNA complexes in 0.1 M NaCl, 0.003 M EDTA at various temperatures, then resolving the products by electrophoresis through a 2.8% polyacrylamide slab gel. Radioautograms of the dried gels are shown in Figures 2A and 2B. Radioactive bands evident on the films were excised and monitored for their ^{32}P content; Figure 2C depicts the fraction of maximum 5.8S rRNA released from 5.8S–28S rRNA complexes vs. incubation temperature. It is evident that both complexes undergo essentially identical thermal transitions, with T_m values of approximately 52 °C. Both the native and artificial complexes display biphasic denaturation curves, indicating some heterogeneity in the 5.8S–28S rRNA populations. Presumably about 20–30% of the 5.8S–28S rRNA complex populations differ slightly from the majority in the molecular details of the 5.8S–28S association, and these details render the complexes slightly more stable to thermal dissociation. Most important, however, is that the native 5.8S–28S rRNA complex and that reconstructed by annealing 5.8S

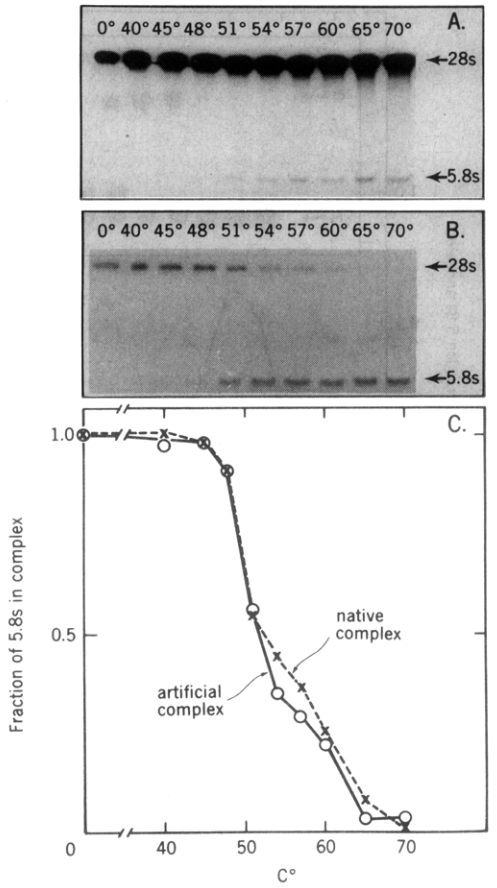

FIGURE 2: Thermal denaturation of native and artificial 5.8S–28S rRNA complexes. Native ^{32}P-labeled 5.8S–28S rRNA complex (125 μg/mL) or ^{32}P-labeled 5.8S rRNA (20 μg/mL) were held under standard annealing conditions in the presence of stripped, nonradioactive 28S rRNA (625 μg/mL). Following quick chilling in an ice bath, the mixtures were diluted with 0.003 M EDTA to a final concentration of 0.1 M NaCl, divided into aliquots, and sealed in capillaries. One aliquot of each was held at the indicated temperatures for 2 min and then added to an equal volume of one-half concentrated E buffer + 0.1% $NaDodSO_4$. The products were resolved by electrophoresis through an 8% polyacrylamide slab gel. Figures 2A and 2B show radioautograms of the dried gels from which bands of radioactivity were excised and monitored for ^{32}P content. The fractions of 5.8S rRNA remaining associated with 28S rRNA were determined for the native complex by defining the maximum fraction of released 5.8S rRNA radioactivity as 1.0 and relating other fractions of released 5.8S rRNA radioactivity to it. The values for the artificial complex are expressed simply as the fraction of the total 5.8S rRNA radioactivity found in the 28S rRNA band at each temperature.

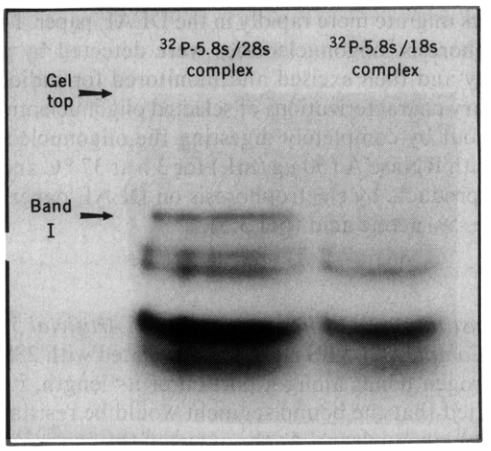

FIGURE 3: Isolation of the ^{32}P-labeled 5.8S–unlabeled 28S rRNA junction complex fragment by polyacrylamide slab gel electrophoresis. As detailed under Materials and Methods, nonradioactive, denatured 28S rRNA or 18S rRNA was held under standard annealing conditions in the presence of ^{32}P-labeled 5.8S rRNA. The NaCl concentration then was adjusted to 0.8 M and RNase T1 digestion was carried out at 0 °C for 30 min. Digestion products were resolved by electrophoresis through a 15% polyacrylamide slab gel. Figure 3 is a radioautogram of such a preparative gel. The fragment indicated as band I, present only in the digestion products of the 5.8S–28S rRNA complex, was excised and eluted from the gel.

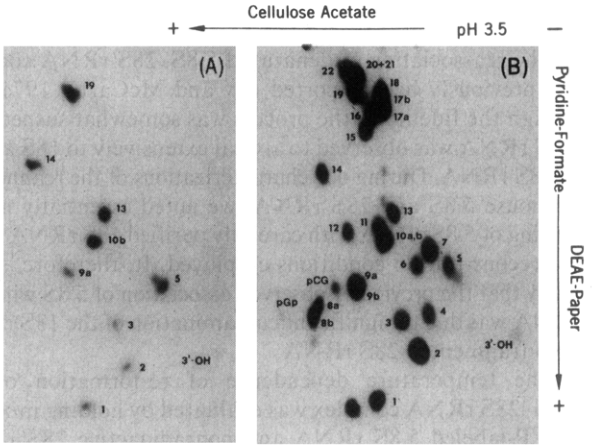

FIGURE 4: Two-dimensional fingerprint analysis of fragment I and 5.8S rRNA. Fragment I was isolated following RNase T1 digestion of the ^{32}P-labeled 5.8S–unlabeled 28S rRNA complex as discussed under Materials and Methods and shown in Figure 3. The fragment then was completely digested with RNase T1 and the resulting oligonucleotides resolved by two-dimensional electrophoresis, also as described under Materials and Methods. Figure 4A presents the radioautogram of the fragment I fingerprint. Figure 4B shows the corresponding fingerprint of intact 5.8S rRNA.

rRNA to 28S rRNA, which had been stripped of its resident 5.8S rRNA, apparently have the same structures; characterization of the mode of association of 5.8S with 28S rRNA in such artificial complexes, therefore, presumably reflects that existing in the native 5.8S–28S rRNA complex and in the ribosome.

Isolation and Characterization of the 5.8S–28S rRNA Junction. The availability of 5.8S–28S rRNA complexes which are radioactive only in the 5.8S moiety permitted the search for the region of association; a ribonuclease-resistant segment recoverable from the 5.8S–28S rRNA complex, but not free 5.8S rRNA, should represent the junction complex. As detailed in Materials and Methods, ^{32}P-labeled 5.8S rRNA was annealed with stripped 28S rRNA, or 18S rRNA as a control, and then digested with RNase T1 at 0 °C in the presence of 0.8 M NaCl. Fragments then were resolved by electrophoresis through a 15% polyacrylamide slab gel and visualized by radioautography, as shown in Figure 3. It is clear that RNase T1 digestion of the 5.8S–28S rRNA complex yields one radioactive band, identified in Figure 3 as band I, which is not generated from free 5.8S rRNA ("annealed" with 18S rRNA). This novel structure was eluted from the appropriate segment of the gel, recovered by precipitation from ethanol, and then digested to completion with RNase T1. Released oligonucleotides were resolved by two-dimensional electrophoresis as described in Materials and Methods; the autoradiogram of the resulting fingerprint is shown in Figure 4A. Figure 4B is an equivalent fingerprint of 5.8S rRNA, for reference. The oligonucleotides were eluted and their molar yields determined. Analyses of the RNase A digestion products of these oligonucleotides, with reference to the nucleotide se-

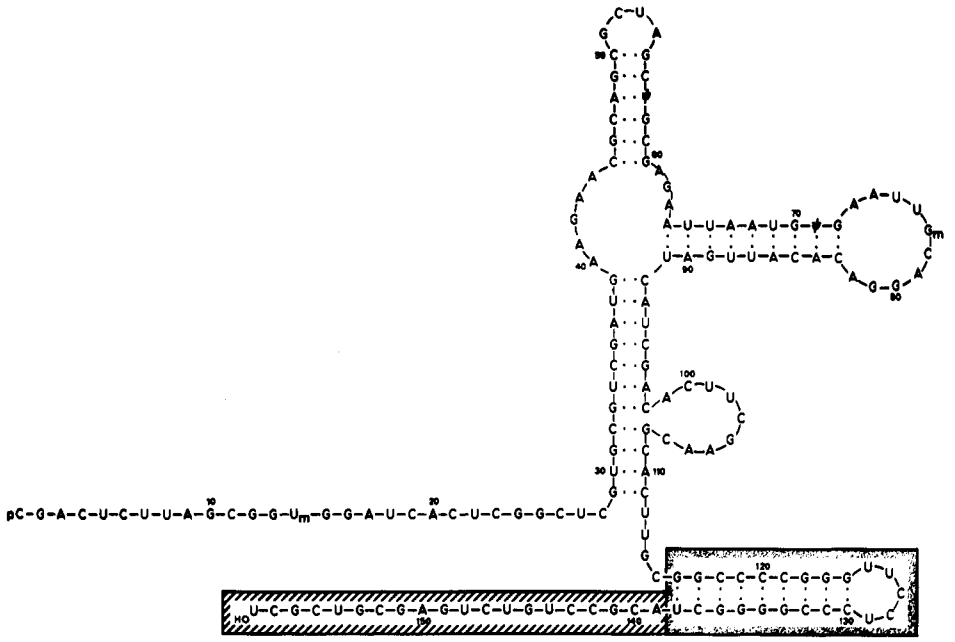

FIGURE 5: A possible secondary structure for mouse 5.8S rRNA. The nucleotide sequence of mouse 5.8S rRNA is drawn in a possible secondary structure. The segment corresponding to fragment I is enclosed within the box. The hatched portion of the box is the region proposed to be associated with 28S rRNA; the stipled portion encloses the intramolecular hairpin which is envisaged to stabilize the 5.8S-28S rRNA junction complex.

TABLE I: RNase T1 Digestion Products of Fragment I.

Oligonucleotide[a]	Sequence[b]	Obsd yield[c]	Expected yield[d]
1	G-	5.4, 5.6, 3.3*, 6.9*	6
2	C-G-	1.2, 1.5, 1.1*, 1.7*	1-2
3	A-G-	0.9, 1.1, 0.8*, 1.2*	1
5	C-C-C-C-G-	0.8, 0.7, 0.2*, 0.8*	1
9a	U-C-G-	1.0, 1.0, 0.9*, 1.1*	1
10b	C-C-U-G-	1.2, 0.6, 1.1*, 1.1*	1
13	C-U-A-C-G-	1, 0.9, 0.9*, 0.9*	1
14	U-C-U-G-	1.1, 1.2, 1.0*, 1.1*	1
19	U-U-C-C-U-C-C-C-G-	1, 0.8, 0.1*, 0.2*	1
3'-OH	C-UOH	0.2, 0.6, n.d., n.d.	1

[a] The numbers refer to oligonucleotides in Figure 4. [b] Oligonucleotides resolved in Figure 4 were excised from the DEAE paper and digested completely with RNase A and the products were analyzed by electrophoresis on DEAE paper at pH 3.5. The sequences given are those uniquely indicated by the resulting analyses, with reference to the 5.8S rRNA sequence. [c] Observed molar yields for four independent preparations of fragment I, including two resulting from RNase T1 digestion of 5.8S-28S rRNA complex and two (denoted by an asterisk) resulting from digestion with RNase T1 plus RNase A. [d] Expected molar yields on the basis of the nucleotide sequence shown in Figure 5.

quence of 5.8S rRNA, indicated the probable nature of the junction complex.

The nucleotide sequence of rat 5.8S rRNA has been determined by Nazar et al. (1975). We find that the 5.8S rRNA of mouse is identical in all respects, including heterogeneity at the 5′ end (pCG or pG). Nazar et al. (1976) also have observed that the mouse 5.8S rRNA is essentially identical with that of rat, although they did not recover pG- 5′ termini. The nucleotide sequence of the 5.8S rRNA molecule is drawn in Figure 5 with complementary regions juxtaposed in a possible secondary structure. The characterization and relative yields of the RNase T1 oligonucleotides from fragment I are summarized in Table I. These oligonucleotides constitute the 42-43 nucleotide, 3′ terminus of the 5.8S molecule, as indicated in the nucleotide sequence of 5.8S rRNA presented in Figure 5. There is some uncertainty regarding the inclusion of G_{116} in the complex; we recover between 1 and 2 mol of C-G- per mol of fragment I. If G_{116} were excluded, the complex should yield only 1 mol of C-G-. Although the fragment I complex contains 42-43 nucleotides of 5.8S rRNA, we propose that only 20-21 of these are associated by complementary base pairing with 28S rRNA. This is because the 21-22 nucleotides residing at the 5′ end of the 5.8S rRNA segment of fragment I contain a self-complementary region and so likely constitute an intramolecular hairpin of importance to the integrity of fragment I (see Discussion section). This hairpin almost certainly exists in free 5.8S rRNA (our unpublished observations). Evidence suggesting that it as well occurs in the 5.8S-28S rRNA junction complex was gained by examination of fragment I following digestion of the 5.8S-28S rRNA complex with RNase T1 plus RNase A. If in fact the hairpin depicted in Figure 5 exists, its apex should be at least partially accessible to RNase A digestion. The result would be a diminution of T1 oligonucleotide 19, and the appearance of the appropriate fragments.

The two-dimensional "fingerprint" of a complete RNase digest of fragment I, prepared as detailed in Materials and Methods by digestion of reconstituted 5.8S-28S rRNA with both RNase T1 and RNase A, is shown in Figure 6. Noteworthy is that oligonucleotide 19 (U-U-C-C-U-C-C-C-G-), the apex of the proposed hairpin, is present in only trace quantities (0.2 and 0.1 mol/mol of fragment I in two independent experiments). Instead, two novel T1 oligonucleotides, indicated in Figure 6 as A and B, are generated. These proved to have the composition of UC_3G (0.8 and 0.9 mol/mol fragment I in two experiments) and UC_4G (0.1 mol/mol fragment I detected in one experiment). Although we have not obtained a sufficient quantity of these fragments to characterize their sequences in detail, their compositions (determined following RNase A digestion of the isolated oligonucleotides) are such that they must derive from oligonucleotide 19 by RNase A cleavage at the susceptible apex of the proposed hairpin

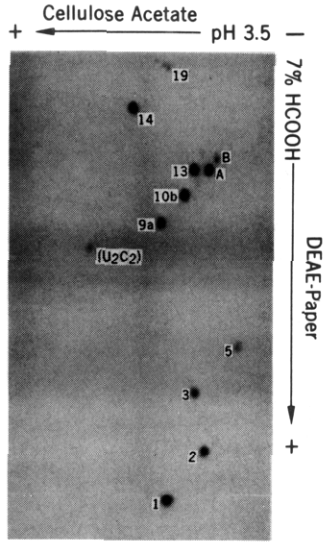

FIGURE 6: Two-dimensional fingerprint analysis of fragment I released by digestion with both RNase T1 and RNase A. The radioautogram shown is a two-dimensional fingerprint of a complete Rnase T1 digest of fragment I which was isolated following digestion of reconstituted ^{32}P-labeled 5.8S–nonradioactive 28S rRNA complex with both RNase T1 and RNase A instead of RNase T1 alone. Note, in contrast to Figure 4A, the low yield of oligonucleotide 19 and the presence of three novel oligonucleotides: (A) UC_3G; (B) UC_4G; and (C) U_2C_2. These represent fragments of oligonucleotide 19, which in the complex is susceptible to RNase A cleavage, presumably because it resides at the apex of the proposed hairpin in fragment I.

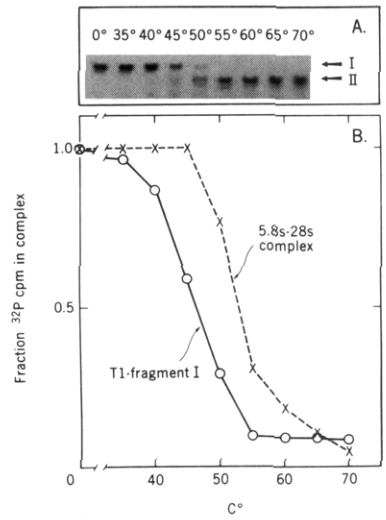

FIGURE 7: Thermal denaturation of fragment I and 5.8S–28S rRNA complex. Fragment I and reconstituted ^{32}P-labeled 5.8S–nonradioactive 28S rRNA complex were individually equilibrated by passage through Sephadex G-25 columns in 0.1 M NaCl and 0.003 M EDTA. Neither was subsequently precipitated from ethanol in order to minimize denaturation of fragment I. Aliquots of each were held at the indicated temperatures for 2 min in sealed capillaries, then immediately chilled in an ice bath, added to sucrose crystals, and applied to polyacrylamide slab gels. The fragment I samples were resolved by electrophoresis through a 15% gel; the radioautogram of this dried gel is shown in A. The 5.8S–28S rRNA complex samples were run on a 3.5% gel, the radioautogram of which was similar to that shown in Figure 2. Bands of radioactivity in both gels were excised and monitored for ^{32}P content; the resulting denaturation curves are presented in Figure 7B.

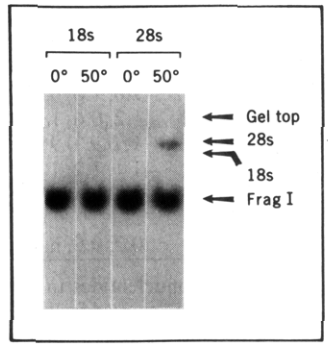

FIGURE 8: Annealing of denatured fragment I to unlabeled, denatured 28S rRNA. A solution of fragment I in 0.01 M EDTA (pH 7.3) was heated to 80 °C for 2 min and then chilled immediately in an ice bath. Aliquots were mixed with denatured 28S rRNA (10 μg) or 18S rRNA (5 μg) at a final NaCl concentration of 0.4 M. The annealing mixtures were held at 50 or at 0 °C for 1 h; the resulting products were resolved by electrophoresis through a 3.5% polyacrylamide slab gel in the cold, following the addition of an equal volume of ½ E buffer + 0.1% $NaDodSO_4$ and sucrose crystals to each sample. The radioautogram of the dried gel is shown.

structure. The residuum of the cleaved oligonucleotide 19, whose structure should be U-U-C-C-, is indicated in Figure 6. Limited quantities of this fragment have precluded determination of the precise sequence. However, complete RNase A digestion released equimolar C- and U-, and the oligonucleotide occupies the position in the fingerprint expected for the composition U_2C_2. The oligonucleotide is recovered in molar yields only 0.1–0.2 of expected. Presumably, cleavage at the apex of the hairpin labilizes this portion of the complex to transient denaturation and hence subsequent cleavage by RNase A or RNase T1 during the digestion interval.

If in fact 5.8S rRNA is associated with 28S rRNA as proposed, the isolated junction complex, fragment I, should be denatured by heat to yield a single fragment smaller than fragment I. The temperature dependence of the process, relative to the denaturation of the native complex, should suggest whether features additional to those discussed are involved in stabilizing the native 5.8S–28S rRNA complex. In pursuing these expectations, purified ^{32}P-labeled fragment I and reconstituted ^{32}P-labeled 5.8S–unlabeled 28S complex were held in parallel at various temperatures as detailed in the legend to Figure 7 and then submitted to electrophoresis through a 15% slab gel. The radioautogram of the dried gel containing fragment I is shown in Figure 7A; the radioautogram of the gel containing the 5.8S–28S rRNA complex was similar to that shown in Figure 2. It is evident that fragment I in fact undergoes temperature-dependent dissociation, to yield a single, electrophoretically homogeneous RNA fragment. Except for the intramolecular hairpin discussed above, fragment I is not obviously capable of forming dimeric structures by complementary base interactions. Presumably, then, the observed decrease in apparent molecular size of fragment I is a consequence of dissociation of the radioactive 5.8S rRNA-derived fragment from its nonradioactive, 28S-derived complement. Figure 7B presents the fractions of fragment I and 5.8S–28S rRNA complex dissociated by incubation at the indicated temperatures. As shown above (Figure 2), the T_m of the 5.8S–28S rRNA complex is about 52–53 °C. Fragment I, the presumed 5.8S–28S rRNA junction complex, is rather more unstable, dissociating in 0.1 M NaCl with a T_m of about 45 °C. This observation suggests that secondary or tertiary structural interactions in addition to those elucidated here assist in maintaining the native 5.8S–28S rRNA complex.

A second expectation of the involvement in the 5.8S–28S rRNA junction complex of the 5.8S-derived segment associated with fragment I is that [^{32}P]RNA derived from fragment I should be capable of specifically annealing to intact 28S rRNA. As detailed in the legend to Figure 8, denatured fragment I was mixed with purified 18S or stripped 28S rRNA,

then held at 0 or 50 °C under appropriate annealing conditions. All products were resolved by slab gel electrophoresis; the resulting radioautogram is presented in Figure 8. It is clear that the segment of 5.8S rRNA released from fragment I is capable of reassociating with 28S but not 18S rRNA. However, we find that the efficiency at which the 5.8S-derived segment from fragment I anneals to 28S rRNA is very much lower than observed with the intact 5.8S rRNA molecule. This lower annealing efficiency, coupled with the observations that fragment I dissociates at lower temperatures than intact 5.8S-28S complex and is rather unstable to manipulation (see Materials and Methods), suggests that at least part of the above-noted secondary or tertiary interactions which enhance the stability of the 5.8S-28S rRNA complex resides in the portions of the 5.8S rRNA molecule which are degraded during the isolation of fragment I.

Discussion

Because of the considerable molecular complexity of the native 5.8S-28S rRNA complex (ca. 5000 nucleotides), we were unable to identify directly a RNase-resistant "junction complex", containing both 5.8S-derived and 28S-derived segments; abundant digestion products obscured the relevant regions of polyacrylamide gels. However, it proved possible to reconstitute, by appropriate annealing procedures, the 5.8S-28S rRNA complex from radioactive 5.8S and nonradioactive 28S rRNA which had been stripped of resident 5.8S rRNA by heat denaturation. The mouse L-cell 5.8S rRNA contains only 158 nucleotides, so the RNase-resistant junction complex, which contains 42 nucleotides derived from the labeled 5.8S rRNA, was readily identified among the digestion products. The 5.8S-28S rRNA complex generated and characterized in this report is very probably identical with the native complex. This identity is suggested by several pieces of information, including: (a) the temperature dependence of the 5.8S-28S rRNA annealing reaction is quite narrow, indicative of a specific, hydrogen-bonding process; (b) the thermal denaturation properties of the artificial and native 5.8S-28S rRNA complexes are identical; (c) an RNase-resistant complex involving part of the 5.8S rRNA molecule is retrievable following annealing to 28S rRNA, but not 18S rRNA; (d) the RNase-resistant 5.8S fragment isolated from the artificial 5.8S-28S complex could be reannealed to intact 28S but not 18S rRNA.

The 5.8S rRNA segment associated with the 28S rRNA in the complex could be unambiguously identified by oligonucleotide fingerprint analysis, with reference to the 5.8S rRNA nucleotide sequence. The associated region, which is indicated in Figure 5, includes the 3′-terminal 42 nucleotides; however, we propose that only about 20-21 of these in fact are associated by hydrogen bonding to a complementary sequence in 28S rRNA. The remaining nucleotides in fragment I constitute a self-complementary sequence and so probably exist as an intramolecular hairpin. The existence of this hairpin is indicated by the susceptibility of the predicted apex of the hairpin to cleavage by RNase A, but not RNase T1 (the apex contains no G residue). We propose that this intramolecular hairpin permits a tertiary structural interaction which serves to stabilize the presumed 5.8S-28S rRNA double helix by coaxial helix stacking. The envisaged 5.8S-28S rRNA junction complex is diagrammatically depicted in Figure 9.

Stabilization of double helical regions by coaxial stacking likely occurs quite commonly in RNA-RNA interactions. The feature certainly occurs in transfer RNA (Kim et al., 1974; Robertus et al., 1974), and may also play an important role in stabilizing the putative interaction of 5S and 23S rRNA in the

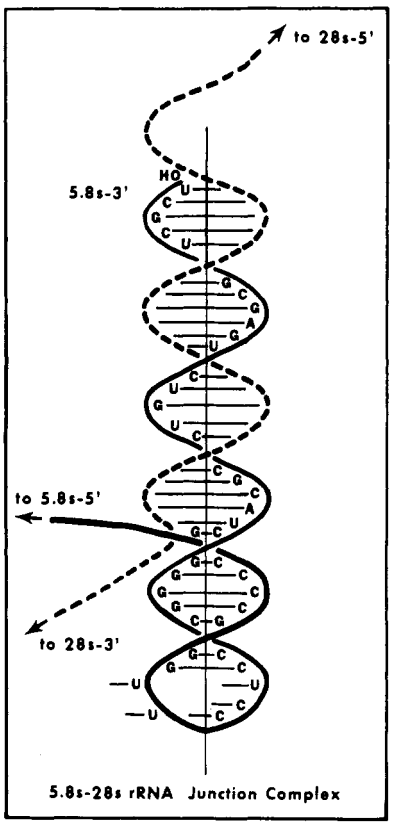

FIGURE 9: The proposed 5.8S-28S rRNA junction complex. As described in the text, the 3′-terminal 20-21 nucleotides of 5.8S rRNA are depicted associated by complementary base interaction with 28S rRNA. The 21-23 nucleotide intramolecular hairpin is aligned coaxially on the 5.8S-28S rRNA duplex.

prokaryotic ribosome. The 5S rRNA of *Escherichia coli* contains a 12-nucleotide complement to 23S rRNA (Herr and Noller, 1975) immediately 5′ adjacent to the "prokaryotic loop", a hairpin common to all examined prokaryotic 5S rRNA molecules (Fox and Woese, 1975). The 5S rRNA is not extracted from ribosomes as a 5S-23S rRNA complex, but such a complex can be generated by annealing (J. E. Dahlberg, personal communication). It may be that during the ribosome cycle the 5S rRNA is transiently bound to the 23S rRNA and that cyclic, coaxial alignment-displacement of the hairpin adjacent to the 5S-23S junction serves to coordinate the 5S-23S association-dissociation process. Still another example of coaxial RNA duplex stacking likely stabilizes the 16S rRNA-mRNA interaction, which apparently transiently occurs during an RNA selection by prokaryotic ribosomes (Shine and Dalgarno, 1971; Steitz and Jakes, 1975). As with the possible 5S-23S interaction, the 16S rRNA-mRNA association-displacement reactions could be strongly influenced by the geometry of a double helical hairpin immediately adjacent to the nucleotide sequence in 16S rRNA which binds its complement in the mRNA. Also, the elegant "reciprocating ratchet" model, proposed by Woese (1970) to be the essential machination of mRNA translocation during protein synthesis, utilizes coaxial stacking of tRNA anticodon loops as an important stabilizing element. Grosjean et al. (1976) have offered experimental evidence that double helical regions adjacent to complementary, intermolecular associations may enhance the intermolecular binding constants several orders of magnitude. The increase in binding constant is not due to a more favorable association rate constant; rather, the rate of dissociation is dramatically reduced.

The secondary structure drawn for mouse 5.8S rRNA in Figure 5 is at best approximate; it is generated by alignment of relatively extensive (and therefore relatively stable) complementary segments within the 5.8S molecule. The actual structure of 5.8S rRNA is very likely much more compact than shown. The relative insensitivity of the molecule to digestion by S1 nuclease (Khan and Maden, 1976) suggests that in fact the majority of the 5.8S rRNA is involved in secondary or tertiary interactions. Since regions of complementarity in addition to those shown in Figure 5 are not present in the molecule, other secondary structural features probably are due to non-Watson–Crick hydrogen bonding. Numerous examples of such have been shown to be involved in the maintenance of tRNA structure (Quigley and Rich, 1976). Nazar et al. (1975) have suggested that, in addition to those duplex regions shown in Figure 5, the 5′- and 3′-terminal 15–20 nucleotides are juxtaposed by complementary base alignments. However, this alignment probably does not exist in the native 5.8S–28S complex, since half of the proposed duplex (the 3′ terminus) apparently is associated with the 28S rRNA. Moreover, the proposed terminal duplex contains sufficiently abundant G-U pairs and unpaired bases that it likely could not exist as a stable feature in solution.

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