Complementarity of sequences in low molecular weight RNAs to regions of messenger and ribosomal RNAs

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ABSTRACT

Total low molecular weight nuclear RNAs of mouse ascites cells have been labeled in vitro and used as probes to search for complementary sequences contained in nuclear or cytoplasmic RNA. From a subset of hybridizing lmm RNAs, two major species of 58,000 and 35,000 mol. wt. have been identified as mouse 5 and 5.8S ribosomal RNA. Mouse 5 and 5.8S rRNA hybridize not only to 18 and 28S rRNA, respectively, but also to nuclear and cytoplasmic poly(A) RNA. Northern blot analysis and oligo-dT cellulose chromatography have confirmed the intermolecular base-pairing of these two small rRNA sequences to total poly(A<sup>+</sup>) RNA as well as to purified rabbit globin mRNA. 5 and 5.8S rRNA also hybridize with positive (coding) but not negative (noncoding) strands of viral RNA. Temperature melting experiments have demonstrated that their hybrid stability with mRNA sequences is comparable to that observed for the 5S:18S and 5.8S:28S hybrids. The functional significance of 5 and 5.8S rRNA base-pairing with mRNAs and larger rRNAs is unknown, but these interactions could play important coordinating roles in ribosome structure, subunit interaction, and mRNA binding during translation.

INTRODUCTION

Low molecular weight RNAs of 50-250 nucleotides comprise a significant percentage of the total RNA found in all eukaryotic cells (1-12). This diverse population of RNA sequences is composed of several different classes specifically located in either the nuclear or cytoplasmic compartment. The cytoplasm contains the better studied species such as the tRNAs and the small 5 and 5.8S ribosomal RNAs as well as several other minor species (3-6). Included in the nucleus are several categories of lmmRNAs (7-11), among them the best studied class, designated small nuclear RNA (snRNA) or the U-rich RNAs (U<sub>1</sub>-U<sub>6</sub>) (1,2,9-11). Many of these nuclear and cytoplasmic species have been well characterized including their rates of turnover, nucleotide sequence, and in most cases, secondary structure (1,10,11). Unfortunately, the precise cellular function that many of these lmmRNAs play in cell structure and metabolism is at present poorly understood. However, their sequence conservation among evolutionarily divergent organisms (11,12)
strongly indicates that they serve similar functions in all eukaryotes and that these functions are vital for cell survival and growth. Both experimental evidence and theoretical considerations have indicated that many of these eukaryotic lmwRNAs may base pair in vivo with other cellular RNA sequences. In the cytoplasm at the level of the ribosome, tRNA interacts through codon-anticodon base pairing with mRNA sequences while 5 and 5.8S ribosomal RNA hydrogen-bond to the larger 18 and 28S ribosomal RNAs, respectively (13,14). Such intermolecular base pairing is undoubtedly important in ribosome structure and protein synthesis. In the nucleus, a small 4.5S RNA originally described by Jelinek et al. (15) has been shown to hybridize to poly(A+) mRNA sequences while the small nuclear RNAs, most notably snRNA U1, has been shown to participate in pre-mRNA splicing events via intermolecular base-pairing with the splice junctions (16-18).

Emerging from these observations is the general concept that intermolecular hydrogen-bonding between eukaryotic lmwRNAs and larger cellular RNA sequences is an important mechanism in the functioning of many of these small nuclear and cytoplasmic RNAs. The precise sequences involved in some of these interactions are still unclear, and present data suggest that a great deal of flexibility in this type of hybrid formation is allowed (15,16,19). Despite a present lack of understanding, RNA:RNA interactions of this type are considered likely to play important roles in cell functions such as RNA processing, transport, and mRNA translation.

To investigate further this phenomenon of intermolecular RNA:RNA hydrogen-bonding, we have isolated total lmwRNA from mouse Taper ascites cell nuclei and then used these 32p-labeled species to search for lmwRNA complementary sequences contained in various other cellular RNA populations. From a subset of four hybridizing lmwRNA sequences, two RNAs identified as ribosomal 5 and 5.8S have been investigated in greater detail. In this paper we show that these two RNAs not only hybridize with the larger ribosomal 18 and 28S RNAs but also hydrogen-bond with mRNA sequences. These observations suggest that RNA:RNA interactions of this type might play important roles in ribosome structure, subunit association, and mRNA translation events.

MATERIALS AND METHODS

Materials

Cytosine 3',5'-[5'-32p]bisphosphate (2000-3000 Ci/m mole), [32p]adenosine triphosphate (3000 Ci/mmole), and [3H]uridine (25-30 Ci/mmole) were purchased from Amersham. Ultrapure formamide, urea, oligo-dT cellulose, bacterial
alkaline phosphatase, and T4 polynucleotide kinase were obtained from BRL, poly(A) and poly(U) from Collaborative Research, micrococal nuclease from Sigma, T4 polynucleotide ligase and RNA sequencing nucleases from PL biochemicals, DNase I (verified RNase free) and proteinase K from Boehringer Mannheim, and N-(3-nitro-benzyloxymethyl)-pyridinium chloride from Gallard-Schlesinger. Phenol was redistilled before use and all glassware was heat-treated to remove RNase contamination. Tobacco mosaic virus RNA, prepared according to the procedure of Gooding and Hebert (20), was a gift from Dr. James Pullman.

Preparation of Nuclei

Mouse Taper ascites cells were maintained by serial passage in the peritoneal cavity of Swiss-Webster mice as previously described by Martin and McCarthy (21). Cells in exponential growth phase were obtained from the mouse and collected by centrifugation. In some experiments, collected cells were radioactively labeled in culture at 37°C for 30 minutes in the presence of 5 μCi/ml of [3H]uridine as previously described (22). All subsequent procedures were carried out at 4°C unless otherwise noted. Harvested cells were washed in 15 vol. of 0.25 M sucrose, 10 mM Tris-HCl, pH 7.6, 10 mM NaCl, 3 mM MgCl₂, 1 mM MnCl₂ (TNM buffer) and collected by centrifugation. Cells were resuspended in 15 vol. of TNM containing 0.4% NP-40 and after centrifugation washed an additional 2 times in TNM buffer. This NP-40 wash did not disrupt the resilient cell membrane of Taper ascites cells, but did eliminate any red blood cell contamination. Pelleted ascites cells were resuspended in 10 vol. of TNM and a 1/10 vol. of 10% Tween 80, and 3% deoxycholate was added while continuously vortexing. After a 3-minute incubation on ice, cells were broken in a Dounce homogenizer with 10-15 passages of a tight-fitting pestle. This suspension was immediately diluted with 3 vol. of TNM buffer, and nuclei were collected by centrifugation. Isolated nuclei were subsequently washed (x3) in TNM buffer before being phenol-extracted.

Preparation of RNA

Total nuclear RNA was isolated from purified nuclei as previously described (23). Briefly, nuclei were digested with DNase I and then proteinase K (in the presence of SDS) before hot-phenol-extraction at pH 5.0 in the presence of SDS. Ethanol precipitated RNA was digested with DNase I to remove remaining traces of contaminating DNA and then phenol-extracted again. Total nuclear RNA was fractionated on linear 5-25% sucrose gradients containing 1 mM EDTA and 0.2% SDS as previously described (24); various size fractions of RNA were pooled and ethanol precipitated. Individual lmmRNAs
were purified from 10% polyacrylamide gels containing 7 M urea by excising appropriate RNA species (revealed by EtBr staining) and eluting the RNA (25).

Cytoplasmic polyribosomes were prepared from cytoplasmic extracts (22) and total polyribosomal RNA purified by hot phenol-extraction at pH 7.8 as previously described (23). Poly(A\(^+\)) RNA was prepared from either total nuclear RNA or total polyribosomal RNA by chromatography on oligo-dT cellulose (26). Bulk ribosomal RNA (used in dot blot hybridization experiments) was prepared by digesting isolated polyribosomes with 15 units/ml of micrococcal nuclease in the presence of 25 mM Tris-HCl, pH 7.6, 40 mM KCl, 7 mM MgCl\(_2\), and 1 mM CaCl\(_2\) for 15 minutes at 25°C. After addition of EGTA to a final concentration of 5 mM, monosomes were prepared by centrifugation of the digested polyribosome preparation on 15-30% sucrose gradients containing 25 mM Tris-HCl, pH 7.6, 40 mM KCl, 7 mM MgCl\(_2\), in an SW 27 rotor at 4°C for 16 hours at 22,000 rpm. Isolated monoribosomes were phenol-extracted and rRNA ethanol precipitated. Rabbit globin 9S mRNA was prepared by phenol-extracting at pH 5.0 total rabbit reticulocyte lysate purchased from Pel Freeze (23). Poly(A\(^+\)) RNA was isolated by oligo-dT cellulose chromatography, fractionated on 5-25% sucrose gradients as detailed above, and the 9S peak pooled and ethanol precipitated.

 Sindbis and VSV virus, grown in BHK cells, were pelleted from a cleared lysate and then purified on a 15-35% (w/v) potassium tartrate equilibrium density gradient in 10 mM Tris-HCl, pH 7.6, 0.1 M NaCl, 1 mM EDTA buffer. Collected virus particles were phenol-extracted at pH 8.0 (23) and the RNA ethanol precipitated. Agarose gel electrophoresis demonstrated that both viral RNA samples were free of cellular contamination.

Total cellular RNA of mouse ascites cells, yeast, or *E. coli* was prepared by hot phenol-extraction of isolated cells at pH 5.0 in the presence of SDS (23). To aid in the breakage of yeast cell walls, glass beads were included in the initial phenol-extraction buffer. Ethanol precipitated RNA was digested with DNAse I as described above, phenol-extracted again, and then ethanol precipitated.

**In vitro RNA Labeling, Hybridization, and RNA Sequencing**

Isolated 1mw nuclear RNA or individual purified species were labeled at the 3' terminus with \[^{32}P\]pCp using T\(_4\) RNA ligase similar to the procedure originally described by Bruce (27). Approximately 2-4 \(\mu\)g of RNA and 40 \(\mu\)Ci of \[^{32}P\]pCp were lyophilized to dryness and resuspended in 30 \(\mu\)l of reaction mixture containing 50 mM HEPES, pH 7.5, 20 mM MgCl\(_2\), 3.5 mM DTT, 10% DMSO, 20 \(\mu\)M ATP, and 2.5-5 units of ligase enzyme. This mixture was incubated for
16 hours on ice and then ethanol precipitated. Specific activity of the RNA labeled in this way was typically on the order of 1-4x10^7 cpm/μg.

For dot blot hybridizations, selected RNA populations were covalently linked to 1 cm² squares of activated DBM paper (28). After RNA coupling, filters were washed with H₂O and then preincubated for 6-16 hours at 37°C in the bottom of siliconized glass vials in 50% formamide, 3 x SSC, 0.02% ficoll, 0.02% polyvinylpyrrolidone (PVP), 1 mg/ml yeast tRNA, and 1% glycine (w/v). Filters were then washed in hybridization buffer consisting of 50% formamide, 3 x SSC, 0.02% ficoll, 0.02% PVP, blotted dry, and approximately 40 μl of hybridization buffer containing labeled lmwRNA was layered on each filter. Vials were sealed and incubated for 36 hours at 37°C. Filters were rinsed repeatedly in wash buffer of 50% formamide, 3 x SSC, 5 mM EDTA, and 0.2% SDS until all unhybridized RNA was removed. Hybridized lmwRNA species were subsequently eluted at 65°C in 90% formamide containing 50 mM Tris-CH₃, pH 7.6, 5 mM EDTA, 0.2% SDS, and then ethanol precipitated in the presence of tRNA carrier. Hybridized RNAs were analyzed on 10% polyacrylamide gels with 7 M urea as described above. For Northern blot transfers, RNA populations were separated on 1.6% agarose or 10% acrylamide gels containing 10 mM methyl mercury and then transferred to activated DBM paper as previously described (29). After RNA hybridization under the conditions described above, Northern blots were washed in 2 x SSC (pH 7.0), 5 mM EDTA, 0.2% SDS at 37°C, blotted dry, and exposed under Kodak XAR-5 film.

Purified lmwRNA species were sequenced according to the chemical method described by Peattie (25). To complete 5S RNA sequencing, unlabeled 5S was purified from total lmwRNA by excision from preparative 10% polyacrylamide gels, labeled at the 5' terminus with T₄ polynucleotide kinase (30), and sequenced enzymatically as previously described (31).

RESULTS

Nuclei were purified from ascites cells under osmotic conditions chosen to minimize RNA degradation, and after phenol extraction and DNase I treatment, total nuclear RNA was fractionated on sucrose gradients. Shown in Figure 1 is the A₂₅₄ and radioactivity profile of one such nuclear preparation. While not resolved under completely denaturing conditions, RNA sequences of up to 45S in size with prominent peaks of 18 and 28S ribosomal RNA were observed. Analysis of rapidly labeled RNA revealed newly synthesized sequences of between 10 and 40S with a mean sedimentation of approximately 20S.

Low molecular weight RNA fractions (as designated in Figure 1) were
Fig. 1. Isolation of mouse ascites cell total nuclear RNA. Taper mouse
ascites cells were labeled in culture for 30 minutes at 37°C in the presence
of 5 μCi/ml of $[^3 H]$uridine. Total nuclear RNA was fractionated on 5-25%
sucrose gradients (sedimentation from left to right); the $A_{254}$ profile was
monitored during gradient fractionation and a typical profile is shown above.
Aliquots of each fraction were TCA precipitated and counted. In addition,
poly(A$^+$) nuclear RNA was isolated from total nuclear RNA by oligo-dT-cellulose
chromatography and fractionated on a separate gradient; the radioactive
profile is also shown. $A_{254}$; radioactivity profile of total
nuclear RNA; radioactivity profile of nuclear poly(A$^+$) RNA.

pooled, ethanol precipitated, and subsequently labeled in vitro with $[^{32}P]$pCp. Analysis of both labeled and unlabeled lmrRNA on acrylamide gels containing
7 M urea is shown in Figure 2A. Approximately 10 discrete species of lmrRNA
varying from 4 to 7S were seen with both EtBr staining and autoradiography. Partial or total sequencing of selected species (those marked with arrows except for 4S) identified many of these lmrRNAs. All species seen in EtBr
staining appeared to be labeled with $[^{32}P]$pCp although some disproportionate
labeling (i.e. 4S) was observed. Lack of RNA sequences below 4S in size or
general smearing in the autoradiogram indicated that little RNA degradation
had occurred either during RNA isolation or during the prolonged incubation
period required for in vitro labeling.

These radioactively-labeled lmrRNAs were subsequently used as probes to
search for sequences complementary to lmrRNA contained in various other
fractions of nuclear or cytoplasmic RNA. (In this experiment, the total
nuclear lmrRNA was used so as to survey both nuclear and pre-cytoplasmic
Fig. 2. Low molecular weight RNA labeling and hybridization. The lmwRNA fraction of total nuclear RNA, similar to that in Fig. 1, was pooled and a small portion labeled in vitro at the 3' terminus with $^{32}$P-pCp. Shown are both the autoradiogram and the EtBr staining pattern of the $^{32}$P-labeled lmwRNA analyzed on a 10% polyacrylamide-7 M urea gel (Fig. 2A). All designated lmwRNA species (with the exception of 4S) have been partially or totally sequenced for positive identification. This $^{32}$P-labeled lmwRNA was hybridized to various populations of nuclear or cytoplasmic RNA as detailed in Materials and Methods. Bound lmwRNAs were analyzed on 10% polyacrylamide-7 M urea gels and $^{32}$P-labeled hybridizing species revealed by autoradiography (Fig. 2B). Molecular weight markers of 5.8S, 5S, and 4S are indicated. Individual DBM filters to which the $^{32}$P-labeled lmwRNA was hybridized contained the following covalently attached RNA populations: c.) Control filter--no RNA. 1.) Mouse ascites cell nuclear poly(A+) RNA. 2.) Mouse ascites cell nuclear poly(A+) RNA. 3.) Mouse ascites cell cytoplasmic poly(A+) RNA. 4.) Purified tobacco mosaic virus RNA. 5.) Poly(A) homopolymer. 6.) Poly(U) homopolymer.

Labeled lmwRNA was hybridized in 50% formamide and 3 x SSC at 37°C to RNA populations covalently bound to activated DBM paper. After hybridization, unbound RNA was removed by washing and hydrogen-bonded species were then removed in 90% formamide at 65°C. Subsequent analysis of hybridized lmwRNAs on polyacrylamide-urea gels revealed four principal species which hybridized to many of the selected RNA fractions (Figure 2B). All species (58,000, 35,000, 29,000, and 25,000 mol. wt.) hybridized to both nuclear and cytoplasmic poly(A+) RNA as well as to total nuclear (A−) RNA comprised primarily of ribosomal RNA (gel analysis not shown). Only the 35,000 mol. wt. species hybridized to tobacco mosaic viral RNA, while none of the four lmwRNAs hydrogen-bonded to the poly(A), poly(U), or control filters.

Subsequent sequencing of each hybridizing species was carried out to
Fig. 3. RNA sequence of hybridizing ImwRNAs. The two larger hybridizing ImwRNAs of approximately 58,000 and 35,000 mol. wt. were isolated from multiple DBM squares (containing covalently-attached mouse ascites cell total nuclear RNA) and subsequently purified of acrylamide-7 M urea gels. Each ImwRNA was then sequenced according to the method described by Peattie (25). Shown are the 3' terminal portions of the 58,000 and 35,000 mol. wt. RNAs; these sequences correspond to published sequences of 5.8S and 5S cytoplasmic ribosomal RNAs, respectively.

unambiguously identify each RNA. Hybridization experiments similar to those described above, but carried out on a larger scale using total nuclear RNA covalently attached to DBM paper, were performed, and specific sequences were resolved on polyacrylamide gels, excised and sequenced. Shown in Figure 3 are the 3' terminal sequences of the 58,000 and 35,000 mol. wt. RNAs. Quite unexpectedly these two RNAs were revealed to be nuclear-associated copies of cytoplasmic 5 and 5.8S ribosomal RNA. More complete sequencing of these two RNA species has demonstrated that they are bona fide small ribosomal RNAs. Indeed the completed sequence of mouse 5S rRNA presented in Table 1 is 100% homologous to human KB cell and rat liver 5S RNA (12). The two smaller hybridizing ImwRNAs of 29,000 and 25,000 mol. wt. were not degradation products of either 5 or 5.8S ribosomal RNA but were unique RNA sequences not previously described. A more complete description including their nucleotide
Table 1. The Nucleotide Sequence of Mouse 5S Ribosomal RNA

<table>
<thead>
<tr>
<th></th>
<th>120</th>
<th>110</th>
<th>100</th>
<th>90</th>
<th>80</th>
<th>70</th>
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<td>UACCACCCUG</td>
<td>AACGCCGCCC</td>
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<td>AUGUCGGGAAG</td>
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<td>60</td>
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<td>CGCCUGGGAA</td>
<td>UACCCGGUGC</td>
<td>UGUAGCCUUUOH</td>
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</tbody>
</table>

Mouse 5S rRNA was purified from total LmW RNA and sequenced from the 3' and 5' terminal ends using chemical and enzymatic sequencing, respectively. The sequence and unusual hybridization characteristics will be published elsewhere. The remainder of this paper will focus on the intermolecular hybridization characteristics of 5 and 5.8S ribosomal RNA.

Since the interaction of these small ribosomal RNAs with poly(A\(^+)\) RNA was not anticipated, we felt it necessary to examine more closely the hybridization specificity of both 5 and 5.8S ribosomal RNA. Purified species were labeled in vitro, repurified on acrylamide gels, and then hybridized to Northern blots containing various RNA populations. Shown in Figure 4 are the EtBr stained gels before RNA transfer to DBM paper and the subsequent autoradiograms of these same blots probed with either \(^{32}\)P-labeled mouse 5.8S (Figure 4A) or mouse 5S (Figure 4B) RNA. Close examination of these Northern blots confirms earlier findings demonstrating that 5.8S hybridizes to 28S ribosomal RNA (14) while 5S hybridizes with 18S ribosomal RNA (13). In these experiments we also observed some hybridization of 5.8S and 5S to 18S and 28S, respectively. However, the amount of hybridization in this case was considerably lower, as well as being less stable at elevated temperatures (see below). Of particular interest was the cross-species hybridization of mouse 5S and 5.8S rRNA to yeast 18 and 26S. These results suggest that the nucleotide sequences involved in this intermolecular RNA:RNA hybridization are at least partially conserved among widely divergent organisms.

More surprising was the hybridization of 5 and 5.8S RNA to cytoplasmic mRNA sequences. These intermolecular hybrids occurred not only with specific mRNAs tested such as the purified rabbit globin 9S mRNA, but also with most if not all the diverse mRNAs contained in the mouse cytoplasm, as shown by the smear of hybridization to total cytoplasmic poly(A\(^+)\) RNA (Figure 4, lane E'). Finally, unlike 5S RNA, 5.8S rRNA hybridized very strongly to an RNA sequence contained in total LmW nuclear RNA (Figure 4, lane D'). Analysis of this
Fig. 4. Hybridization of 5 and 5.8S RNA to cellular RNAs. Various cellular RNA preparations resolved on 1.6% agarose gels containing 10 mM methyl mercury were transferred to activated sheets of DBM paper and subsequently probed with purified $^{32}$P-labeled 5.8S or 5S ribosomal RNA. Shown are the EtBr stained gel before transfer as well as the resultant autoradiogram of the Northern blot probed with labeled RNA. Fig. 4A is the blot probed with $^{32}$P-labeled 5.8S RNA. The lanes correspond to: A.) Total E. coli RNA. B.) Total yeast RNA. C.) Total mouse RNA. D.) Mouse nuclear 4mRNA. E.) Mouse cytoplasmic poly(A$^+$) RNA. F.) Purified rabbit globin 9S mRNA. Fig. 4B is the blot probed with $^{32}$P-labeled 5S RNA. The lanes correspond to: A.) Total E. coli RNA. B.) Total yeast RNA. C.) Mouse nuclear 4mRNA. D.) Total mouse nuclear RNA (> 10S). E.) Mouse cytoplasmic poly(A$^+$) RNA. F.) Purified rabbit globin 9S mRNA.
Fig. 5. Self-hybridization of ribosomal 5.8S RNA. Total nuclear 1mwnRNA from mouse ascites cells was resolved on a 10% polyacrylamide gel containing 10 mM methyl mercury and subsequently transferred to activated DBM paper. This Northern blot was then probed with 32P-labeled 5.8S RNA, and complementary sequences revealed by autoradiography. Lane A: Total 1mwnRNA stained with EtBr. Lane A': Northern blot analysis of Lane A probed with labeled 5.8S RNA. Lane B: Total polysomal RNA stained with EtBr. Lane B': Northern blot analysis of Lane B probed with labeled 5.8S RNA. RNA markers of 5.8S, 5S, and 4S are indicated.

fraction resolved on a 10% polyacrylamide gel (Figure 5) revealed this 1mwnRNA to be 5.8S and the hybridization a result of self-annealing. An additional 8S RNA species complementary to 5.8S seen in total mouse RNA (Figure 4, lane C') is at present of unknown identity.

In view of the somewhat unexpected nature of the above results, we felt it necessary to characterize further the hybridization of 5 and 5.8S RNA to both classes of cytoplasmic RNA. First, the stability of hybridization to large ribosomal RNA was examined under different washing temperatures (Figure 6). Both mouse 5.8S (Figure 6A) and mouse 5S (Figure 6B) RNA hybridized to their homologous 28 or 18S RNAs, respectively, at elevated temperatures of up to and including 60°C in 2 x SSC washing buffer. Hybridizations of 5.8S to 18S and 5S to 28S, while not as strong, were nonetheless present in these experiments as well. Surprising was the strong hybridization of mouse 5.8 to
Fig. 6. Hybridization of 5.8S and 5S RNA to 28S and 18S ribosomal RNA. Total mouse RNA (M) or total yeast RNA (Y) was resolved on agarose-methyl mercury gels and then transferred to activated DBM paper. Northern blots were probed with either $^{32}$P-labeled mouse 5.8S RNA (6A) or $^{32}$P-labeled mouse 5S RNA (6B). The individual filters were initially washed in 1 x SSC buffer at 37°C, and then washed in 1 x SSC at elevated temperatures of 40, 50, 60, or 70°C and the remaining hybridized 5.8S or 5S RNA detected by autoradiography.
Fig. 7. Hybridization and melting profile of 5.8S or 5S RNA with messenger RNA. Aliquots of mouse 18 and 28S ribosomal RNA (x--x), nuclear poly(A') RNA (o--o), cytoplasmic poly(A') RNA (□--□), or rabbit globin 9S mRNA (•••••) were covalently attached to DBM filters and then hybridized with either 32P-labeled mouse 5.8S or 5S RNA in hybridization buffer. After washing filters in 50% formamide and 3 x SSC at 37°C, individual filters were subsequently washed in either 50% formamide and 3 x SSC or in 1 x SSC at selected elevated temperatures. The released radioactivity was plotted as a function of temperature. A. 32P-labeled 5.8S RNA eluted in 50% formamide, 3 x SSC buffer. B. 32P-labeled 5S RNA eluted in 50% formamide, 3 x SSC buffer. C. 32P-labeled 5.8S RNA eluted in 1 x SSC buffer. D. 32P-labeled 5S RNA eluted in 1 x SSC buffer.

yeast 26S RNA seen in the comparison of mouse and yeast at 70°C. This result demonstrates that cross-species hybridization can occur between these apparently conserved intermolecular RNA:RNA base-pairing sequences. These sequences are, however, likely to have been somewhat altered during evolution of the RNA of the large ribosomal subunit. The self-hybridization of 5.8S RNA around the 4S region is again evident in Figure 6.

The hybridization of these two small ribosomal RNAs to mRNA sequences was also examined in melting curve experiments. Shown in Figure 7 are the temperature melting profiles of such 5 or 5.8S hybrids to nuclear or cytoplasmic poly(A') RNA as well as to purified globin 9S mRNA. In melting buffer of 50% formamide and 3 x SSC (Figure 7A and 7B), similar $T_m$ values of between 43° and 47°C were observed. No large differences were seen between
Table 2. Oligo-dT Cellulose Chromatography of 5 and 5.8S rRNA Hybrids with Globin 9S mRNA

<table>
<thead>
<tr>
<th>32P-labeled rRNA</th>
<th>globin 9S mRNA</th>
<th>32P cpm retained on oligo-dT cellulose in 0.5 M KCl*</th>
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</thead>
<tbody>
<tr>
<td>5S</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>5S</td>
<td>+</td>
<td>13,820</td>
</tr>
<tr>
<td>5.8S</td>
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</tr>
<tr>
<td>5.8S</td>
<td>+</td>
<td>8,970</td>
</tr>
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</table>

*Results have been normalized to 100,000 cpm of labeled 5 or 5.8S RNA used in the hybridization to 9S mRNA.

The different populations of mRNA sequences. The $T_m$ values obtained in this manner were very reproducible, varying amongst individual experiments by no more than 1°C. These melting profiles were comparable to the melting profiles of either 5 or 5.8S RNA to mouse large ribosomal RNAs (Figure 7A and 7B). In order to facilitate comparison of the stabilities of these hybrids with previous studies, these experiments were repeated using a different buffer system under less stringent conditions than those above. In 1 x SSC buffer all $T_m$ values (with the exception of 5S to 9S mRNA) were shifted approximately 15-20°C higher to between 60° and 65°C (Figure 7C and 7D). The shift in $T_m$ is consistent with that expected for true RNA:RNA hybrids under the conditions employed in this experiment. The values are also comparable to those previously reported for melting of 5.8S RNA from 28S ribosomal RNA (14), an intermolecular hybrid that has also been demonstrated in vivo (32). Therefore, under both hybridization conditions, the 5 and 5.8S hybrids to mRNA were as stable as those between 5 or 5.8S RNA and the respective large ribosomal RNAs.

Two additional independent experimental approaches to confirm the hybridization of small ribosomal RNAs to mRNA were also undertaken. First, labeled 5 or 5.8S RNA was hybridized in 50% formamide and 3 x SSC buffer at 37°C in the presence or absence of globin 9S poly(A+) mRNA and then subsequently passed over oligo-dT cellulose in 0.5 M KCl. The results of such an experiment are shown in Table 2. In the absence of globin mRNA, neither 5 nor 5.8S RNA was bound to oligo-dT cellulose. However, both small ribosomal RNAs were retained on the column when prehybridized to poly(A+) mRNA; the retention was presumably the result of hydrogen bonding to globin mRNA. Second, labeled 1mwRNA was hybridized with purified Sindbis (positive coding strand) and VSV (negative coding strand) viral RNAs. If the hydrogen bonding
Fig. 8. 5 and 5.8S rRNA hydrogen-bonding with positive-strand viral RNA. Total 1mRNA was labeled at the 3' terminus with [$^{32}$P]pCp. This RNA was incubated on dot blot filters with covalently attached RNA populations of: C.) Control filter--no RNA. 1.) Sindbis viral RNA. 2.) Vesicular stomatitis viral RNA. After 24 hours of incubation, filters were washed and hybridized RNA eluted as previously detailed. Hydrogen-bonded 1mRNAs were analyzed on a 10% polyacrylamide-7 M urea gel and revealed by autoradiography.

of 5 and 5.8S to mRNA sequences is a general feature of these two small rRNAs, then they should base-pair with coding but not non-coding RNA strands. Dot blot analysis of labeled 1mRNA with covalently attached Sindbis or VSV viral RNA revealed that indeed 5 and 5.8S rRNA, as well as several other 1mRNA species, were able to hybridize with Sindbis but not VSV RNAs (Figure 8).

DISCUSSION

Hybridization of total nuclear 1mRNA isolated from Taper ascites cells to various populations of nuclear or cytoplasmic RNA revealed four species of 1mRNA capable of intermolecular RNA:RNA hybridization. Subsequent sequencing of the two largest species identified them as nucleus-associated copies of the
small ribosomal 5 and 5.8S RNAs. Each of these small rRNAs hydrogen-bonded to both messenger RNA and large ribosomal RNAs, which together comprise greater than 90% of total cellular RNA. The results initially suggested the random base-pairing of 5 and 5.8S RNA with most other ribonucleic acid sequences. However, Northern blot analysis demonstrated the sequence specificity of the intermolecular RNA:RNA hybrids formed.

The results presented in this paper confirm previous findings concerning the hybridization of 5 and 5.8S RNA to 18 and 28S ribosomal RNA, respectively (13,14). These small rRNA:large rRNA interactions have been postulated to function in ribosome structure (3,14) and subunit interaction (13); the 5.8S:28S hybrid appears to have been at one time intramolecular in nature (33,34). Of particular interest in the present study was the cross-species hybridization of mouse small rRNAs with yeast 18S and 26S RNA. This is not surprising in light of the sequence conservation of rRNAs during evolution (3,12). Nonetheless, the primary sequences of rRNAs are not identical and this suggests that the nucleotide sequences responsible for the hybridization have perhaps been preferentially conserved. However, the apparent differences in Tm 1/2 values for the different 5.8S:28S rRNA hybrids indicates that even in these presumably conserved sequences, some drift has occurred. In view of the strength of the heterologous mouse 5.8S:yeast 26S hybrid relative to the homologous mouse hybrid, it is possible to suggest that there is a great degree of flexibility in the stringency of pairing, as long as the same regions of the RNA molecules are involved in duplex formation thus permitting their presumed in vivo functions. Clearly, more extensive comparative studies of the interactions of small and large ribosomal RNAs are required to determine the significance of our preliminary findings.

The more novel RNA:RNA interactions reported in this study are the hybridizations of both 5 and 5.8S RNA to mRNA sequences. In fact, detailed analysis of previously reported 5S:18S and 5.8S:28S hybrids was performed primarily to establish the stringency of the hybridization conditions and to have a well established RNA:RNA hybrid as a control with which to compare these small rRNA:mRNA interactions. Comparison of the Tm 1/2 for 5 and 5.8S hybrids with mRNA to those observed for 5 and 5.8S to large ribosomal RNAs gave similar values, indicating a comparable degree of stability for both. Additional melting experiments under less stringent conditions (1 x SSC versus 50% formamide and 3 x SSC) demonstrated an increase in Tm 1/2 of approximately 15-20°C, as would be expected for true RNA:RNA hybrids. The values are comparable to those previously reported under similar conditions for the
The additional experiments using oligo-dT cellulose chromatography and dot blot analysis with viral mRNAs confirmed the ability of 5 and 5.8S rRNA to hydrogen-bond with mRNA sequences. It could be argued that hybridization of 5 and 5.8S rRNA to both total and globin mRNA populations is due to contamination of each by fragments of large ribosomal RNA. This would require that the large rRNA fragments in the globin preparation exactly coelectrophorese with 9S mRNA. In addition, the retention of the small rRNAs by the globin mRNA preparation on oligo-dT cellulose would then have to arise from the binding of large rRNA fragments to oligo-dT via oligo(A) tracts. These are possible but to us unlikely sources of artefactual results. However, two lines of experimental evidence also argue against binding arising from large rRNA contamination. First, 5 and 5.8S rRNA hybridize to the purified Sindbis (positive strand) viral RNA but not VSV (negative strand) viral RNA. Both fractions were isolated from purified virus particles and shown to be free of contaminating large rRNA. Second, work in progress mapping the regions responsible for 5S interaction with 18S rRNA and mRNA sequences has shown that different regions of the 5S molecule are responsible for the respective interactions (Maxwell and Liu, unpublished results).

The hybridization of 5 and 5.8S rRNA to a great diversity of mRNA sequences suggests a general function for this interaction. At the present time, the role such small rRNA:mRNA base pairings could play in vivo is unclear. It seems likely that these interactions would occur at the ribosome level, functioning in mRNA translation. Perhaps one or both of the small rRNAs is important in the binding and correct positioning of mRNA sequences for translational initiation or termination events. Potential base-pairing sites might exist in the 3' or 5' non-coding regions of mRNAs. Consistent with this idea is the recent finding that the nucleotides surrounding the initiator codon in eukaryotic mRNAs are arranged in a non-random fashion (35). This same consensus sequence has been recently suggested as the site for another possible intermolecular base-pairing interaction between mRNA and 18S rRNA (36). As was observed for the Shine-Delgarno interaction between mRNA and 16S rRNA in prokaryotes (37), the significant sequences are difficult to identify on a theoretical basis and require further hybridization and mapping studies. Indeed, computer analysis of potential 5 and 5.8S complementary sequences contained in various mRNA sequences has thus far been difficult to interpret. This approach has been complicated by the diverse population of mRNAs and their apparent lack of obvious homologous regions [with the exception of the poly(A) addition site (38)], coupled with the diverse
background of short complementary sequences found in most RNAs. In spite of this, a preliminary comparison of 5S rRNA and several mRNA sequences does suggest a potential for 5S base-pairing in the 3' non-coding region of mRNA adjacent to the termination codon (Maxwell and Martin, unpublished results). However, such an interaction remains to be confirmed experimentally.

We have attempted to map potential 5S base-pairing sites within globin 9S mRNA by hybridizing labeled 5S RNA to restriction endonuclease cleaved fragments of cloned globin genes. While 5S hybridizes to 9S globin mRNA as demonstrated in this study, no hybridization of 5S RNA to any of the cloned globin DNA fragments was observed (Maxwell and Martin, unpublished results). A similar result was also obtained in attempting to map hybridization sites of 5.8S RNA with 28S rRNA using cloned DNA fragments of the 28S rRNA gene (T.A. Walker and N.R. Pace, personal communication). Both these observations strongly suggest that hybridization between RNA sequences is significantly different than base-pairing of DNA:DNA or DNA:RNA sequences.

Recent molecular models for RNA structure and RNA processing have been presented which utilize intra- and intermolecular RNA hydrogen-bonding (3,16-18,37,39). In all of these proposed RNA:RNA interactions, a considerable amount of flexibility in base-complementarity is often required. Some proposed RNA:RNA interactions utilize non-Watson-Crick base-pairing such as G:U (16,39) or the more recently suggested G:A base-pair (39,40). While G:U base-pairing has been demonstrated not only in synthetic polymers but in cellular RNA molecules as well (41,42), many of the other proposed interactions and complementary base-pairings remain to be verified experimentally.

Nevertheless, the observations presented here coupled with those of other laboratories suggest that a considerable amount of base-pairing flexibility may be an important aspect of RNA function in cells. The understanding of RNA:RNA interactions, both intra- and intermolecular, is likely to be a prerequisite for the elucidation of the mechanism of both RNA processing and mRNA translation.

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