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A low-molecular-weight RNA from mouse ascites cells that hybridizes to both 18S rRNA and mRNA sequences

(RNA·RNA hybrid/intermolecular base pairing)

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ABSTRACT **A low-molecular-weight RNA species from mouse ascites cells has been selected and purified by its intermolecular RNA·RNA hybridization capabilities. This 4.5S RNA is able to base pair with poly(A)$^+$ mRNA sequences and with 18S rRNA. Melting experiments have shown that the intermolecular hybrids formed with this complementary low-molecular-weight RNA are of comparable stability to other RNA·RNA interactions. Analysis has shown that this hybridizing RNA is 87 nucleotides long and has an unusual sequence structure. Located near the 3′ terminus is an alternating pyrimidine dinucleotide region of UUCCUUCCUU. This region along with the 3′-adjacent nucleotides form a 14-nucleotide sequence that exhibits perfect complementarity with 18S rRNA. An additional region of 10 nucleotides at the 3′ terminus is perfectly homologous to a similarly located sequence in 5.8S rRNA. An obvious RNA polymerase III binding site is not found internally in this low-molecular-weight RNA sequence. The complementary and homologous character of hybridizing RNA with respect to rRNA and mRNA sequences suggests a potential regulatory role for this RNA in the coupling of ribosome and mRNA functions.**

Eukaryotic cells contain a diverse population of low-molecular-weight RNAs found in both the nucleus and cytoplasm (1). Theoretical and experimental analyses have indicated that many of these sequences can hydrogen bond to other, larger ribonucleic acid sequences. Such observations suggest that a common molecular mechanism of perhaps most eukaryotic low-molecular-weight RNAs is the formation of intermolecular RNA·RNA hybrids. Examples of this intermolecular base pairing include the anti-codon–codon interaction between mRNA and tRNA, 5S and 5.8S rRNA base pairing with 18S (2) and 28S rRNA (3), respectively, snRNAs and the intron boundaries of pre-mRNA molecules (4), and the Alu I low-molecular-weight RNA sequences and mRNA (5). The interaction of various low-molecular-weight RNA sequences with pre-mRNA, mRNA, and rRNA has implicated these RNAs in important roles in RNA processing, transport, and translation.

To investigate further this phenomenon of intermolecular RNA·RNA hybridization involving low-molecular-weight RNA sequences, we have used *in vitro*-labeled low-molecular-weight RNAs isolated from Taper mouse ascites cells as probes to search for other low-molecular-weight RNA-complementary sequences contained in nuclear and cytoplasmic RNA populations. Using the *in vitro* hybridization assay (6), we have selected four such low-molecular-weight RNA sequences that are capable of base pairing with poly(A)$^+$ mRNA sequences. One of these isolated species is the complementary RNA described in this work. It hybridizes not only to mRNAs but also to 18S rRNA. Sequence determination has also revealed homology with 5.8S rRNA. Because of its hybridizing ability and unusual sequence organization, a partial hybrid structure, we have termed this species hybridizing RNA. The intermolecular hydrogen bonding capabilities of hybridizing RNA and its complementary/homologous regions with ribosomal or ribosomal-associated RNAs suggests possible regulatory roles.

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MATERIALS AND METHODS

Materials. Cytosine 3′,5′-[5′-^{32}P]bisphosphate (2000–3000 Ci/mmol; 1 Ci = 37 GBq) and adenosine 5′-[γ-^{32}P]triphosphate (1000–3000 Ci/mmol) were purchased from Amersham and New England Nuclear, respectively. T4 RNA ligase, T4 kinase, and deoxydideoxyribonucleotides were from P-L Biochemicals, avian myeloblastosis virus reverse transcriptase from Life Sciences (St. Petersburg, FL), acrylamide and agarose from Bio-Rad, sucrose from Schwarz/Mann, and formamide from Bethesda Research Laboratories. Phenol was redistilled before use, and all glassware was heat treated.

RNA Isolation. Mouse Taper ascites cells were maintained by serial passage in the peritoneal cavity of Swiss-Webster mice as described (7). Cells in exponential growth phase were harvested, pelleted by centrifugation, and washed in 15 vol of TNM4 buffer (0.25 M sucrose/10 mM Tris·HCl, pH 7.6/10 mM NaCl/3 mM $MgCl_2$/1 mM $MnCl_2$). All isolation procedures were carried out at 4°C. Pelleted cells were resuspended in 15 vol of TNM4 containing 0.4% Nonidet P-40, pelleted by centrifugation, and subsequently washed twice in TNM4 buffer. Collected cells were resuspended in 10 vol of TNM4, and Tween 80 and deoxycholate were then added to final concentrations of 1% (wt/vol) and 0.3% (wt/vol), respectively, while Vortex mixing. After a 3-min incubation on ice, cells were broken in a Dounce homogenizer with 10–15 strokes of a tight-fitting pestle. This suspension was immediately diluted with 3 vol of TNM4, and the nuclei were collected by centrifugation for 10 min at 1000 × *g*. Isolated nuclei were subsequently washed twice in TNM4 before being phenol extracted as described (8). Total nuclear RNA was fractionated on linear 5–25% sucrose gradients containing 1 mM EDTA and 0.2% $NaDodSO_4$ (9).

RNA Labeling, Hybridization, and Low-Molecular-Weight RNA Isolation. Aliquots of nuclear low-molecular-weight RNA collected from sucrose gradients were labeled *in vitro* with [^{32}P]pCp using T4 RNA ligase as originally described (10). The labeled RNA was hybridized in buffer of 50% (vol/vol) formamide, 3× SSC, 0.02% polyvinylpyrrolidone, and 0.02% Ficoll at 37°C for 18–30 hr to various fractions of nuclear or cytoplasmic RNA covalently attached to 1-cm squares of activated DBM paper (11). (1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7.0.) After washing in hybridization buffer, bound sequences were eluted in 90% (vol/vol) formamide at 65°C and subsequently analyzed on 10% polyacrylamide/7 M urea gels. For temperature-melting experiments, gel-purified low-molecular-weight RNAs were

rehybridized to appropriate DBM filters, and bound RNA subsequently was eluted in hybridization buffer at increased temperatures. The radioactivity in collected aliquots was measured, and the amount of eluted low-molecular-weight RNA was determined. For large-scale isolation of the hybridizing 4.5S RNA, similar annealing was carried out on 10 × 20 cm dot blots containing covalently-attached mouse ascites cell nuclear RNA. RNA gel blot analysis of RNA populations resolved on 1.6% agarose gels containing 10 mM methylmercury was carried out as detailed (12).

RNA Sequencing. Hybridizing 4.5S RNA isolated by hybridization-selection was sequenced by the Peattie method (13) and the 5′ end completed by primer extension. For primer extension, an oligonucleotide complementary to nucleotides 46–87 was synthesized (Applied Biosystems, Foster City, CA) and used to prime DNA synthesis with reverse transcriptase. Reaction buffer (50 mM Tris·HCl, pH 8.0/50 mM KCl/5 mM MgCl/10 mM dithiothreitol) contained 100 mM concentrations of the three unsubstituted deoxyribonucleotides and 50 mM/10 mM concentrations of the one-substituted deoxyribonucleotide/dideoxyribonucleotide. Fifteen micrograms of total low-molecular-weight RNA was mixed with 3 ng of 5′-end-labeled primer in 4 μl of H_2O and heated for 2 min at 90°C. After quick cooling to 65°C, 4 μl of 2.5× reaction buffer was added and slowly cooled to 37°C. Dideoxyribonucleotides and 20 units of reverse transcriptase were added for a final volume of 10 μl, and the reaction was carried out at 42°C for 15 min. Reaction mixes were diluted with an equal volume of 95% (vol/vol) formamide/10 mM EDTA/0.01% (wt/vol) bromphenol blue, boiled for 3 min, and then applied to a sequencing gel.

RESULTS

Isolation of Hybridizing Low-Molecular-Weight RNAs. Low-molecular-weight RNA, isolated from total mouse ascites cell nuclear RNA fractionated on sucrose gradients, was labeled *in vitro* with [^{32}P]pCp using T4 RNA ligase. Polyacrylamide gel analysis of this radiolabeled low-molecular-weight RNA fraction revealed approximately 10 major RNA species (Fig. 1) whose labeled profile was similar if not identical to the ethidium bromide-stained RNA pattern. In the initial screening of these low-molecular-weight RNA species for intermolecular hybridization capabilities, ^{32}P-labeled low-molecular-weight RNAs were hybridized to nuclear or cytoplasmic poly(A)$^+$ RNA that had been covalently attached to DBM paper. This analysis revealed four RNAs of approximately 58, 35, 29, and 26 kDa that were able to base pair with nuclear and cytoplasmic poly(A)$^+$ RNA (Fig. 1). Experiments identifying the two largest low-molecular-weight RNAs as 5S and 5.8S rRNA, their hybridization characteristics, and possible significance is presented elsewhere (27). The work reported here investigates the identity and hybridization capabilities of the 29-kDa species, the 4.5S RNA designated in Fig. 1. This 4.5S RNA hybridizes to both nuclear and cytoplasmic poly(A)$^+$ RNA but not to tobacco mosaic viral RNA or the ribohomopolymer poly(A) or poly(U) controls.

Intermolecular RNA·RNA Hybridization Capabilities of a 4.5S RNA. More detailed analysis of the intermolecular hybridization characteristics of 4.5S RNA was carried out using RNA gel blot procedures. Large dot blots containing poly(A)$^+$ RNA were used to hybrid-select larger quantities of the 4.5S RNA that were then purified on polyacrylamide gels. RNA gel blots containing various RNA populations were then probed with the purified 4.5S RNA. Results shown in Fig. 2 confirmed the ability of the 4.5S RNA to hydrogen bond with poly(A)$^+$ mRNA sequences. This low-molecular-weight RNA sequence hybridized to purified rabbit globin 9S mRNA, but perhaps more importantly, a general hydrogen bonding of 4.5S RNA to total mouse cytoplasmic mRNA (lane 5) was observed, indicating that the base pairing of this low-molecular-weight RNA sequence to mRNAs is not gene specific but a more general phenomenon.

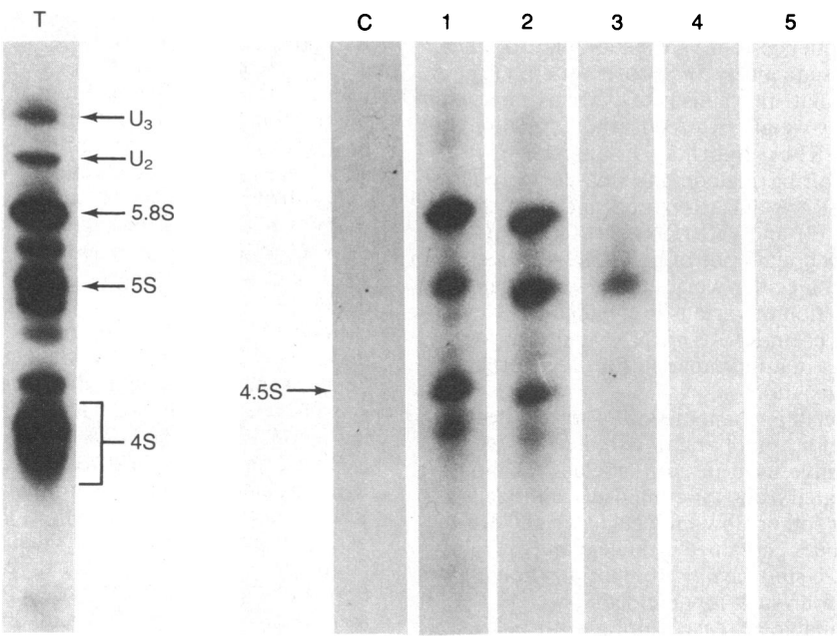

FIG. 1. Selection of mouse ascites cell hybridizing RNA. Total low-molecular-weight RNA was labeled *in vitro* at the 3′ terminus with [^{32}P]pCp and then hybridized to DBM filters containing various covalently attached RNA populations. After filter hybridization and washing, bound low-molecular-weight RNA sequences were eluted and analyzed on 10% polyacrylamide/7 M urea gels. Shown are lane T, the total labeled low-molecular-weight RNA fraction used in hybridization experiments, and hybrid-selected low-molecular-weight RNA species isolated from filters containing covalently attached RNA populations of lane C, control filter, no RNA; lane 1, nuclear poly(A)$^+$ RNA; lane 2, cytoplasmic poly(A)$^+$ RNA; lane 3, tobacco mosaic virus RNA; lane 4, homopolymer poly(A); and lane 5, homopolymer poly(U). Specific low-molecular-weight RNA markers are designated in the total population, and 4.5S hybridizing RNA is designated in the hybrid-selected species.

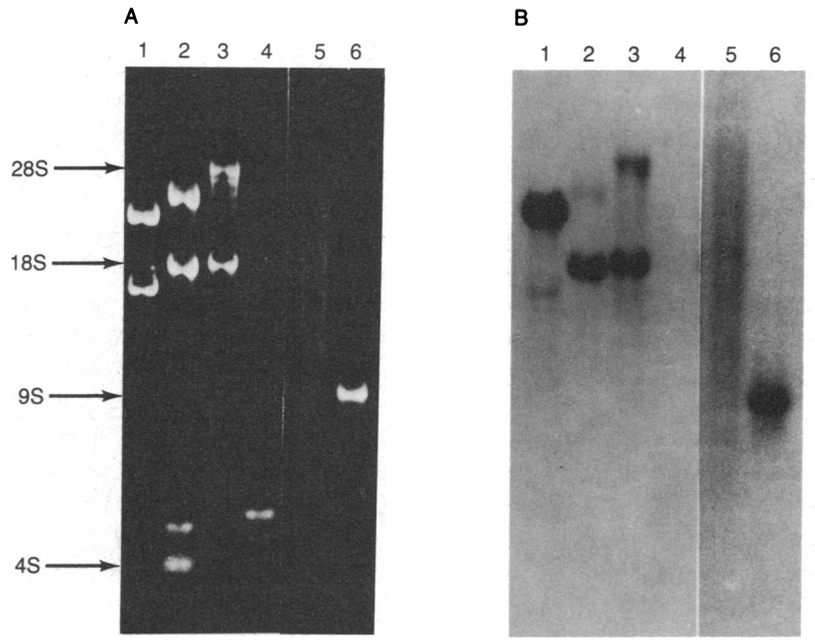

FIG. 2. RNA gel blot analysis of the intermolecular base-pairing capabilities of mouse hybridizing RNA. Hybrid-selected mouse 4.5S hybridizing RNA was used to seek complementary regions in various other cellular RNAs. (*A*) Ethidium bromide-stained agarose gel. Lanes: 1, total *E. coli* RNA; 2, total yeast RNA; 3, total mouse ascites cell RNA; 4, mouse ascites cell low-molecular-weight RNA fraction; 5, mouse ascites cell cytoplasmic poly(A)$^+$ RNA; 6, rabbit globin 9S mRNA. Molecular size markers are designated. (*B*) Autoradiogram of the agarose gel shown in *A* blotted to activated DBM paper and then probed with hybrid-selected mouse hybridizing RNA. Lanes correspond to those in *A*.

The 4.5S RNA also hybridized strongly to eukaryotic 18S rRNA. Hybridization to yeast 18S rRNA indicated that the nucleotide sequence(s) responsible for 4.5S interaction has been conserved during 18S rRNA evolution. On the other hand, no base pairing was observed with prokaryotic 16S rRNA, although some hybridization was seen with 23S rRNA. The reduced hybridization observed with both large rRNAs of *Escherichia coli* (23S) and mouse (28S) is consistent with the 5.8S rRNA homologous sequences observed in this low-molecular-weight RNA (see below).

The stability of 4.5S RNA·mRNA and 4.5S RNA·rRNA hybrids was studied in temperature-melting experiments. All hybrids between 4.5S and mRNA or rRNA exhibited similar melting profiles with melting temperature of 50% of the hybrid values at approximately 45°C in buffer of 50% (vol/vol) formamide and 3× SSC (Fig. 3). These melting temperature values are representative of moderately stable RNA·RNA hybrids, comparable to those observed (under the same buffer conditions) for 5.8S·28S and 5S·18S rRNA hydrogen bonding (unpublished results).

4.5S Hybridizing RNA Sequence Analysis. Purified 4.5S hybridizing RNA was sequenced using the chemical method of Peattie (13) on the 3′ end of 4.5S (nucleotides 87–15) and then the primer method extension using a synthetic oligonucleotide (41mer; nucleotides 87–46) to sequence the 5′ region (nucleotides 45–1). The lack of difficulty sequencing hybridizing RNA suggested that large numbers of modified bases are not contained in this RNA species, and preliminary studies have indicated the absence of any 5′ cap structure (unpublished data). Shown in Fig. 4 is the primary sequence of this 87-nucleotide low-molecular-weight RNA species.

Sequence analysis and comparisons have revealed that mouse 4.5S hybridizing RNA is a sequence not previously described. It is not a degradation product of known larger RNA molecules as determined by sequence comparison. No apparent RNA polymerase III binding site is observed, although a region similar to the extra arm and box B sequence (14) is found in the middle of the 4.5S hybridizing RNA between nucleotides 26 and 43 (Fig. 4). RNA gel blot analysis of mouse RNA during the synthetic oligonucleotide used for sequencing as a probe has demonstrated that only one RNA sequence of 4.5S in size is recognized by this probe, indicating that hybridizing RNA is not processed from a larger transcript. Southern blot analysis has also revealed that, unlike RNA polymerase I and polymerase III transcripts, hybridizing RNA is encoded by a single copy gene (Q. Trinh-Rohlik and E.S.M., unpublished results).

Analysis of our 4.5S hybridizing RNA at the 3′ end

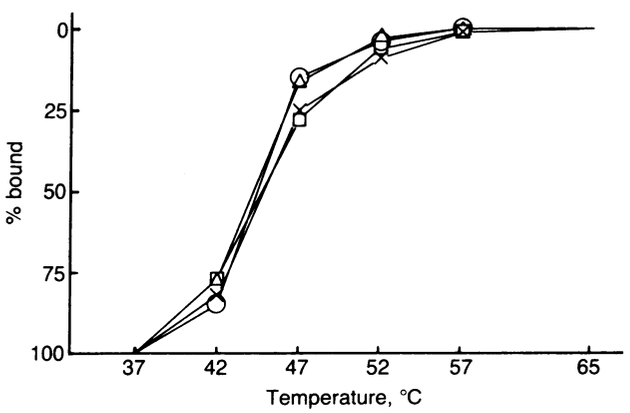

FIG. 3. Mouse 4.5S hybridizing RNA hybrid stabilities. Mouse hybridizing RNA was incubated at 37°C in buffer containing 50% (vol/vol) formamide and 3× SSC to DBM filters containing covalently attached RNAs. After hybridization and washing at 37°C in the same buffer, hybridized RNA was thermally eluted from these filters (in hybridization buffer) by gradually increasing the temperature and collecting eluted aliquots. Aliquots were counted and plotted as % originally bound hybridizing RNA vs. temperature. DBM filters contained covalently attached RNA populations of mouse nuclear poly(A)$^+$ RNA (circles), mouse cytoplasmic poly(A)$^+$ RNA (squares), purified mouse 18S rRNA (crosses), and purified rabbit globin mRNA (triangles).

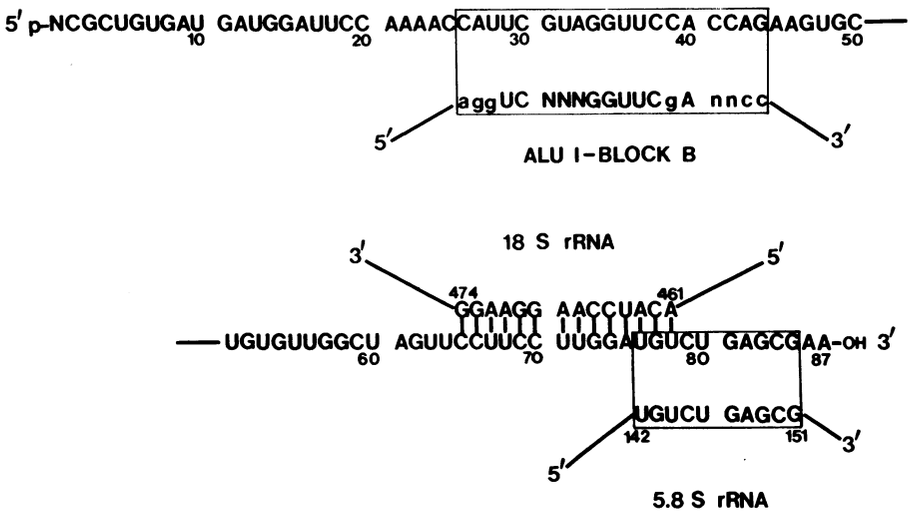

FIG. 4. Nucleotide sequence of mouse 4.5S hybridizing RNA and potential complementary/homologous regions. Hybrid-selected 4.5S RNA, repurified on polyacrylamide gels, was sequenced. The sequence of this 87-nucleotide RNA is presented, and potentially complementary/homologous regions with other RNA species are indicated.

revealed several regions of particular interest with respect to nucleotide sequence and their homology/complementarity to other RNA species. Nucleotides 76–85 are homologous to an identical nucleotide region found at the 3′ end of the 5.8S rRNA (Fig. 4). Each is located within several nucleotides of the 3′ terminus of their respective molecules. Sequence analysis upstream from this homologous region clearly reveals, however, that 4.5S hybridizing RNA is not a pseudogene of 5.8S RNA or related to 5.8S in any other immediately apparent way. This 5.8S-homologous sequence in 4.5S hybridizing RNA explains the observed hybridization to both 28S and 23S rRNAs. In 5.8S rRNA this sequence is part of the intermolecular hybridizing region with 28S rRNA (3) and, therefore, 4.5S hybridizing RNA containing this nucleotide sequence will also base pair with 28S rRNA. This same sequence, as well as the rest of the 5.8S molecule, exhibits homology with the 5′ end of 23S rRNA (15). The intramolecular base pairing of the 5′ terminus of 23S with the remainder of the molecule would explain how 4.5S hybridizing RNA, containing 5.8S sequences, could hybridize to 23S rRNA as well.

Located slightly further upstream to the 5.8S sequence is a pyrimidine stretch of alternating cytosine and uridine dinucleotides between positions 63 and 72. Computer analysis of potentially complementary regions between 4.5S and 18S rRNA has indicated that this region is most likely responsible for the observed intermolecular RNA·RNA base pairing between these two RNAs. Most of this pyrimidine-rich region along with some 3′ sequence forms a 14-base-pair region of perfect complementarity with nucleotides 474–461 in 18S rRNA. However, confirmation of this interaction awaits further experimentation.

DISCUSSION

Hybrid selection of mouse low-molecular-weight RNA sequences that are capable of hydrogen bonding to poly(A)$^+$ mRNA sequences has resulted in the isolation of a low-molecular-weight RNA species (hybridizing RNA). This 87-nucleotide RNA hybridizes not only to mRNA sequences but with 18S rRNA as well, as shown by RNA gel blot analysis. These base-pairing interactions are comparable in strength to other intermolecular RNA·RNA hybrids.

The function of the hybridizing RNA *in vivo* is presently unknown. However, its hybridization characteristics suggest that it might play a role in coordinating rRNA and mRNA functions. Its ability to bind to mRNAs and 18S rRNA, sequences both in the small ribosomal subunit initiation complex, implicates 4.5S hybridizing RNA as a potential translational regulator molecule. Such hydrogen bonding of hybridizing RNA to the ribosome would likely alter overall ribosome structure affecting mRNA binding and thus rates of translation or levels of protein synthesis. The potentially competitive interaction of hybridizing RNA with 28S at the site of 5.8S rRNA hybridization could also be involved in altering ribosome structure and, hence, function. It is important to point out that the initial selection of this RNA has been from a nuclear RNA preparation. Results have shown that 4.5S hybridizing RNA is concentrated in the nucleus in actively growing cells. Nevertheless, significant amounts are found in the cytoplasm cosedimenting with polyribosomes (Q. Trinh-Rohlik, G. Shanab, and E.S.M., unpublished data). The nuclear location of 4.5S hybridizing RNA is not surprising if one postulates the sequestering of this species within the nucleus until cellular conditions require its transport to the cytoplasm for interaction with ribosomes and protein synthesis events. However, the nuclear location of hybridizing RNA might also be indicative of additional or alternative functions. For example, the intermolecular hybridization capabilities of hybridizing RNA could be utilized during transport of mRNA and rRNA from the nucleus to the cytoplasm or in the coordination of mRNA and rRNA accumulation with cellular protein synthesis. Clearly, any proposed function(s) for 4.5S hybridizing RNA is at this time speculative, and more detailed examination is necessary to distinguish between these different possibilities.

Intermolecular RNA·RNA base pairing of low-molecular-weight sequences within the eukaryotic ribosome has been implicated as an important mechanism in ribosome structure and function. These low-molecular-weight RNA interactions include the hydrogen bonding of 5.8S and 28S rRNAs (3), 5S and 18S rRNAs (2), and of course, the base pairing of tRNAs to mRNA and possibly 5S rRNA (16). If 4.5S hybridizing RNA is involved in translational events, a primary basis for its function would likely be intermolecular base pairing with mRNA and 18S rRNA. Such a mechanism of translational regulation via intermolecular base pairing of low-molecular-weight RNA sequences has been recently demonstrated in prokaryotes for the mRNA-interfering complementary RNA of *E. coli* (17).

The hybridization characteristics of mouse hybridizing RNA are similar in many respects to other reported low-

molecular-weight RNA sequences and their hybridization with large RNA sequences associated with polyribosomes. The Alu I transcripts of CHO cells have been shown to base pair with mRNA sequences (5). Adenovirus VA I and VA II RNAs also hybridize with viral mRNA sequences (18), and subsequent work has convincingly demonstrated that VA I is directly involved in the regulation of viral protein synthesis (19, 20). In addition, Epstein–Barr viral low-molecular-weight RNA sequences (EBV I and EBV II) have been implicated in protein synthesis and intermolecular base pairing with 18S rRNA (21) (the same 18S sequence proposed in this work to interact with mouse 4.5S hybridizing RNA).

The adenoviral and Epstein–Barr viral low-molecular-weight RNAs, as well as several mouse (Y1 and Y2) and human (HY1 and HY2) low-molecular-weight RNAs, possess pyrimidine-rich sequences reminiscent of the 3′-terminal sequence (UUCCUUCCUU) of mouse hybridizing RNA (21–23). However, they differ from mouse hybridizing RNA in two respects. First, they are different in size as well as nucleotide sequences from mouse hybridizing RNA. Second, they are all transcribed by RNA polymerase III and, therefore, contain polymerase III binding sites in their gene sequence. Sequence analysis indicates lack of an apparent polymerase III binding site in the hybridizing RNA sequence, suggesting transcription by another RNA polymerase. Therefore, mouse 4.5S hybridizing RNA is a low-molecular-weight RNA possessing intermolecular RNA·RNA hybridization capabilities but differing from those sequences with similar intermolecular base-pairing capacities. At present it is not known whether mouse hybridizing RNA is part of a small ribonucleoprotein complex (RNP). Adenoviral VA I, VA II, Epstein–Barr viral EBV I and EBV II, and the mouse and human "Y" low-molecular-weight RNAs are precipitated by one or the other of the autoimmune antibody classes La and Ro (24–26).

It remains a possibility that hybridizing RNA is a chimeric molecule resulting from splicing events; resolution of this possibility awaits the analysis of the gene structure for this RNA species. Most pressing, however, is the use of sensitive sequence probes to determine the *in vivo* associations of this 4.5S hybridizing RNA, which we expect to shed light on its cellular functions.

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