Homologous genes for mouse 4.5S hybRNA are found in all eukaryotes and their low molecular weight RNA transcripts intermolecularly hybridize with eukaryotic 18S ribosomal RNAs

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ABSTRACT

Previous work (1) has reported the isolation and sequencing of a mouse low molecular weight RNA species designated 4.5S hybridizing RNA or hybRNA because of its ability to intermolecularly hybridize with mouse mRNA and 18S rRNA sequences. Using synthetic DNA oligonucleotide probes we have examined the conservation of this gene sequence and its expression as a 1mwRNA transcript across evolution. Southern blot analysis has shown that homologous genes of single or low copy number are found in all eukaryotes examined as well as in *E. coli*. Northern blot analysis has demonstrated 4.5S hybRNA transcription in all mouse tissues as well as expression in yeast and *Xenopus laevis* as 1mwRNAs of approximately 130 and 100 nucleotides, respectively, as compared with mouse/rat/hamster species of approximately 87 nucleotides. Yeast and *X. laevis* 4.5S hybRNA homologs, isolated by hybrid-selection, were shown by Northern blot analysis to intermolecularly hybridize with homologous as well as heterologous 18S rRNA sequences. The conservation of 4.5S hybRNA homologous genes and their expression as 1mwRNA transcripts with common intermolecular RNA:RNA hybridization capabilities in fungi, amphibians, and mammals argues for a common, conserved and required biological function for this 1mwRNA in all eukaryotes and potential utilization of its intermolecular RNA:RNA hybridization capabilities to carry out this function.

INTRODUCTION

Diverse populations of low molecular weight RNA sequences are found in both prokaryotic and eukaryotic organisms. Investigations of their structure and function have strikingly demonstrated the frequent ability of many of these 1mwRNAs to intermolecularly base-pair with other larger RNA sequences (1-18). This common or shared molecular mechanism of 1mwRNA:RNA hybridization is an essential aspect of their function *in vivo*. Such intermolecular RNA:RNA hybrids have been shown to play important roles in regulating DNA replication (2), RNA processing (3,4) and splicing (5-8), ribosome structure (9-17), and mRNA translation (18).

In previous work, we have reported the isolation and characterization of a new mouse 1mwRNA species of 87 nucleotides in length (1), different from any previously reported eukaryotic or prokaryotic 1mwRNA. This 1mwRNA has been

designated 4.5S hybridizing RNA, or hybRNA, because of its ability to intermolecularly base-pair with mRNA and 18S rRNA sequences in vitro. While the function of mouse 4.5S hybRNA is unknown at present, this 1mwRNA species may carry out its assigned biological function in the cell via this apparently common mechanism of intermolecular RNA:RNA hybrid formation.

The work presented here describes the analysis of 4.5S hybRNA gene organization and expression in evolutionarily divergent organisms and examines the ability of these diverse 4.5S hybRNA homologs to intermolecularly base-pair with 18S rRNA. We have shown that the gene for this 1mwRNA species is found in all eukaryotes examined and is expressed as a 1mwRNA transcript in such diverse organisms as fungi, amphibia, and mammals. In addition, analysis of the yeast and Xenopus laevis 4.5S hybRNA homologs has shown that the ability to intermolecularly hybridize with eukaryotic 18S rRNA has been conserved in each of these homologs as well.

MATERIALS AND METHODS

Materials

Adenosine 5'-[γ -³²P]triphosphate (3000 Ci/mmol) and Gene Screen hybridization filters were purchased from New England Nuclear. Agarose, acrylamide, SDS, and Zeta Probe hybridization filters were purchased from Bio-Rad. Ultra-pure sucrose, urea, and formamide were obtained from Bethesda Research Laboratories (BRL). Restriction endonucleases were from BRL, New England Biolabs, and Boehringer Mannheim Biochemicals. Swiss-Webster white mice and Syrian hamsters were obtained from Harlan Sprague Dawley, and DB strain rats were kindly provided by Dr. Clement Markert. Xenopus laevis and Drosophila melanogaster (wild type) were obtained from Carolina Biological Supply, and flies were grown as suggested by the supplier. E. coli strain HB101 was obtained from BRL, and Saccharomyces cerevisiae (X2180-1A, a SUC2 mal⁻ gal2 CUP1) was kindly provided by Dr. Leo Parks. Phenol was redistilled and all glassware was heat-treated before use. Oligonucleotides were synthesized with an Applied Biosystems synthesizer.

DNA Isolation

Mouse, rat, hamster, and Xenopus laevis DNA was prepared by phenol/chloroform extraction of isolated nuclear pellets. Mouse nuclei were prepared from mouse Taper ascites cells as previously described (11). Nuclei were prepared from rat, hamster, and Xenopus laevis liver by mincing dissected livers in ice-cold homogenization buffer consisting of 20 mM Tris-HCl, pH 7.6, 0.32 M sucrose, 50 mM NaCl, 5 mM MgCl₂, and 1 mM CaCl₂. Washed tissue was

homogenized at 4°C with a Potter-Elvehjem homogenizer and nuclei collected by centrifugation at 700xg for 10 min. The nuclear pellets were washed (x2) in homogenization buffer and resuspended in 10 mM Tris-HCl, pH 7.7, 0.75 M NaCl, and 25 mM EDTA (TSE buffer). The pellets were digested with proteinase K (100 µg/ml) in the presence of 0.5% SDS for 3 h at 37°C. DNA was then phenol extracted, RNA contaminants removed by RNase digestion and subsequent phenol/chloroform extraction, and purified DNA dialyzed against TE buffer (10 mM Tris-HCl, pH 7.6, 1 mM EDTA) as previously described (19). Yeast DNA was prepared from Saccharomyces cerevisiae spheroplasts (20). Briefly, yeast grown on YEPD medium was digested with Lyticase (Sigma Chemical Co.) to yield prepared yeast spheroplasts. Isolated spheroplasts were resuspended in TNE buffer (10 mM Tris-HCl, pH 7.6, 10 mM NaCl, 1 mM EDTA), lysed with 1% SDS and contaminant RNA removed by RNase digestion. Yeast DNA was then prepared by phenol and phenol/chloroform extraction as described (19). E. coli DNA was prepared from E. coli HB101 cells grown on LB medium, lysed in TSE buffer containing 0.5% SDS, digested with proteinase K, and DNA phenol/chloroform extracted as described above. Human DNA was kindly supplied by Dr. Russel Kaufman, Duke University.

RNA Isolation

Total nuclear RNA from mouse Taper ascites cells and isolated 1mRNA fractions were prepared as previously described (11). Total RNA samples from selected mouse tissues were prepared by freezing isolated organs in liquid nitrogen and then grinding the frozen tissue with a mortar and pestle (maintained at -80°C). Powdered tissue was then rapidly resuspended in extraction buffer (50 mM Na-acetate, pH 4.5, 1 mM EDTA, and 1% SDS) and immediately extracted with hot phenol as previously detailed by Scherrer (21). Total RNA from rat, hamster, or Xenopus laevis liver, and Drosophila melanogaster was prepared from frozen tissue by phenol extraction as described above. Total RNA from E. coli (HB101), yeast spheroplasts (prepared as detailed above), and HeLa cells was prepared by lysing isolated cells in extraction buffer and immediately extracting the cell lysates in hot phenol as previously described (21). Low molecular weight RNA fractions of yeast and Xenopus laevis RNA were prepared by resolution of total RNA samples on 5-25% sucrose gradients as previously described (11).

Southern/Northern Blotting and Hybridization

Isolated DNA samples were digested at 37°C overnight with appropriate restriction endonucleases according to manufacturer's recommendations at an enzyme-to-DNA ratio of 4 units/µg. After phenol/chloroform extraction and

ethanol precipitation (19), DNA was resuspended overnight in TE buffer and then resolved on 0.8% agarose gels in TBE buffer (89 mM Tris-HCl/89 mM boric acid, pH 8.3, 2 mM EDTA). DNA samples (25-40 µg/lane) were transferred to Gene Screen (New England Nuclear) or Zeta Probe (Bio-Rad) hybridization filters by capillary blotting or electroblotting, respectively. Blotting protocols followed were those supplied by the respective manufacturers. Vacuum-dried filters were prehybridized at 42°C for 6-16 h in 50% formamide, 3xSSC, 0.04% ficoll, 0.04% polyvinylpyrrolidone (PVP), and 100 µg/ml denatured salmon sperm DNA. Filters from electroblotting were washed at 65°C in 0.1xSSC and 0.1% SDS. Blots were prehybridized at 42°C for 6-16 h in buffer of 25 mM sodium phosphate, pH 6.5, 5xSSC, 0.2% ficoll, 0.2% PVP, 200 µg/ml of denatured salmon sperm DNA, and 50% formamide. Hybridization was carried out at 37°C for 24-36 h in buffer of 25 mM sodium phosphate, pH 6.5, 5xSSC, 0.04% ficoll, 0.04% PVP, and 50% formamide. After hybridization, blots were washed first in 2xSSC, pH 7.0, and 0.1% SDS at 23°C (x4) and then with the same buffer at 55°C (x2). The hybridization probe for Southern blot analysis was a synthetic DNA oligomer of 39 nucleotides complementary to the 3' end (nucleotides 49-87) of mouse 4.5S hybRNA labeled at the 5' terminus with ³²P as previously described (19).

RNA samples (15-25 µg/lane) were resolved on 10% polyacrylamide gels containing 7 M urea (11) or 1.5% agarose-formaldehyde gels (19) and then electroblotted to Zeta Probe hybridization filters according to manufacturer-supplied protocols. Prehybridization/hybridization was as detailed above. Filters were first washed in 2xSSC, pH 7.0, buffer containing 0.1% SDS at 23°C (x4). Then filters were washed (x2) under low or high stringency conditions as detailed in the figure legends. Hybridization probes utilized for Northern blot analysis were either of two synthetic DNA oligonucleotides complementary to the 5' (nucleotides 1-31) or 3' (nucleotides 49-87) ends of mouse 4.5S hybRNA which had been radiolabeled at the 5' terminus with ³²P as previously described (19).

Primer Extension Sequencing of 4.5S hybRNA Homologs

Rat, hamster, Xenopus laevis, and yeast hybRNA homologs were verified by primer extension/dideoxynucleotide sequencing the 5'-terminal region of each hybRNA homolog. Total 1mwRNA fractions of either rat, hamster, Xenopus laevis, or yeast (10 µg/sequencing reaction) were annealed with 2-5 ng of 5'-radiolabeled 39mer primer and sequenced as previously detailed (1), except that the concentration of the three unsubstituted deoxyribonucleotides was 100 µM and the substituted deoxyribonucleotide/dideoxyribonucleotide concentra-

tions were both 20 μ M. In sequencing the yeast hybRNA homolog, Klenow fragment (10 μ /rxn) was first added to the sequencing reactions to remove the unbase-paired 3' end of the 39mer primer before extension/sequencing with reverse transcriptase.

Hybrid-Selection of 4.5S hybRNA Homologs

4.5S hybRNA homologs from Saccharomyces cerevisiae and Xenopus laevis were isolated by dot blot hybrid-selection. The synthetic DNA oligonucleotide 39mer was covalently attached to activated APT paper as previously described (22). Approximately 15-20 μ g of DNA oligomer was attached per 4x4-cm dot blots, and multiple dot blots were used in hybrid-selection. Filters were prehybridized at 42°C as previously described (11) and then hybridized 36-40 h at 30°C with radioactively-labeled 1mwRNA fractions in hybridization buffer containing 50% formamide, 5xSSC, 25 mM sodium phosphate, pH 6.5, 0.04% ficoll, and 0.04% PVP. Isolated yeast or X. laevis 1mwRNA fractions were labeled at the 3' terminus with 32 P as previously described (23). Dot blots were washed extensively at 30°C in 2xSSC, pH 7.0, buffer containing 0.1% SDS before eluting hybridized 1mwRNAs at 65°C in elution buffer of 90% formamide, 50 mM Tris-HCl, pH 7.6, 5 mM EDTA and 0.1% SDS. Collected eluates were ethanol precipitated and 4.5S hybRNA homologs purified on 10% polyacrylamide gels containing 7 M urea (11). Gel slices containing yeast or X. laevis 4.5S hybRNA homologs were placed in hybridization bags with appropriate Northern blots and hybridization in 50% formamide, 5xSSC, 25 mM sodium phosphate, pH 6.5, 0.04% ficoll and 0.04% PVP carried out at 30°C for 36 h. Filters were washed (x2) at 23°C in 2xSSC and 0.1% SDS and then washed (x2) in the same buffer at 50°C.

RESULTS

Figure 1 shows the primary sequence of mouse 4.5S hybRNA previously determined by RNA sequencing (1). [Recent analysis of the genomic clone for this 1mwRNA transcript (data to be presented elsewhere) has corrected nucleotide position No. 35, determining it to be U instead of G as previously reported.] Indicated in Figure 1 is a 14-nucleotide sequence (nucleotides 65-78) located at the 3' end of 4.5S hybRNA which exhibits complementarity with mouse 18S rRNA. The complementarity of this region of 4.5S hybRNA with 18S rRNA may explain, as previously suggested (1), the observed intermolecular RNA:RNA hybridization between these two RNA species. Also indicated are the complementary oligonucleotides (5'-31mer and 3'-39mer) used in this study for Southern and Northern blot analysis.

Fig. 1. Primary sequence of mouse 4.5S hybRNA. Designated are the synthetic DNA oligonucleotides complementary to mouse 4.5S hybRNA used in the hybridization experiments of this study.

Genomic Organization and Expression of Mouse 4.5S hybRNA

The 39mer oligonucleotide complementary to the 3'-terminal region of mouse 4.5S hybRNA was radiolabeled and used as a probe in Southern blot analysis to examine the genomic organization of this 1mRNA gene in mouse as well as other rodents. Cleavage of the mouse DNA with restriction endonucleases *Bam*HI and *Pst*I produced single restriction fragments recognized by this 39mer probe (Figure 2). Digestion with restriction endonucleases *Bgl*II, *Eco*RI, and *Hind*III similarly produced single, recognized restriction fragments of 7.5, 9.4, and 7.0 kbp, respectively (data not shown). Digestion with restriction endonuclease *Mae*I, an enzyme which cuts the 4.5S hybRNA gene sequence near the middle of the recognition sequence for this probe, yielded no DNA restriction fragments recognized by this probe. These combined results suggest a single copy gene for 4.5S hybRNA in the mouse genome. Similar Southern blot analysis of rat and hamster genomic DNA revealed recognition of single restriction fragments by the 39mer probe, again suggesting a single copy gene for 4.5S hybRNA in these related rodents (Figure 2). The differing size of the recognized *Pst*I restriction fragments of mouse, rat, and hamster indicated that this 1mRNA gene is embedded within or surrounded by non-conserved DNA sequence.

The expression of mouse 4.5S hybRNA in various mouse tissues was examined by Northern blot analysis (Figure 3). Results demonstrated 4.5S hybRNA expression in brain, heart, intestine, kidney, liver, and spleen, suggesting that this 1mRNA is expressed in all tissue/cell types and is not a tissue-specific 1mRNA. Longer exposure of this autoradiogram or others examining 4.5S hybRNA expression never revealed any larger RNAs recognized by the 4.5S

Fig. 2. Restriction analysis of mouse, rat, and hamster 4.5S hybRNA genes. Isolated mouse, rat, and hamster DNA was digested with designated restriction endonucleases, resolved on agarose gels, and blotted to nitrocellulose paper. Southern blots were probed with the DNA oligomer (39mer) complementary to the 3' end of mouse 4.5S hybRNA as detailed in Materials and Methods. Molecular weight markers are designated. Restricted mouse (lanes 1-3), hamster (lanes 4,5), and rat (lane 6).

hybRNA probes, indicating that 4.5S hybRNA is not a degradation product nor a processed species of a larger RNA transcript.

Conservation of 4.5S hybRNA and Its Expression as a 1mRNA Transcript in Evolution

The existence of homologous genes in other organisms was subsequently examined by Southern blot analysis of restricted genomic DNA from a variety of eukaryotic organisms as well as *E. coli*. The results of Figure 4 demonstrate the existence of 4.5S hybRNA-homologous genes in all eukaryotes examined as well as recognition of restriction fragments in the digested *E. coli* DNA. The recognized restriction fragments of mouse are more difficult to see in this Southern blot because of the lower stringency wash conditions used to reveal those hybridizing fragments of evolutionarily distant organisms. The molar ratio of bacterial-to-mammalian-to-amphibian DNA in this blot varies by approximately 1000 fold. Even so, the recognition by the 39mer oligonucleotide probe of only one or a few restriction fragments in each sample suggests

Fig. 3. Northern blot analysis of mouse 4.5S hybRNA expression in different mouse tissues. Total RNA samples from mouse brain (B), heart (H), intestine (I), kidney (K), liver (L), and spleen (S) were prepared, resolved on a 10% polyacrylamide-7 M urea gel, electroblotted to Zeta Probe paper, and probed with the 39mer DNA oligonucleotide complementary to the 3' end of mouse 4.5S hybRNA as detailed in Materials and Methods. The final washes (x2) for this Northern blot were in 0.1xSSC buffer, pH 7.0, containing 0.1% SDS at 55°C.

Fig. 4. Restriction analysis of evolutionarily diverse genomes for the presence of the 4.5S hybRNA gene sequence. Isolated DNA was digested with restriction endonucleases *Pst*I (P) or *Eco*RI (E), resolved on an agarose gel, electroblotted to Zeta Probe paper, and probed with the radiolabeled DNA 39mer oligonucleotide as described in Materials and Methods. DNA samples and molecular weight markers are designated.

that the 4.5S hybRNA-homologous genes in each organism are present in single or low copy number.

Expression of these homologous genes was then examined by Northern blot analysis. Samples of total RNA from *E. coli*, yeast, *Drosophila melanogaster*, *Xenopus laevis*, HeLa cells, mouse, rat, and hamster were probed with either the 3'-terminal 39mer or 5'-terminal 31mer 4.5S hybRNA-complementary probes (Figure 5). 4.5S hybRNA-homologous 1mwRNA transcripts of approximately 130 and 100 nucleotides in length were detected in yeast and *Xenopus laevis*, respectively, as compared with the mouse and rat/hamster 4.5S hybRNAs of 87 nucleotides in length. (The appearance of apparently larger 4.5S hybRNA transcripts in the mouse sample is due to the smearing of this particular RNA sample on this particular RNA blot.)

Differential recognition of the 31mer vs. 39mer probes to the various 4.5S hybRNA homologs after blot washing under different stringency conditions indicated, not surprisingly, a variation in primary sequence of the different hybRNA species. Comparison of hybrid strength of the 31mer vs. 39mer indicated that the primary sequence of the 3' end of each 4.5S hybRNA homolog was more conserved than the 5' end. The 5'-31mer did not hybridize with the yeast sequence even under low stringency washing conditions. Hybridization of the 3'-terminal 39mer to the yeast and *Xenopus laevis* 4.5S hybRNA homologs was either lost or reduced upon washing the blot at higher stringency conditions, indicating, as one might expect, divergence of primary sequence between fungal, amphibian, and mammalian 4.5S hybRNA homologs. The three rodent 4.5S hybRNAs were recognized by the 5'-31mer or 3'-39mer probes even under very stringent washing conditions (data not shown), indicating a strong conservation of primary sequence for this 1mwRNA in rodents. It is not clear at the present time why 4.5S hybRNA 1mwRNA transcripts are not observed in the total RNA samples of HeLa cells, *D. melanogaster*, and *E. coli*. It is possible the growth or culture conditions used in preparing these organisms for RNA isolation were not those which induce the synthesis of this 1mwRNA species.

To verify that the rat, hamster, *Xenopus laevis*, and yeast 1mwRNA transcripts recognized by the mouse 39mer probe were indeed hybRNA homologs, the region immediately upstream to the annealed 39mer probe for each homolog was sequenced by primer extension using reverse transcriptase. Figure 6 shows that the rat and hamster homologs exhibit almost identical sequences to the mouse 4.5S hybRNA. The *Xenopus laevis* and yeast homologs, consistent with their different sizes as compared to the mouse hybRNA, exhibit lower sequence

Fig. 5. Expression of 4.5S hybRNA genes as 1mRNA transcripts in evolutionarily diverse organisms. Total RNA samples isolated from *E. coli*, yeast (*Saccharomyces cerevisiae*), *Drosophila melanogaster*, *Xenopus laevis*, HeLa cells, mouse, rat, and hamster were resolved on 10% polyacrylamide-7 M urea gels, electroblotted to Zeta Probe paper, and hybridized as detailed in Materials and Methods with either the DNA 31mer or 39mer oligonucleotides complementary to the 5' end and 3' end of mouse 4.5S hybRNA, respectively. Northern blots were initially washed (x2) at low stringency (2xSSC, pH 7.0, 0.1% SDS at 37°C). The blot probed with the 3'-39mer probe was then rewashed (x2) under higher stringency conditions (0.5xSSC, pH 7.0, 0.1% SDS at 55°C).

homology but still are clearly recognizable as hybRNAs. The low homology of the yeast hybRNA in this 5' region is consistent with the Northern blot hybridization results of Figure 5.

Conservation of 4.5S hybRNA Intermolecular RNA:RNA Hybridization with Eukaryotic 18S rRNA

To examine the ability of 4.5S hybRNA homologs to intermolecularly hybridize with 18S rRNA as has been observed for the mouse 4.5S hybRNA sequence, yeast and *Xenopus laevis* homologs were isolated using the 3'-39mer oligonucleotide for 1mRNA hybrid-selection. Shown in Figure 7 are the hybrid-selected 1mRNA sequences obtained from yeast and *Xenopus laevis* total 1mRNA fractions. Arrows indicate the 4.5S hybRNA homologs of each selected RNA population. To maximize the amount of homolog selected, low stringency wash conditions were used which led to contamination of the homologs by other 1mRNA transcripts (non-specific hybridization/selection). Each hybrid-

5'ACGCUGUGAUGAUGGAUUC AAAACCAUUCGUAGUUUCCACCAGAAGU — MOUSE
 |||||
 5'nCGCUGUGAUGAUGGAUUC AAAACCAUUCGUAGUUUCCACCAGAAa — RAT
 |||||
 5'nCGCUGUGAUGAUGGAUUCnAAAACCAUUCGUAGUUUCCACnAGAAa — HAMSTER
 |||||
 5' +~40 AC GGUA-GGAGUACGC-UUAC-GAACCCAU-CGUUAGUACUCACGGUGA — YEAST
 |||||
 5'ACGCUGUGAUGAUGGA-UUCCAAAACC-AUUCGU-AGUUUCCACCA-GAAGU — MOUSE
 |||||
 5' +~20 GGAAUUCCAAAGCC-AUUCGU-AGUUUCCACCA-GAAGU — XENOPUS
 |||||

Fig. 6. Primary sequence of the 5' regions of rat, hamster, *Xenopus laevis*, and yeast hybRNA homologs.

Fig. 7. Hybrid-selection of yeast and *Xenopus laevis* 4.5S hybRNA 1mwRNA homologs. Isolated fractions of yeast and *Xenopus laevis* 1mwRNA were labeled at the 3' terminus with [32 P]pCp using T4 RNA ligase. Labeled 1mwRNAs were then hybridized to the 39mer DNA oligonucleotide complementary to the 3' terminus of mouse 4.5S hybRNA that had been covalently attached to dot blots as outlined in Materials and Methods. Hybridized 1mwRNAs containing the yeast and *Xenopus laevis* 4.5S hybRNA homologs were eluted and analyzed on a 10% polyacrylamide-7 M urea gel. A) Total 1mwRNA fractions of *Xenopus laevis* (lane 1) and yeast (lane 2) 3'-terminally labeled with [32 P]pCp. Molecular weight markers are designated. B) Hybrid-selected *Xenopus laevis* 1mwRNAs (lane 1), yeast 1mwRNAs (lane 2), and mouse 4.5S hybRNA marker (lane 3). Arrows designate the *Xenopus laevis* and yeast 4.5S hybRNA homologs which were cut from the gel and used in intermolecular RNA:RNA hybridization experiments.

Fig. 8. Intermolecular RNA:RNA hybridization between yeast and *Xenopus laevis* 4.5S hybRNA homologs and 18S rRNA. Total RNA samples isolated from mouse (lanes 1), rat (lanes 2), hamster (lanes 3), HeLa cells (lanes 4), *Xenopus laevis* (lanes 5), yeast (lanes 6), *E. coli* (lanes 7) were resolved on 1.5% agarose-formaldehyde gels and electroblotted to Zeta Probe paper. Northern blots were then probed with yeast or *Xenopus laevis* 4.5S hybRNA homologs isolated by hybrid-selection under hybridization conditions detailed in Materials and Methods, and the intermolecular RNA:RNA hybrids detected by autoradiography. A) EtBr-stained gel of resolved RNA samples. Large rRNA species are designated. B) Northern blot of resolved RNA samples probed with the *Xenopus laevis* 4.5S hybRNA homolog. C) Northern blot of resolved RNA samples probed with the yeast 4.5S hybRNA homolog.

selected 4.5S hybRNA homolog represents less than 0.5% of the applied radio-labeled 1mRNA fraction. Based upon their deduced molecular weights, the yeast and *Xenopus laevis* homologs were purified by excision from the gel and then hybridized to total RNA populations isolated from mouse, rat, hamster, human, *Xenopus laevis*, yeast, and *E. coli* resolved on Northern blots. Each 4.5S hybRNA homolog hybridized to heterologous as well as homologous eukaryotic 18S rRNA sequences demonstrating the conservation of complementary sequences in each RNA across the evolutionary scale of eukaryotes (Figure 8). This recognition was specific for the 18S rRNA as evidenced by the lack of intermolecular RNA:RNA base-pairing with any other cellular RNA. (The hybridization of each 4.5S hybRNA to transcripts smaller than 18S rRNA in the hamster sample is due to a partial degradation of this RNA population, as can be seen in the EtBr-stained gel of this RNA in Figure 8, Panel A). The yeast 4.5S hybRNA homolog uniquely recognized *E. coli* 16S and 23S rRNA species. The

observed hybridization of the yeast hybRNA homolog to 18S rRNA is not due to 5S rRNA (a possible contaminant of the hybrid-selected material which migrates very closely to the yeast hybRNA sequence in the purification gel), since we have shown that this yeast 1mw rRNA species does not intermolecularly base-pair with 18S rRNA, unlike the situation for other eukaryotic 5S rRNAs (9,11).

DISCUSSION

Southern blot analysis of isolated genomic DNAs has shown the presence of 4.5S hybRNA-homologous genes in widely divergent eukaryotic organisms from fungi to mammals. The single or low number of hybridizing restriction fragments in all these organisms suggests a single or low copy number for this 1mwRNA gene. The presence of 4.5S hybRNA-homologous genes in fungi and amphibians, as well as mammals, is supported by Northern blot analysis demonstrating the transcription of these genes to produce 1mwRNA species. Initial genomic analysis has also indicated that a 4.5S hybRNA-homologous gene(s) is present in *E. coli*, suggesting its possible presence in prokaryotes as well as eukaryotes. However, at the present time we are cautious about this interpretation for two reasons. First, Northern blot analysis has not revealed an apparent 1mwRNA/RNA transcript synthesized from this potential gene(s). Second, it is possible that since both the mouse (1) and yeast hybRNA sequences are able to intermolecularly base-pair with *E. coli* 16S and/or 23S rRNAs, the restriction fragments recognized by the mouse 4.5S hybRNA probe may simply be cross-hybridization with the 16S/23S rRNA genes. Further screening of other prokaryotic DNAs, as well as additional investigation of the recognized *E. coli* restriction fragments, is necessary before any conclusions can be reached.

The conservation of 4.5S hybRNA genes and their expression as 1mwRNA transcripts in such evolutionarily divergent eukaryotes as yeast, *Xenopus laevis*, and mice strongly suggest that this 1mwRNA sequence is retained in all eukaryotes to carry out a common and biologically required function. Its expression in all mouse tissues examined indicates a function that is required in all cell types. We originally suggested that because of its intermolecular hybridization capabilities with mouse mRNA and 18S rRNA sequences, two cellular RNAs involved in protein synthesis, that mouse 4.5S hybRNA may be involved in regulating mRNA translational events and the synthesis of new cellular proteins. Such examples of 1mwRNAs regulating protein synthesis have been reported previously in both prokaryotes (18) and eukaryotes (24). However, initial cell biology experiments with mouse ascites cells have demonstrated

18S rRNAs	5' ————— 3'
<i>HOMO SAPIENS</i>	A C A U C C A A G G - A A G G
<i>RATTUS NORVEGICUS</i>	A C A U C C A A G G - A A G G
<i>RATTUS NORVEGICUS</i>	A C A U C C A A G G - A A G G
<i>ORYCTOLAGUS CANICULUS</i>	A C A U C C A A G G - A A G G
<i>XENOPUS LAEVIS</i>	A C A U C C A A G G - A A G G
<i>ARTEMIA SALINA</i>	A C A U C C A A G G - A A G G
<i>SACCHAROMYCES CEREVISIAE</i>	A C A U C C A A G G - A A G G
<i>DICTYOSTELIUM DISCOIDEUM</i>	A C U U C U A C G G - A A G G
<i>OXYTRICHA NOVA</i>	A C A U C U A A G G - A A G G
<i>STYLONYCHIA PUSTULATA</i>	A C A U C U A A G G - A A G G
<i>TETRAHYMENA THERMOPHILA</i>	A C A A C U A C G G U U C G G
<i>ORYZA SATIVA</i>	A C A U C C A A G G - A A G G
<i>ZEA MAYS</i>	A C A U C C A A G G - A A G G
<i>GLYCINE MAX</i>	A C A U C C A A G G - A A G G
 <u>16S rRNA</u>	
<i>ESCHERICHIA COLI</i>	A C U C C U A C G G - G A G G

Fig. 9. Evolutionary conservation of a potential 4.5S hybRNA-complementary sequence in eukaryotic 18S rRNAs (25).

that mouse 4.5S hybRNA is primarily (>90%) located in the nucleus. While not necessarily inconsistent with our original suggestion, these results put into question the hypothesis of a ribosomal-associated or even cytoplasmic function for mouse 4.5S hybRNA. Current experiments are examining in more detail the cell biology of mouse and yeast hybRNAs to gain more insight into the biological function of this lmrRNA species.

Most striking in these experiments is the demonstration that the yeast and *Xenopus laevis* 4.5S hybRNA homologs, like the mouse 4.5S hybRNA sequence, are able to intermolecularly base-pair with 18S rRNA. Heterologous as well as homologous hybRNA:18S rRNA intermolecular base-pairing demonstrates the conservation of the complementary sequences for hybrid formation in both the 4.5S hybRNA homologs and the eukaryotic 18S rRNA sequences. We have suggested

previously, from computer analysis, that this observed hybrid may be due to base-pairing of a region located at the 3' end of mouse 4.5S hybRNA with a 14-nucleotide sequence (nucleotides 456-469) in mouse 18S rRNA. This 4.5S hybRNA-complementary sequence is evolutionarily conserved in other eukaryotic 18S rRNAs (25), lending support for the involvement of this sequence in 4.5S hybRNA:18S rRNA hybrid formation (Figure 9). The fact that the primary sequence in the 3' region of 4.5S hybRNA sequence, that region which contains the proposed 18S rRNA-complementary sequence, appears to be more conserved than the 5' end is consistent with the involvement of this nucleotide sequence in hybrid formation. The corresponding region in prokaryotes, totally conserved among bacterial 16S rRNA species (25), is sufficiently different from its eukaryotic counterpart to suggest that it would not allow a sufficiently stable RNA:RNA hybrid to be formed with the mouse and Xenopus laevis hybRNA sequences. Explanation of the observed intermolecular base-pairing between prokaryotic 16S rRNA and the yeast hybRNA species awaits further sequence analysis of the yeast hybRNA homolog.

The conservation of intermolecular RNA:RNA hybridization capabilities with 18S rRNA for all the examined 4.5S hybRNA homologs suggests that this interaction is important in the biological function of this 1mwRNA species. Intermolecular RNA:RNA base-pairing is an important feature in the functioning of many if not most 1mwRNA sequences of both eukaryotic and prokaryotic organisms. The extension of this precedent to 4.5S hybRNA sequences is suggested by the data presented here, but will remain uncertain until a cellular function for this RNA is determined.

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