Three novel genes encoding small RNAs homologous to human and mouse RNase P RNA have been isolated from a mouse genomic library. As assessed by Northern blot analysis and nuclease protection assays, transcripts derived from one or more of these genes are expressed in murine cells and tissues. The RNA products of these RNase P RNA-homologous genes are smaller in size (238–248 nucleotides) than the 305-nucleotide transcript previously identified. These smaller transcripts are uniformly less abundant than the larger RNase P RNA, but their expression varies severalfold among different mouse tissues. Similar short homologues of RNase P RNA also are expressed in rat, rabbit, and human cells. We conclude that higher vertebrates express multiple isoforms of RNase P RNA.

RNase P is a site-specific endoribonuclease that cleaves tRNA precursor molecules to generate the 5′ termini of mature tRNAs in both prokaryotic and eukaryotic cells (1–3). The holoenzyme is a ribonucleoprotein, the RNA subunit of which, termed RNase P RNA (RPR), exhibits considerable variability in size among different species, ranging from 140 to 490 nucleotides in length (4–6). The RNA moiety alone, as isolated from Escherichia coli or Bacillus subtilis, is capable of catalyzing the site-specific cleavage reaction in vitro in the absence of its apoprotein (7, 8). Eukaryotic RNase P, in contrast, requires assembly of an RN-holoenzyme for activity (9–12).

In yeasts, RNase P is compartmentalized; both the apoprotein and RNA components of mitochondrial RNase P are distinct and arise from different genes than those encoding components of the nuclear enzyme (4, 13–16). In Saccharomyces cerevisiae, the protein subunit of mitochondrial RNase P is derived from a nuclear gene (15, 16), whereas the mitochondrial RPR is encoded within the mitochondrial genome (4). In mammalian cells, RNase P activity is present in both nuclear and mitochondrial fractions (11, 17), but only a single form of RPR has been identified (18, 19), and no RPR-homologous sequences can be identified within the more compact mammalian mitochondrial genome.

The present study was designed to test the hypothesis that mammals express multiple isoforms of RPRs. We screened a mouse genomic library using a PCR-amplified mouse RPR sequence as the probe. Three new genes encoding homologues of RNase P RNA (RPRH) were isolated and sequenced. All of these encode RNA molecules with a high degree of sequence identity to the human and mouse RPR genes published previously (18, 19) but are smaller in size. Transcripts derived from one or more of these novel genes are expressed in murine cells, and multiple sizes of RPR-related transcripts are expressed in other mammalian species.

**EXPERIMENTAL PROCEDURES**

Cloning—The 305 base pairs of mouse RPR coding region was PCR-amplified with two primers (RPS3, ATAGGGCGGAGGAAGCTCATCA, and RPS3, ACCTATAAGGCGGAGGAAGTCATC) synthesized from the human RPR sequence (18). An 1295SV mouse genomic library (Stratagene, La Jolla, CA) was screened using the primer-extended RPR probe (20). Duplicate nylon membrane filters were lifted from the λ phage library plates, hybridized to the mouse RPR probe in 2× PIPES/0.5% SDS/100 μg/ml of denatured salmon sperm DNA/mL, and washed at low and high stringencies (i.e. 0.1× SSC (0.15 m NaCl, 0.015 m sodium citrate)/0.1% SDS at 57 or 62 °C). The purified clones were further evaluated by a PCR-based assay using the RPR primers, RPS3 and RPS3. The λ clones that were positive by plaque hybridization but negative in a PCR assay (no amplification of a 305-base pair RPR fragment) were chosen for detailed analysis. DNA was prepared from those clones (21) and characterized by restriction mapping and Southern blot hybridization. DNA fragments containing RPRH sequences were subcloned into pBluescript or pUC18 vectors and sequenced using an automatic sequencing system (Applied Biosystems, Inc., Foster City, CA). The sequencing data were compiled using DNASTAR (DNASTAR, Inc., Madison, WI) and GGCG programs (Genetic Computer Group, Inc., Madison, WI).

Northern Blot Hybridization—Total RNAs were isolated from cultured cells or animal tissues by CsCl gradient precipitation following direct lysis in 4 m guanidinium thiocyanate and 10 m Tris-HCl, pH 8.0 (22). The RNA samples were denatured in 50% formamide at 85 °C, electrophoresed through 6% denaturing polyacrylamide gels containing 1× TBE buffer (0.89 m Tris boric acid, 0.02 m EDTA, pH 8.3) and 8 m urea, electroblotted onto nylon membranes in 0.5× TBE at 25 volts for 1 h using an electrotransferring apparatus (PROTEAN II, Bio-Rad, Hercules, CA), and hybridized with RPR or RPRH cDNA probes as described previously (23).

Southern Blot Hybridization—Genomic DNA was isolated from mouse Sd 8 myogenic cells, rat and rabbit kidney, and human WI-38 cells (a diploid cell line), digested with restriction enzymes, and electrophoresed through 0.8% agarose gels. Hybridization was performed in the same buffer as Northern blot hybridization. Filters were washed in 0.1× SSC/0.1% SDS at various temperatures.

S1 Nuclease Mapping—Single-stranded DNA probes were prepared from the RPRH4 gene using a unique EcoRI site for end labeling. The probe for 5′ end mapping was made from a PCR fragment encompassing the RPRH4 gene, which was digested with EcoRI and labeled at the 5′ end by T4 kinase. The antisense strand extending from nt 207 to nt 417 (numbered as in Fig. 2) was purified through a denaturing polyacrylamide gel. The probe for 3′ end mapping was made from a 950-base pair PvuI fragment containing the RPRH4 gene, which was used as the template for unidirectional PCR with an M13 forward primer. The antisense strand of the DNA fragment was replaced by the newly synthesized PCR strand that is 70 nucleotides shorter than the

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) U31003 (RPRH2), U31227 (RPRH3), and U31228 (RPRH4).

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with EcoRI and labeled at 3' ends using Klenow DNA polymerase and [\alpha^32P]dATP. The 3' labeled antisense strand DNA spanning the 3' region of RPRH4 (nt 416–700) was purified on a denaturing polyacrylamide gel. S1 nuclease mapping was performed following published methods (24). Briefly, the DNA probes (10^6 cpm) were hybridized overnight with 30–40 µg of total cellular RNA in 5 µl of buffer containing 2 x PIPES/0.4 M NaCl/80% formamide at 30°C. The hybridization mixture was diluted and subjected to S1 nuclease digestion at 30°C for 1 h. The protected DNA fragments were resolved on sequencing gels.

RESULTS

Identification and Cloning of RPR-related Genes—To assess the possibility that the mouse genome includes multiple genes homologous to RNase P RNA, we performed genomic Southern blot hybridization under various stringencies. Following digestion with each of several restriction enzymes, multiple bands remained after washing under high stringency conditions (Fig. 1). Because the cleavage sites of the restriction enzymes used in Fig. 1 are not present in the mouse 305-nt RPR sequence (19), the RPRH genes that differ from the published mouse RPR sequence are mapped by S1 nuclease protection assays. Nucleotide positions in the flanking region of RPRH genes that differ from the published mouse RPR sequence are shown in lowercase, and nucleotides within the 5' and 3' flanking regions at which complete divergence begins are italicized.

Fig. 1. Mouse genomic DNA blot. 10 µg of mouse DNA were digested with each of five restriction enzymes, as indicated, and hybridized with the mouse RPR cDNA probe. kb, kilobase pairs.

Sequence Analysis of Three RPRH Genes—As shown in Fig. 2, all three RPR genes include a putative RNA coding region that is closely related to human and mouse RNase P RNA (18, 19). The putative RPRH transcripts start immediately following a TATA-like motif and end at a putative termination signal (TTTT) for transcription by RNA polymerase III (25, 26) and present in the human RPR gene (18, 27) could not be identified in any of the three RPRH genes, suggesting that expression of these genes may be controlled in a distinctive manner.

Identification and Cloning of RPR-related Genes

Characterization of RPRH Transcripts—Northern blot hybridization of total RNAs isolated from mouse tissues showed three major RNA species estimated to be 305, 248, and 238 nucleotides in length, respectively (Fig. 3). The largest RNA species (305 nt) for the published mouse RPR sequence are shown in lowercase, and nucleotides within the 5' and 3' flanking regions at which complete divergence begins are italicized.

Characterization of RPRH Transcripts

Fig. 3. Southern blot analysis of mouse genomic DNA. 

Identification and Cloning of RPR-related Genes—To assess the possibility that the mouse genome includes multiple genes homologous to RNase P RNA, we performed genomic Southern blot hybridization under various stringencies. Following digestion with each of several restriction enzymes, multiple bands remained after washing under high stringency conditions (Fig. 1). Because the cleavage sites of the restriction enzymes used in Fig. 1 are not present in the mouse 305-nt RPR sequence (19), the RPRH genes that differ from the published mouse RPR sequence are mapped by S1 nuclease protection assays. Nucleotide positions in the flanking region of RPRH genes that differ from the published mouse RPR sequence are shown in lowercase, and nucleotides within the 5' and 3' flanking regions at which complete divergence begins are italicized.
RNAs are at least 3-fold more abundant in kidney and liver than in heart and skeletal muscle, whereas the 305-nt RNA is expressed at relatively high levels in all tissues.

To verify that the 238- and 248-nt RNAs are indeed transcribed from RPRH gene(s) and not generated by partial degradation of the 305 nt of RNase P RNA, we performed S1 nuclease protection mapping using two single-stranded antisense DNA probes prepared from the RPRH4 gene (Fig. 4). The 5' end protection probe, extending 210 nucleotides upstream from the EcoRI site, detected two transcription start sites (Fig. 4A). One of the start sites is located at 127/126 nt upstream from the EcoRI site, a position that corresponds to the 5' end of the 238-nt RNA transcript predicted from the RPRH4 gene. The other start site is located 135–138 nt from the EcoRI site, corresponding to the 5' end of a 248-nt RNA transcript predicted from RPRH4 gene. The 3' end protection probe, extending 347 nucleotides downstream from the EcoRI site, detected a RNA transcript ending 112 nt downstream from the EcoRI site (Fig. 4B), consistent with the predicted 3' end of RPRH4 transcript.

These fragments of probes based on the RPRH4 gene, when bound to their complementary RNA sequences from murine cells, were resistant to high concentrations of S1 nuclease, providing evidence that the 238- and 248-nt RNA species are not degradation products of the 305-nt RPR transcript but bona fide products of the RPRH4 gene. The 3' end analysis further supports this conclusion in that the predicted hybridization product formed between the RPRH4 probe, and the 305-nt RPR transcript (115 nt) is present and abundant at low concentrations of S1 nuclease (Fig. 4B) but disappears at the higher concentrations of S1 nuclease necessary to digest single base mismatches with the probe. Spatial relationships between these probes and transcripts derived from the RPR and RPRH4 genes are summarized in Fig. 4C.

Multiple Isoforms of RPR-related Genes and RNAs in Other Mammals—To investigate the distribution of the RPR-homologous genes in other mammalian species, we performed genomic Southern blot hybridization by using restriction enzymes that do not cut the published RPR sequences (18, 19).
Southern blot analyses demonstrated that multiple bands are present in rat and rabbit DNAs digested by various enzymes (Fig. 5, A and B). We estimate that rat, like mouse, has four RPR isogenes and that rabbit has at least two RPR isogenes. The human RPR cDNA detected at least two bands (Fig. 5C): a strong band corresponding to the RNase P RNA gene and a weak band that may represent a RPR-related gene.

Northern blot hybridization using the mouse and human RPR sequence to probe RNAs isolated from rat, rabbit, and human cells also revealed at least two major RPR isoforms in each of these mammalian species: a larger RPR that corresponds to the 305 nt form in the mouse, and a smaller and less abundant band that corresponds to the RPRH transcripts we have identified in murine cells (Fig. 6). When human RNA and genomic DNA were probed with the mouse, instead of human, RPR sequence, only the RNase P RNA but not the RPR homologues was detected (data not shown), suggesting that the human RPRH is more diverged from the RNase P RNA than those in mouse, rat, and rabbit. In summary, these data indicate that most, if not all, mammalian species contain and express multiple genes encoding isoforms of RNase P RNA.

DISCUSSION

Genes encoding an RNase P RNA were cloned previously from human and mouse and encode transcripts ranging from 305 nt in mice to 341 nt in humans (18, 19). We have cloned three new murine genes homologous to the published RPR sequences, but predicted to generate shorter RNA transcripts. Within the group of RPRH genes, RPRH2 has diverged from the RPRH3 and RPRH4 genes both within the transcribed regions and within flanking sequences, resulting in transcriptional termination signals. Within the group of RPRH genes, RPRH2 has diverged from the RPRH3 and RPRH4 genes both within the transcribed regions and within flanking sequences, suggesting that these genes are derived from a cloning artifact. First, BamHI fragments of lambda clones that contain RPRH genes are matched in size to BamHI fragments detected in a mouse genomic Southern blot. Second, two of the novel RPRH gene sequences (RPRH2 and RPRH4) were present in two or more independent lambda clones containing overlapping mouse genomic DNA fragments and an identical RPRH sequence. Other data demonstrate that the RPRH sequences that we have identified are not pseudogenes. Transcripts corresponding in size to the products predicted from RPRH genes are present in murine cells, and nuclease protection experiments confirm that the shorter forms of RPR-homologous RNA observed in Northern blots are not degradation products of the 305-nt RPR. Short forms of RPR-homologous RNA also are present in rat, rabbit, and human cells.

The structure and function of RNase P RNAs have been studied by computer modeling (28), chemical cleavage (29), nuclease protection (30), and mutational or phylogenetic analyses (31–34). Although the size and sequence of RNase P RNAs have diverged considerably during evolution, a similar three-dimensional structure appears to be conserved (1–3, 19, 28, 32, 33). The 305-nt mouse RPR is predicted to have the core structure common to other RNase P RNAs, which includes three major rings formed by internal base pairing (19). The tertiary structure is established through base pairing between the rings (19). Because of their shorter length, mouse RPRH transcripts may form only two of the three rings of the common RNase P RNA core structure.

The functional properties of RPRH transcripts in mammalian cells have not yet been determined. Truncated forms of human RPRs retain enzymatic activity in reconstitution assays in vitro (35), suggesting that RPRH transcripts potentially serve as functional components of holoenzyme complexes. An interesting alternative possibility is that these naturally occurring truncated forms of RNase P RNA may be capable of binding to the apoprotein constituents of RNase P ribonucleoprotein complexes but are enzymatically inactive due to the disruption of the RNA tertiary structure. In this way, RPRH gene products could function as negative regulators of RNase P. It should be possible, in future studies, to assess the functional characteristics of the RPRH gene products that we have identified, as well as their protein subunit and subcellular distribution. Because yeast expresses different forms of RNase P RNA in mitochondria and the nucleus (4, 13, 14), perhaps one or more of the RPRH transcripts will have a similarly compartmentalized function.

Comparison of the primary sequences of the previously defined mouse RPR gene and the new RPRH genes also suggests certain interesting possibilities concerning their evolutionary origins. An analysis using a Jotun Hein alignment method (DNASTAR), as shown in Table I, indicates that all three RPRH genes are clearly related to each other but diverge considerably from the RPR gene downstream of their 3’ transcriptional termination signals. Within the group of RPRH genes, RPRH2 has diverged from the RPRH3 and RPRH4 genes both within the transcribed regions and within flanking sequences,
whereas the latter two genes are almost identical throughout the entire 1-kilobase pair region we sequenced. These comparisons suggest that the present diversity within this gene family has arisen from sequential gene duplication events.

In summary, three novel genes encoding sequences homologous to RNase P RNA have been isolated from a mouse genomic library. Transcripts derived from one or more of these RPRH genes are expressed in murine cells, and multiple sizes of RPR-related transcripts are present in other mammalian species as well. We conclude that higher vertebrates express multiple isoforms of RNase P RNA.

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| TABLE I |
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| Comparison of the putative RNA-transcribing regions between the three RPRH genes and the previously reported RNase P RNA gene using a Jotun Hein alignment method (DNASTAR) The upper right part shows the percentage of similarity. The lower left part shows the percentage of divergence. |
| | RPR | RPRH2 | RPRH3 | RPRH4 |
| RPR | 90.0 | 92.0 | 93.0 |
| RPRH2 | 51.0 | 88.0 | 89.0 |
| RPRH3 | 47.1 | 13.9 | 92.0 |
| RPRH4 | 46.8 | 12.2 | 7.8 |
Cloning and Characterization of Three New Murine Genes Encoding Short Homologues of RNase P RNA
Kang Li and R. Sanders Williams

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