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Mouse ribonuclease III. cDNA structure, expression analysis, and chromosomal location

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Abstract

Background: Members of the ribonuclease III superfamily of double-stranded(ds)-RNA-specific endoribonucleases participate in diverse RNA maturation and decay pathways in eukaryotic and prokaryotic cells. A human RNase III orthologue has been implicated in ribosomal RNA maturation. To better understand the structure and mechanism of mammalian RNase III and its involvement in RNA metabolism we determined the cDNA structure, chromosomal location, and expression patterns of mouse RNase III.

Results: The predicted mouse RNase III polypeptide contains 1373 amino acids (~160 kDa). The polypeptide exhibits a single C-terminal dsRNA-binding motif (dsRBM), tandem catalytic domains, a proline-rich region (PRR) and an RS domain. Northern analysis and RT-PCR reveal that the transcript (4487 nt) is expressed in all tissues examined, including extraembryonic tissues and the midgestation embryo. Northern analysis indicates the presence of an additional, shorter form of the transcript in testicular tissue. Fluorescent *in situ* hybridization demonstrates that the mouse RNase III gene maps to chromosome 15, region B, and that the human RNase III gene maps to a syntenic location on chromosome 5p13-p14.

Conclusions: The broad transcript expression pattern indicates a conserved cellular role(s) for mouse RNase III. The putative polypeptide is highly similar to human RNase III (99% amino acid sequence identity for the two catalytic domains and dsRBM), but is distinct from other eukaryotic orthologues, including Dicer, which is involved in RNA interference. The mouse RNase III gene has a chromosomal location distinct from the Dicer gene.

Background

The enzymatic cleavage of double-stranded(ds) RNA structures is an essential step in the maturation and decay of many eukaryotic and prokaryotic RNAs. Members of the ribonuclease III superfamily of endoribonucleases [1] are the primary agents of dsRNA cleavage [2]. RNase III orthologues are conserved in eukaryotes and in bacteria, with *Escherichia coli* RNase III [3] as the best characterized member. *E. coli* RNase III is active as a homodimer, and requires a divalent metal ion (preferably Mg^{2+}) to hydrolyze phosphodiesters, creating 5'-phosphate, 3'-hydroxyl product termini [4]. *E. coli* RNase III cleaves rRNA and mRNA precursors as part of the respective maturation

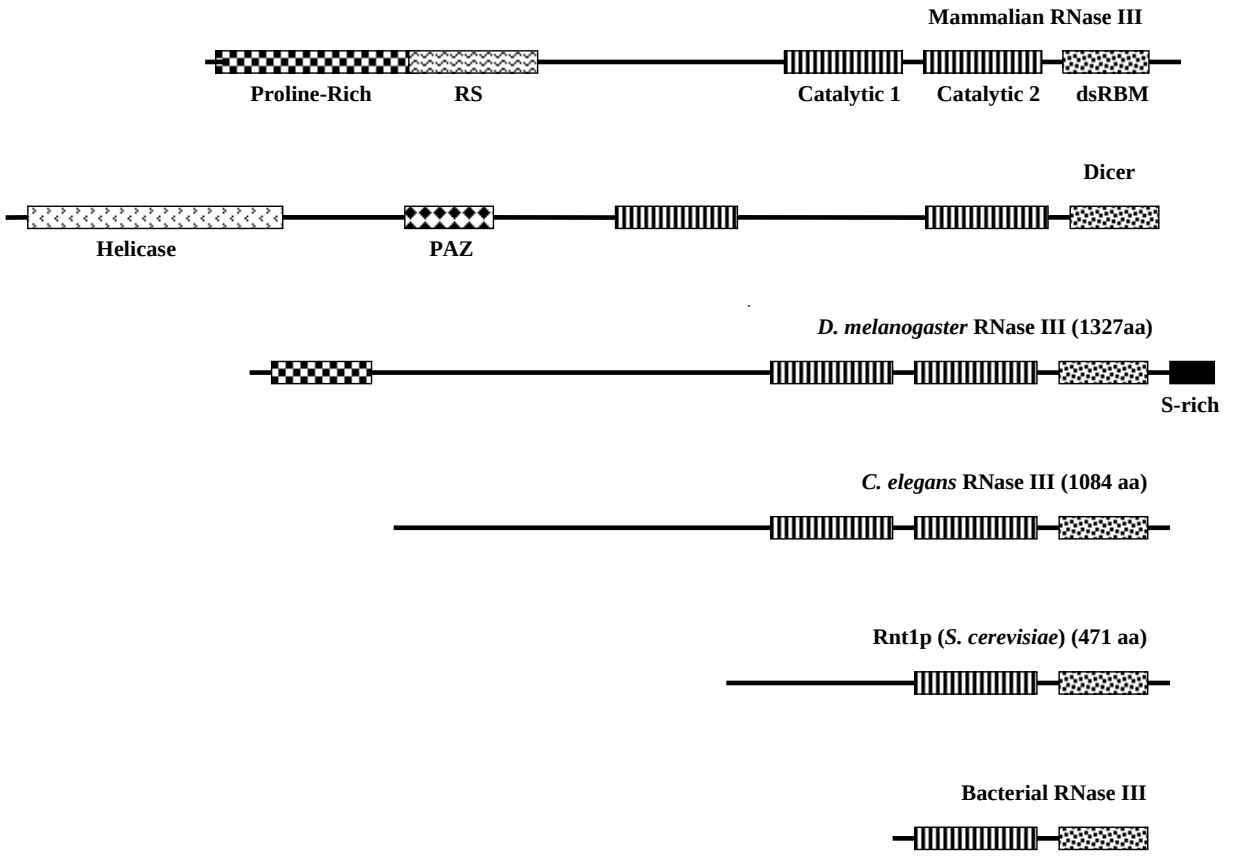

Figure 1
Ribonuclease III superfamily members. The orthologues include bacteria, yeast, *C. elegans*, *Drosophila*, mammalian RNase III, and mammalian Dicer. The textured boxes in the linear diagrams of the orthologues indicate the characteristic domain elements.

pathways. *E. coli* RNase III also initiates mRNA degradation, and participates in antisense RNA action [2,5–8].

Bacterial RNase III orthologues exhibit the simplest primary structure, which includes a C-terminal dsRNA-binding motif (dsRBM), and an N-terminal catalytic (nuclease) domain (Figure 1). The dsRBM is present in many other proteins that bind dsRNA [9,10] and is important for RNase III activity in *in vitro* [11]. The catalytic domain contains a number of conserved residues, including an 11 amino acid signature motif that is a distinguishing feature of RNase III orthologues. The isolated *E. coli* RNase III catalytic domain, which retains homodimeric behavior, can cleave dsRNA substrates under specific conditions *in vitro* [11]. The recently reported crystal structure of the catalytic domain of *Aquifex aeolicus* RNase III reveals an extensive subunit interface, with the cleft between the subunits predicted to bind dsRNA [12]. The two proposed active sites are positioned at each end of the intersubunit cleft and contain conserved residues from each subunit. Several highly conserved carboxylic acids in the active sites bind a single divalent metal ion [12], and one of these residues has been shown to be essential for catalytic activity of *E. coli* RNase III [13].

Two mammalian RNase III orthologues have been identified, and exhibit common domains as well as apparently unique features. The RNase III orthologue "Dicer" (Figure 1) plays a central role in RNA interference (RNAi) by cleaving dsRNAs to ~21 bp fragments, termed small interfering(si) RNAs. The siRNAs are incorporated into a macromolecular complex which carries out degradation of

homologous RNA sequences [14]. Dicer action serves to inhibit viral infection and retroposon movement, and also plays a role in developmental pathways by cleaving precursors to small regulatory RNAs (reviewed in [14,15]). The predicted sequence of the mouse Dicer polypeptide exhibits a single dsRBM and tandem catalytic domains. In addition, the N-terminal region contains a DExH/DEAH RNA helicase motif and a PAZ (Pinwheel-Argonaut-Zwille) domain, which is also present in other proteins involved in RNAi [16,17].

The second mammalian RNase III orthologue also exhibits a single C-terminal dsRBM and tandem catalytic domains, but is otherwise structurally distinct from Dicer as it lacks the helicase and PAZ domains (Figure 1) [18,19]. Preliminary evidence indicates that this RNase III orthologue participates in rRNA maturation. Thus, a reduction in human RNase III levels *in vivo* causes the accumulation of specific rRNA processing intermediates [18]. Consistent with this functional role, human RNase III localizes to the nucleolus in a cell-cycle-dependent manner [18]. A truncated form of human RNase III has been purified and shown to cleave dsRNA *in vitro* [18]. However, little else is known of the functional roles or the mechanistic features of this enzyme. We report here (i) the characteristics of the cDNA sequence and predicted polypeptide of mouse RNase III, (ii) demonstrate transcript expression patterns, and (iii) report the chromosomal locations of the mouse and human RNase III genes.

Results and Discussion

Features of the mouse RNase III cDNA sequence

The mouse RNase III cDNA structure was determined by sequence analysis of individual cDNA clones (see Materials and Methods), whose sequences relative to the full-length sequence are shown in Figure 2. The length of the mouse RNase III cDNA is 4487 nt (see Additional File 1), and the predicted initiation codon is at position 243. This prediction is based on the occurrence of three in-frame stop codons upstream of the indicated ATG, within the 242 nt 5'-untranslated region (5'-UTR) (see Additional File 1). It is not firmly established if the reported 5' end nucleotide corresponds to the true 5' end of the mRNA. However, several independent partial cDNA clones (see Figure 2) contain the same 5' nucleotide. There is no significant sequence similarity of the mouse 5'-UTR with that of human RNase III (245 nt). The 3'-UTR is 123 nt in size, and contains a consensus polyadenylation signal (AAUAAA) 12 nucleotides from the poly(A) tail. The 3'-UTR exhibits 87% identity with the human 3'-UTR (372 nt) over a ~76 nt region, corresponding to positions 37–113 of the mouse 3'-UTR and positions 39–115 of the human 3'-UTR. A search of the NCBI mouse EST database yielded five independent clones which exhibit 100 percent sequence identity with the 3'-UTR (data not shown). The predicted 3'-UTR of the mouse RNase III mRNA and that of the rat RNase III mRNA share 85% nucleotide identity, and have the same lengths (data not shown).

Features of the predicted RNase III polypeptide

The dsRBM and catalytic domains

Conceptual translation of the mouse RNase III cDNA, using the AUG at position 243 as the initiation codon, yields a polypeptide of 1373 amino acids with a predicted molecular mass of 158.8 kDa and a pI of 7.99. The polypeptide contains a single dsRBM and two RNase III catalytic domain elements (see Additional File 1). The RNase III domain (i.e. the two catalytic domains and dsRBM) of the mouse and human sequences exhibit 99% amino acid identity and 88% nucleotide sequence identity. Figure 3 provides an alignment of the RNase III domains of the mouse, human, *Drosophila* and *C. elegans* RNase III orthologues. The latter two sequences also exhibit strong conservation of sequence over the entire length of the region. Note also that *Drosophila* RNase III has a serine-rich C-terminal extension [19]. There are two catalytic domain signature sequences present in these orthologues. The single signature sequence of bacterial RNase III orthologues not only provides residues essential for catalysis, but contributes to the subunit interface [12]. It is therefore likely that mouse RNase III is active as a homodimer. If so, the holoenzyme may contain four active sites.

The Proline-rich region

A proline-rich region (PRR) is present in the N-terminal portion of the polypeptide (see Additional File 1). Of the 63 prolines occurring in the PRR, 62 (98%) are conserved in the corresponding region of human RNase III. Repetitive proline sequences tend to adopt a polyproline II (PPII) helix, consisting of an extended structure with three residues per turn. A proline at every third position serves to stabilize the structure [20], and also participates in hydrogen bonds as well as in hydrophobic interactions [21]. The presence of a PRR in mouse RNase III suggests protein-protein interactions important for function. PRR-mediated interactions are relatively weak and reversible, and occur with the PPII helix in the C-terminal domain of RNA polymerase II during transcription initiation and elongation [22].

The Arginine/Serine (RS) domain

The mouse RNase III polypeptide contains an RS domain adjacent to the PRR (see Additional File 1). The 13 positions containing the RS/SR dipeptide motif are shared between the mouse and human polypeptides, with an overall 87% sequence identity, with the human sequence containing an additional SR dipeptide. RS domains participate in protein-protein interactions, and RS domain-containing proteins play essential roles in constitutive or alternative mRNA splicing [23–25]. RS proteins bind RNA

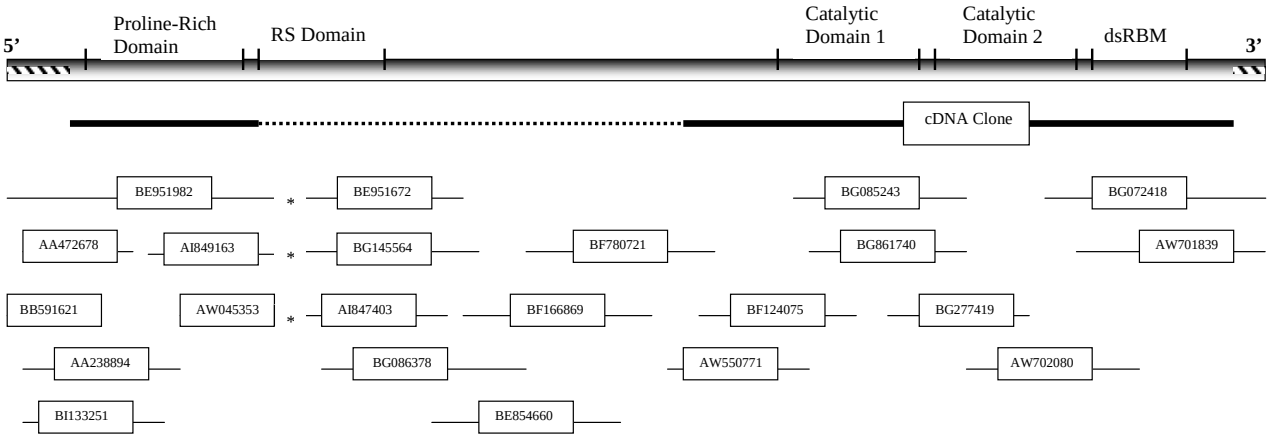

Figure 2
Positions of partial cDNA clones used to determine the complete cDNA sequence of mouse RNase III. The full-length cDNA is represented by the shaded bar at the top, with specific domains indicated, and the 5' and 3' UTRs marked by the hashed lines. The cDNA clones are identified by their accession numbers. The cDNA clone generated in-house from kidney cDNA is represented by a solid black line and is labeled "cDNA clone" (GenBank Accession #AF533013). The dotted line indicates the internal sequence missing from the clone. The Not1 restriction site is indicated by the asterisk and is the site of plasmid ligation for a number of the cDNA clones. That there is no missing sequence at this position is indicated by (i) the continuity of the open reading frame across this site, and (ii) an exact, continuous alignment with the human sequence across this site [18].

via an RNA recognition motif (RRM), allowing subsequent recruitment of splicing components *via* RS domain interactions. The presence of this domain in mouse RNase III suggests similar protein-protein interactions involved in RNA maturation, which may be functionally associated with components of the RNA splicing and transport machinery.

Potential sites of post-translational modification

Psort analysis of the predicted mouse RNase III amino acid sequence indicates three potential nuclear localization signals positioned at residues 254, 355 and 508 (see Additional File 1). A Prosite scan identified multiple potential phosphorylation sites for protein kinase C, casein kinase II and cAMP-dependent protein kinase (data not shown). Some of these signals may be involved in protein localization, as it has been shown that human RNase III localizes to the nucleolus during the cell cycle S phase [18].

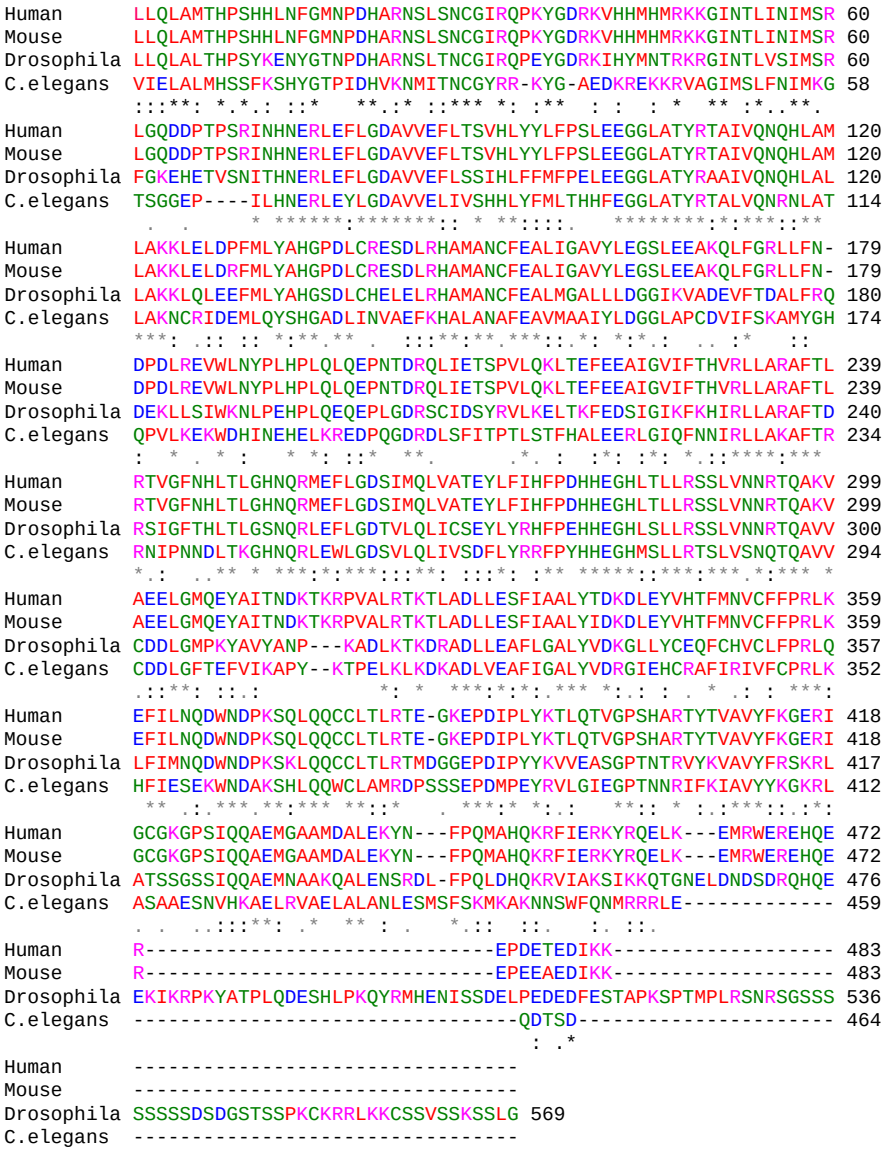

Figure 3
Similarities of the RNase III domain (catalytic domains + dsRBM) of the human, mouse, *Drosophila* and *C. elegans* RNase III orthologues. Multiple amino acid sequence alignment was performed using the CLUSTALW and Align programs. For the mouse orthologue, the first amino acid shown corresponds to amino acid 892 in the complete sequence (see Additional File 1). Blue: acidic residues; Pink, basic residues; Green, hydrophilic residues; Red, hydrophobic residues. The symbols below the sequence refer to level of conservation: asterisk, completely conserved; colon and period: decreasing levels of conservation.

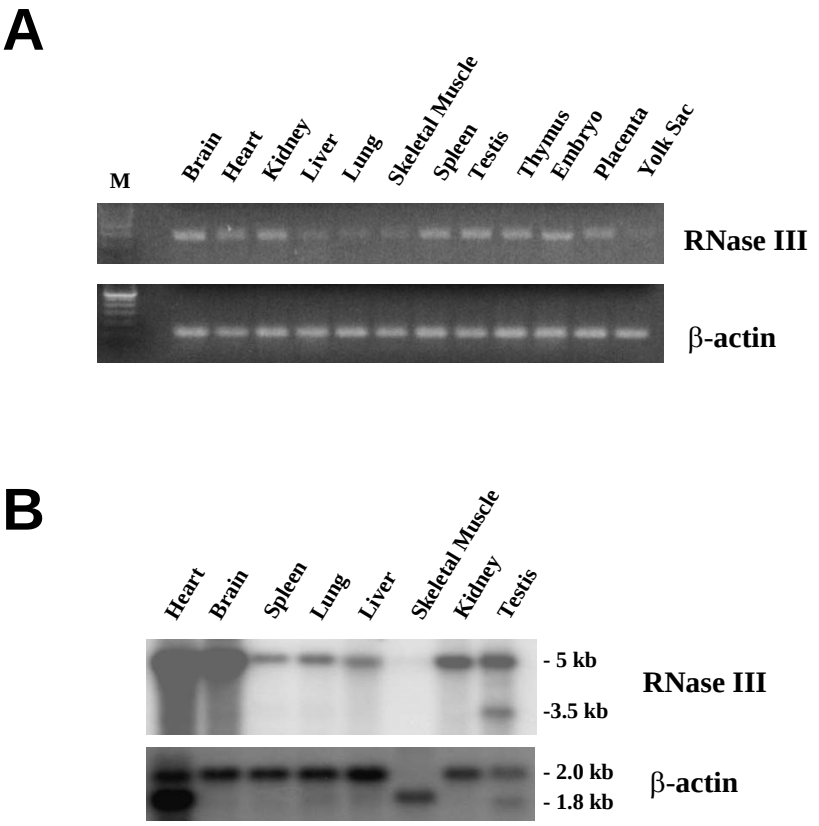

Figure 4
Expression analysis of the mouse RNase III transcript. **A.** RT-PCR analysis of the RNase III transcript in adult mouse organs, in day 13.5 (E13.5) mouse embryo ("Embryo"), and in extra-embryonic organs ("Placenta" and "Yolk Sac"). Analysis was performed as described in Materials and Methods. The 272 bp PCR product was generated using an oligodeoxynucleotide pair that localized to a sequence in the dsRBM (forward primer) and 3'-UTR (reverse primer). For each lane, a separate RT-PCR analysis which omitted reverse transcriptase did not provide a DNA product (data not shown). The lower panel displays a control experiment showing uniform production of a β-actin mRNA-specific PCR product (100 bp). DNA size markers are provided in lane M. **B.** Northern analysis of the RNase III transcript. A ^{32}P-labeled, 1.2 kb probe specific to the 3' region of the RNase III sequence (cDNA clone accession no. AA549506) was hybridized to a multiple tissue northern blot. Each lane contained 2 μg of poly(A)$^+$ RNA from the indicated adult mouse organs (upper panel). The blot was visualized by phosphorimaging. The actin control blot is shown in the lower panel. The 1.8 kb transcript in the heart, skeletal muscle and testis lanes represents an alternatively processed actin RNA.

Mouse RNase III transcript expression patterns

The transcript expression patterns were analyzed by RT-PCR and by northern blot (Figures 4A,4B). The analyses show that the mouse RNase III transcript is expressed in all adult organs examined, as well as in the E13.5 midgestation embryo, the extraembryonic yolk sac, and the placenta. Northern analysis (Figure 4B) reveals a transcript size of ~4.5 kb, which is consistent with the predicted length of the cDNA, including a poly(A) tail. The northern analysis also reveals that the transcript is heavily expressed in brain tissue and perhaps also heart tissue, while only lightly expressed in skeletal muscle. The reason(s) for the differential levels of expression is unclear, but it is of interest to note that the mouse Dicer transcript is also prominently expressed in brain and heart tissue, while only lightly expressed in skeletal muscle [17]. Interestingly, an

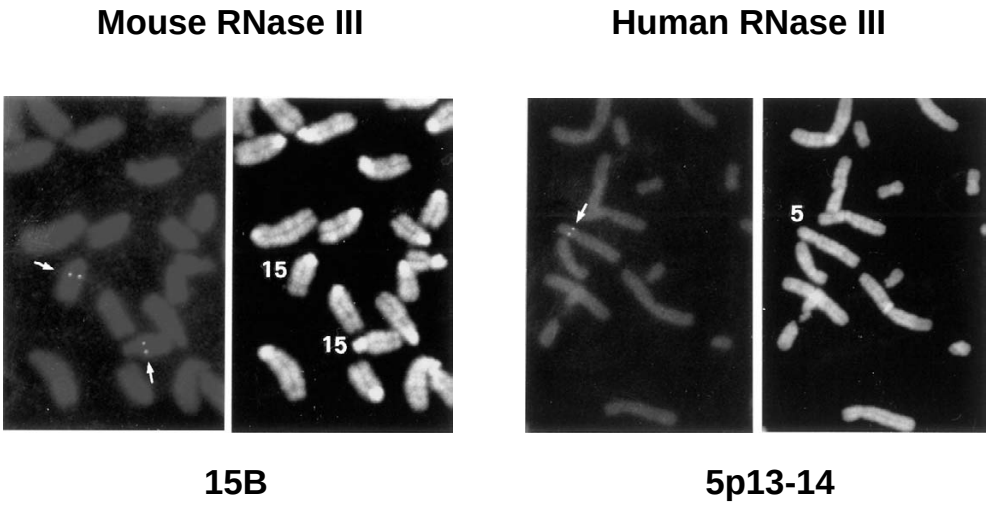

Figure 5
Chromosomal locations of the mouse and human RNase III genes. Fluorescent *in situ* hybridization (FISH) analysis was performed according to published procedures [36,37] by SeeDNA Biotech, Inc. (Windsor, Ontario, Canada). The mouse gene mapping result is shown in panel A, and the human gene mapping result shown in panel C. For the mouse gene mapping experiment, the probe was a mouse cDNA clone (Accession No. AA549506; ~1.2 kb insert). For the human gene mapping experiment, the probe was a human cDNA clone (Accession No. AA460045; 1.26 kb insert).

additional species (~3.5 kb) is present in testis (Figure 4B), which may represent an alternatively processed form of the transcript. This species is not seen in the testis lane of the RT-PCR analysis, since the PCR primers are targeted to the 3'-UTR, which is most likely the same for the two transcripts.

Chromosomal locations of the mouse and human ribonuclease III genes

The mouse RNase III gene was mapped using fluorescent in situ hybridization (FISH), employing a partial cDNA clone as probe. The gene is located in the proximal segment (region B) of chromosome 15 (Figure 5A). The human ribonuclease III gene was mapped to chromosome 5p13-p14 (Figure 5B), a region that is syntenic to mouse chromosome 15B.

Conclusions

Sequence database searches and other studies [17] have revealed only two RNase III orthologues (Dicer and RNase III) in the mouse and human genomes. However, the presence of an additional RNase III-specific transcript in at least one tissue indicates the possibility of alternative forms of RNase III. The broad expression pattern suggests a conserved cellular function for mouse RNase III. In this regard, sequence database searches and a southern "zoo" blot (data not shown) indicate significant conservation of the RNase III gene among many other vertebrates, including rat, rabbit and cow. However, functional roles for mammalian RNase III have yet to be fully defined. A role in rRNA maturation is suggested by the observation that antisense oligodeoxynucleotide-mediated reduction in human RNase III levels also causes a decrease in the

amount of 5.8 S rRNA and a concomitant increase in the 12 S precursor [18]. A similar defect in rRNA maturation is seen upon U8 snoRNA depletion in *Xenopus* oocytes [26]. As U8 snoRNA participates in rRNA maturation, it is possible that an RNase III-dependent step also involves U8 snoRNA. Alternatively, a role for RNase III in the maturation of U8 snoRNA (as well as other snoRNAs) is a possibility. In this regard, the *S. cerevisiae* RNase III orthologue Rnt1p not only cleaves the 35 S rRNA precursor within the 3'-ETS [27–29], but also processes snoRNA and snRNA precursors [30–33]. Further biochemical studies are required to identify the RNA targets for mammalian RNase III and to determine its involvement in RNA maturation pathways.

Methods

Materials

Chemicals and reagents were molecular biology grade or reagent grade and were purchased from Fisher Scientific or Sigma Chemical Company. Restriction enzymes were purchased from New England Biolabs and were used according to the supplied instructions. The radiolabeled nucleotide [α-^{32}P]dCTP (3,000 Ci/mmol) and nick translation kits were purchased from Amersham-Pharmacia. Oligodeoxynucleotides used for DNA sequencing and PCR were synthesized by Invitrogen. The Multiple Tissue Northern (MTN) blot was obtained from Clontech. I.M.A.G.E. Consortium cDNA clones were obtained from Invitrogen/Research Genetics. The cDNA used as template for PCR cloning and sequencing was obtained from Clontech (Marathon-Ready cDNA), or prepared from mouse liver or kidney RNA as previously described [34]. Bacterial plasmids used in DNA sequencing reactions were purified using Qiagen plasmid purification kits. DNA sequencing reactions employed an ABI 3700 automated DNA Analyzer, and Big Dye terminator kits. Sequences were assembled with the ABI DNA sequencing analysis software (v3.6).

Determination of the mouse ribonuclease III cDNA sequence

Several avenues were followed to obtain the complete cDNA sequence for mouse RNase III. One strategy was based on a previous study [35] which identified eukaryotic RNase III orthologues by a BLAST search of the NCBI translated mouse EST database, using *E. coli* RNase III as query sequence. We carried out a similar search against the NCBI mouse EST database, using the *Drosophila* and human RNase III [18,19] as query sequences. Specific clones were identified, obtained and sequenced. The clone positions and accession numbers are given in Figure 2.

In a second approach, mouse kidney cDNA (Clontech Marathon-ready cDNA) served as a template for PCR which used the Advantage-2 Polymerase Mix (Clontech). The PCR reaction (50 µl volume) also included: 10 µM each primer (sequences available by request), and 5 µl of the cDNA preparation. The PCR conditions were: 94° for 30 sec, then 5 cycles of 94° (5 sec) and 72° (4 min.). This was followed by 5 cycles of: 94° (5 sec) and 70° (4 min.). The final steps consisted of 20–25 cycles of 94° (5 sec.) and 68° (4 min.). The products were purified and cloned into plasmid pPICZ-C (Invitrogen) at the SfiI and SnaB1 sites. The recombinant clone (indicated in Figure 2) was sequenced (Accession Number AF533013). All sequences obtained as described above were subjected to a series of CLUSTALW alignments. Additional clone sequences obtained from the NCBI database were used for further verification of the assembled sequence. The assembled cDNA sequence was subjected to hypothetical translation using the "Translate" program available on the EXPASY website [www.expasy.ch]. Functional domains were determined using CLUSTALW and manual methods. The molecular weight was calculated using the primary structure analysis program available from ExPASY proteomics tools. Potential post-translational modification sites and nuclear localization signals were identified using PROSITE and Psort programs, respectively [www.expasy.ch].

Expression analyses

Total RNA was obtained from mouse embryonic tissue or adult mouse organs and reverse transcription carried out as described [34]. For each tissue analysis, PCR was carried out in a 25 µl reaction volume using 1 µl of the first-strand cDNA reaction, according to the Superscript Preamplification System for First Strand cDNA Synthesis (Invitrogen). Primer sequences were (forward) 5'-GGGGCCATCCCATGCTAGAA-3' and (reverse) 5'-CCACTCCTGCCCTCGTTTACT-3'. PCR conditions were 95°C × 10 min; 25 cycles of 94°C × 1 min; 55°C × 1 min; 72°C × 1 min; with a final extension of 72°C × 5 min. β-actin was used as an internal standard; primer sequences were (forward) 5'-CCCAACTTGATGTATGAAGG-3' and (reverse) 5'-TTGTGTAAGGTAAGGTGTGC-3'. PCR conditions were 95°C × 1 min, 30 cycles of 94°C × 30 sec, 58°C × 30 sec, 72°C × 1 min, with a final extension of 72°C × 3 min. Reactions were analyzed by electrophoresis in 1% agarose gels, and DNA was visualized by ethidium staining.

Authors' Contributions

Author 1 (KRF) carried out the sequence analyses, cDNA clone construction and *in-silico* analyses. Author 2 (RHN) performed the expression analyses. Author 3 conceived of the study and participated in its design and execution. All authors read and approved the final manuscript.

Additional material

Additional File 1

Mouse RNase III cDNA and amino acid sequences. The proposed translational initiation codon and the polyadenylation signal are underlined. The three in-frame stop codons upstream of the initiation codon are italicized. Nucleotide and amino acid positions are provided on the right. The human RNase III amino acid sequence is also shown in italics. The shaded amino acids indicate residues in the Proline-rich region, the RS domain, and the catalytic domain signature sequences. Three potential nuclear localization signals beginning at positions 254, 355 and 508 are underlined.

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