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Pancreatic-Type Ribonuclease 1 Gene Duplications in Rat Species

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Abstract. Mammalian pancreatic-type ribonucleases (RNases) 1 represent single-copy genes in the genome of most investigated mammalian species, including *Mus musculus* and other murid rodents. However, in six species belonging to the genus *Rattus* and closely related taxa, several paralogous gene products were identified by Southern blotting and PCR amplifications of genomic sequences. Phylogenies of nucleotide and derived amino acid sequences were reconstructed by several procedures, with three *Mus* species as outgroup. Duplications of the RNase 1 occurred after the divergence of *Niviventer cremoriventer* and *Leopoldamys edwardsi* from the other investigated species. Four groups of paralogous genes could be identified from specific amino acid sequence features in each of them. Low ratios of nonsynonymous-to-synonymous substitutions and the paucity of pseudogene features suggest functional gene products. One of the RNase 1 genes of *R. norvegicus* is expressed in the pancreas. RNases 1 were isolated from pancreatic tissues of *R. rattus* and *R. exulans* and submitted to N-terminal amino acid sequence analysis. In *R. rattus*, the orthologue of the expressed gene of *R. norvegicus* was identified, but in *R. exulans*, two paralogous gene products were found. The gene encoding for one of these had not yet been found by PCR amplification of genomic DNA. A well-defined group of orthologous sequences found in five investigated species codes for very basic RNases. Northern blot analysis showed expression of messenger RNA for this RNase in the spleen of *R. norvegicus*, but the protein product could not be identified. Evolutionary rates of RNase 1, expressed as nucleotide substitutions per site per 10^3 million years (Myr), vary between 5 and 9 in the lines leading to *Mus*, *Niviventer*, and *Lepoldamys* (on the basis of an ancestral date of mouse/rat divergence of 12.2 Myr) and between 20 and 50 in the lines to the other sequences after divergence from *Niviventer* and *Leopoldamys* (5.5 Myr).

Key words: Pancreatic-type ribonuclease 1 — Rat — Gene Duplications

Introduction

The pyrimidine-specific ribonuclease A superfamily constitutes a group of homologous proteins of about 125 residues with well-characterized members in many vertebrates (Beintema et al. 1997). The genes coding for these proteins have an intron in the 5′ untranslated region, but there are no introns in the coding sequences. Within the mammals four separate RNase A families occur (Beintema et al. 1997). Members of different families differ at more than 50% of the amino acid positions. Eight RNase genes are found at a relatively short distance from one another on human chromosome 14 (Strydom 1998). These are single-copy genes of RNases families 1, 4, and 5

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(angiogenin) and five belonging to the RNase 2 family [human RNases 2, 3, 6, 7, and 8 (Zhang et al. 2002b)]. Mouse RNase genes have also been allocated to chromosome 14, which is partially homologous to human chromosome 14 (Strydom 1998). In this species, there is also one copy of the RNase 1 gene, which is expressed in several tissues (Samuelson et al. 1991).

The mammalian pancreatic-type ribonucleases (RNases 1) represent the most investigated one of these families and have been much used for deriving phylogenetic relationships between mammals, from both amino acid (Beintema et al. 1997) and DNA (Breukelman et al. 1998; Kleineidam et al. 1999a; Dubois et al. 1999) sequences. These studies were performed assuming that the enzyme represents a single-copy gene in the genome of the majority of mammalian species as established by Southern blotting (Breukelman et al. 1993) and PCR amplification. Members of other RNase A gene families differ in sequence from RNases 1 sufficiently that they are not detected in these experiments.

In four taxa duplications of the RNase 1 gene were observed, which, however, could be easily accommodated in phylogenetic tree reconstructions: a recent duplication in an ancestor of guinea pig resulted in two paralogous pancreatic RNases in this species (Beintema et al. 1988). In ancestral ruminants two gene duplications of RNase 1 (Breukelman et al. 1998) gave rise to paralogous RNases present in pancreas, expressed in brain (Zhao et al. 2001), and isolated from bovine seminal plasma (D'Alessio et al. 1972). In an ancestral tylopod a gene duplication occurred, with one gene product being the pancreatic enzyme in camels, while expression of the other one has not yet been investigated (Kleineidam et al. 1999a). In leaf-eating monkeys two RNase 1 genes were demonstrated, of which one is expressed at a high level in the pancreas, resulting from a recent gene duplication in an ancestor of this primate taxon (Zhang et al. 2002a). The RNase 1 gene duplications demonstrated in ancestors of ruminants, tylopods, and leaf-eating monkeys are very likely adaptations to the digestive physiology of these foregut fermenting mammals (Zhang et al. 2002a).

The presence of duplicated genes introduces a bias when comparing the evolution of a single-copy gene in a species with that of its duplicated orthologues in another one with gene duplications. Which of the duplicated genes are expressed as functional enzymes? and What are the evolutionary differences between paralogous gene copies? But they also may not be expressed and have distinct pseudogene features as observed in sequences of seminal-type ruminant ribonucleases, except in the expressed bovine one (Kleineidam et al. 1999b). It may be expected that if a gene has such pseudogene features, lack of selection will have caused inactivation of its promoter.

In our previous studies on RNases from 15 murid rodent species one RNase 1 gene was encountered in each species (Dubois et al. 1999), which is in agreement with studies by Samuelson et al. (1991) on the gene from mouse (*Mus musculus*). However, to our surprise we found several RNase 1 sequences in the genome of the laboratory rat, *Rattus norvegicus*. Inspection of Southern blots of RNase 1 obtained in preliminary experiments and also published by Rosenberg et al. (1995) showed more than one band. Here we present an analysis of results obtained on the presence and sequences of multiple RNases 1 in several members of the genus *Rattus* and related genera. Trees derived from these sequences do not show distinct early duplicated paralogous groups of orthologous sequences present in each of the investigated species. These results suggest a mechanism described as the birth-and-death process of gene family evolution, which is encountered in a few major gene families of host defense-related mammalian proteins (Nei et al. 1997) and also in rodent eosinophil-associated RNases (Zhang et al. 2000), which belong to the mammalian RNase 2 family (Beintema et al. 1997). Our studies suggest that in the RNase 1 family a similar process occurred in the genus *Rattus* but not in other murid genera.

Materials and Methods

DNA was extracted from 95% ethanol-preserved tissues housed in the collection of mammalian tissues of the Institut des Sciences de l'Evolution, Montpellier (Catzeflis, 1991). Table 1 lists the taxa involved in this study.

RNase Sequences. Amplification of RNase sequences was performed by the hot-start procedure (Ampliwax PCR Gem 100; Perkin Elmer) with different oligonucleotide pairs (Eurosequence BV, Groningen). As forward primers, oligonucleotide MSP (5′-ATGGGTCTGGAGAAGTCCCT-3′), positions 51 to 70, coding for the N-terminal 7 residues of the signal peptide (25 residues) of the *Mus musculus* RNase sequence (Schüller et al. 1990), and primer RRS (5′-GAATCATCGGCCGATAAGTTT-3′), coding for residues ESSADKF in the N-terminal protein sequence of *R. rattus* pancreatic RNase, were used; and as the reverse primer, MC (5′ACATCTCACTGGTTTGGCCT-3′), positions 530 to 511 of *Mus musculus* RNase [residues 135 to 129 in a continued RNase amino acid sequence numbering, located after the stop codon at positions 125, which have identical cDNA sequences in mouse and rat pancreatic RNases (Schüller et al. 1990)]. PCR amplifications were performed in a 100-μl final volume, usually with 100 ng of extracted DNA using a Perkin Elmer DNA thermal cycler 480. Taq polymerase (0.5 U) and buffer (High Expand PCR System; Boehringer; Mannheim, FRG) were added to the mixture with a 10 m*M* concentration of each dNTP (Boehringer). The reaction was carried out with the following 30 cycles: 1 min at 94°C, 1 min at 57°C, and 1 min at 72°C for extension. The amplified fragments were cloned into the PCR vector with the TA cloning kit (Invitrogen Corp.) using standard procedures (Sambrook et al. 1989). For each sequence, at least three independent clones were fully sequenced on

Table 1. Rodents used for pancreatic ribonuclease sequence experiments[a]

Species	Registry No.	Origin	Collector	EMBL No.
Mus musculus	ls	The Netherlands		M27814
Mus saxicola	T-824	India	F. Catzeflis	AJ238700
Mus pahari	T-350 (and ls)	Thailand	F. Bonhomme	AJ238699
Leopoldamys edwardsi	T-864	Vietnam	C. Van Sung	AJ005777
Niviventer cremoriventer	T-1432	Thailand	A. Lynam	AJ005778
Sundamys müelleri	T-754	Borneo	R. Stuebing	AJ315453
Berylmys bowersi	T-861	Vietnam	C. Van Sung	α, AJ315454
				δ, AJ315455
Rattus norvegicus	ls	The Netherlands		β, J00771
				γ, AJ315460
				δ, AJ315462
Rattus rattus	T-812	India	F. Catzeflis	β, AJ315466
				γ, AJ315461
				δ, AJ315463
Rattus exulans	T-1313	Sumatra	M. Ruedi	α, AJ315458
	T-1771	Java	J.J. Beintema	β, AJ005776
				δ, AJ315459
Rattus fuscipes	T-1026	Australia	I. Beveridge	α, AJ315456
				γ, AJ315457
Rattus tiomanicus	T-752	Borneo	R. Stuebing	β, AJ315465
				δ, AJ315464

[a] Tissues samples (T-), geographic origin (*ls,* laboratory strain), name of the collector/donor, and EMBL accession number of the sequences.

both primers using the primer T7 promoter and the reverse primer M13 (Invitrogen Corp.). Sequencing was performed using standard procedures with ^{35}S-labeled nucleotides (Sanger et al. 1977).

Phylogenetic Analyses. Alignment of sequences was done manually. Indels were introduced as multiples of three bases coding for amino acid residues in genes which show no pseudogene features and are expressed or likely expressed as proteins. However, in one of the sequences, the alignment was ambiguous. The phylogenetic analyses were conducted on sequences of mature RNases which include 384 nucleotide positions (128 amino acid positions; residues 1–124 in Fig. 1), of which 174 were variable and 117 phylogenetically informative, when all events [transitions (TS), transversions (TV), and indels] are considered.

The aligned sequences were treated by maximum likelihood, general time-reversible (GTR) model (Lanave et al. 1984; Rodriquez et al. 1990) distances, and parsimony analyses, using the program PAUP Version 4.0b5 (Swofford 1998). Confidence levels were estimated using the bootstrap (BP) (Felsenstein 1985).

Southern Blots (Feinberg and Vogelstein 1983, 1984). Genomic DNA of *R. norvegicus* and *R. exulans* was subjected to restriction enzyme analysis and subsequently to Southern blot analysis (Sambrook et al. 1989). For each restriction analysis 20 μg of genomic DNA was digested for 18 h with 5 U of restriction enzyme in a total volume of 100 μl. After separation of the digests by 0.7% agarose gel electrophoresis in 0.5× TBE, the DNA fragments were transferred to a positively charged nylon membrane (Boehringer). Hybridization was performed for 18 h at 65°C with probes of 440 bp of *R. norvegicus* RNase β or of *M. saxicola* RNase encoding the mature proteins and labeled with the random primed labeling kit (Boehringer).

Expression of R. norvegicus *RNase* δ. Oligonucleotide sequence pairs coding for residues −1/+7 and 67/73 (including the insertion between position 67 and position 68 in *Rattus* RNases δ) of *R. norvegicus* RNase δ were synthesized and used as primers. Five-microgram quantities of total RNA, isolated from different tissues, were reverse-transcribed into cDNA in a 20-μl reaction mixture containing 20 m*M* Tris–HCl (pH 8.3), 50 m*M* KCl, 6 m*M* $MgCl_2$, 1 m*M* dNTP, 1 U/μl RNasin, 1.25 pmol/μl of downstream primer, and 1.25U of M-MuLV reverse transcriptase by incubation at 37°C for 1 h and inactivation at 100°C for 5 min. PCR amplification of cDNA was performed with a thermal cycler in a 100-μl reaction mixture containing 20 m*M* Tris–HCl (pH 8.3), 50 m*M* KCl, 2.5 m*M* $MgCl_2$, 0.2 m*M* dNTP, 1 pmol/μl of both downstream and upstream primers, and 0.025 U/μl of Taq polymerase. The amplification profile was as follows: 3 cycles at 96°C for 90 s, 50°C for 60 s, and 72°C for 60 s and 40 cycles at 94°C for 90 s, 50°C for 60 s, and 72°C for 60 s, and a final step of 5 min at 72°C. The cDNAs were separated by electrophoresis on 1.4% agarose in 1× TBE, transferred to Hybond-N membranes (Amersham), and hybridized to the ^{32}P-labeled cDNA coding for *R. norvegicus* RNase β (final washing conditions, 0.5× SSC, 0.1% SDS at 67°C). The specificity of amplified products from *R. norvegicus* spleen RNase δ was confirmed by restriction analysis with *Bam*HI and *Hpa*II enzymes.

Isolation of RNases 1. Pancreatic tissue from *R. rattus* was kindly provided by J.T. de Jonge (Kennis- & Adviescentrum Dierplagen, Wageningen, The Netherlands) and that from *R. exulans* was collected from animals caught in a forest in the Mount Patuha region (Java, Indonesia). Pancreatic tissue of *R. rattus* or *R. exulans* or spleen tissue from *R. norvegicus* was homogenized in 0.125 *M* sulfuric acid with an Ultratorax. The suspension was centrifuged and the precipitate formed after treating the supernatant with ammonium sulfate between 50 and 100% saturation was resuspended in 0.2 *M* acetic acid. RNases were then isolated by reversed-phase HPLC on a Nucleosil 10 C-18 column (4.6 × 250 mm) with a linear gradient from 0 to 67% of acetonitrile in 0.1% trifluoroacetic acid in 54 min. The flow rate was 1 ml/min. RNase activities in collected peak fractions were determined with yeast RNA as substrate (Shapira 1962; Zhao et al. 1998), and the purity of the active peak was checked by polyacrylamide gel electrophoresis (PAGE) in the presence of SDS (Laemmli 1970). The isolated pancreatic RNases were used for N-terminal amino acid sequence analysis on an Applied Biosystems 477 pulse-liquid sequencer with an on-line 120A phenylthiohydantoin analyzer (Eurosequence BV, Groningen).

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                                +1                    20                  40
                                 !                     !                   !
M. musculus    ILFPLFFLLLGWVQPSLGRES***AAQKFQRQHMDPDGSSINSPTYCNQMMKRRDMTNGSCKPVNTFVHEPLA
M. pahari      ......V...........K..***S....E.....SS...N...........S.S..KE.............E
M. saxicola    M.....V..............***S...........A...S................K...............
Leopoldamys    ...S.LV.V.........K..***S....N.....TE...NS.........TP.G..K............T..
Niviventer     ...S.LV.V........SK..***S.D..K.....TE...NS.........T.G...................
Sundamys       ...S.LV.V.........K..***SPD..K.....TE...KS........RSPQE..K.........L....E
Bbα            ...S.LV.V............***S.E..K.......SIPNS............E..T..............D
Reα            ...S.LV.V............***S.D..K.....TE...KS............E..T..............E
Re prot α                        ...***S.D..K.....TE...KS....
Rfα            ...S.LV.V............***S.D..K.....TE...KS..............................E
Rnβ            F..S.LV.V.........G..RESS.D..K.....TE.P.KS...........QG..K..............E
Reβ            ...S.LV.V.........V..RESS.D..K......ESP.KS...........QG..K..............E
Rrβ                                         K.....S.SP.KS......V..SG.N..KD.............K
Rr prot β                        V..RESS.D..K.....T.SP.KS....
Rtβ            A..S.LV.V........SG..RESS.D..K.......SP.KS...........QG..K....R.........E
Rnγ            F..S.LV.V.........V..RETP....E.....EE.PFPS......E...S.G..S.W..SM.........
Rrγ            L..S.LV.V.........V..RETQT.........EE.HFPS..........S.G..S.....M........E
Rfγ            ...S.LV.V..C.....VG..KESPSE..K.R...EE.PYQS..........D.G..S.R...L......SW.
Bbδ            ...S.LV.V..........KP***S.EE.K.......SPPNSR.....R..R.QG..K....R.......SW.
Rnδ            ...S.LV.V..........KP***SV.D.K.......SPPNSR...........G..K....R....L..SW.
Rrδ            ...S.LV.V..........KP***SV.D.K......GS.PNSR...........G..K....R....L..SW.
Reδ            ...S.LV.V..........KP***SV.D.K.......SPPNSR...........G..K....R.......SW.
Rtδ            ...S.LV.V..........KP***SV.D.K......GSPPNSR..I........GI.KD........L..SGQ

Reγ            ...S.LV.V.........V..RETP.E..K.....EE.PFPS......E...S.G..S.W..SM......S..
Reγ/δ          ...S.LV.V.........V..RETP.E..K.....EE.PFPS......E.....G..K....R.......SW.
Re prot x                             ETQ....K.....EE.DPNS....

                     60                   80                 100                 120
                      !                    !                   !                   !
M. musculus    DVQAVCSQENVTCKN*RKSNCYKSSSALHITDCHLKGNSKYPNCDYKTTQYQKHIIVACEGNPYVPVHFDATV
M. pahari      ....I..........*GNR.........................N.N.N..........D........L....
M. saxicola    ....I..........*G...............................S....Q...................
Leopoldamys    ....I...GQ.....*G........C.......R..............SD......I......S.......SE
Niviventer     ....I..........*G.K.....T........R..............SD......I..D...S.........
Sundamys       ....I...DK....D*G....H....T......C...S.N..K?N.T..ES.....I..D...........S.
Bbα            ..K.I...GQM....*G....H....T....E.R...S........T..DS.....I..D...........SE
Reα            ....I...GQ.....*GRN..H....T......R...S........T..DS.....I..D...........S.
Rfα            ....I...GQ.....*G.........T......R...S........T..DS..QL.I..D........L.DS.
Rnβ            ....I...GQ.....*GRN..H....T.R....R...S........T..DS.....I..D...........S.
Reβ            ....I...GQ.....*GRN..H....T....E.R...S.......E.T..DS.....I......L....Y.D..
Rrβ            ....I...GQ.....*GRN.......T......R...S....K...T..DT.N...I......L....?..S.
Rtβ            ....I...GQ.....*GRN.......T....E.....S........T..DS.....I......L......DS.
Rnγ            T...I...GQ.....*GRN..H....T.R....R...S........T..NS.....I......L......DS.
Rrγ            T...I...GQ.....*G.R..H....T.R....R...S....K...T..DS.....I..D****.*..L....
Rfγ            K.K.I...DK.....*G....H..I.T.N....L.M.S....K...S..AR...S.I..D........Y....
Bbδ            T.K.I...RQM...TT.RN..H....T....E.R...S........T..DS.....I..D...........SE
Rnδ            T.K.I...RQM...TSSRN..H....T......R...S........T..NS.....I......L.......S.
Rrδ            K...I...RQM.R.TSS.K..H....P....E.R...S....K...T..NS.....I......L....L..R.
Reδ            T.K.I...RQM...TSSRN..H....P......R...S........T..NS.....I......L....Y..S.
Rtδ            R...I...RQM...TSSRK..H....T......N...S........T..NR.....I......L....Y..S.

Reγ            T...T
Reγ/δ          T.K.I...RQM...TSSRN..H....P......R...S........T..NS.....I......L....Y..S.
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Fig. 1. Protein sequence alignment. See Table 1 for species identities and abbreviations. *Dots* indicate identical amino acids, *asterisks* indicate indels, *spaces* indicate unknown sequences, and *question marks* indicate undetermined nucleic acids. "+1" indicates the beginning of the mature RNase and the position used as the starting point used for phylogenetic reconstruction. Sequenced proteins are in *italics*. *Squares* indicate recognized amino acid signatures. The last two sequences were not used for phylogenetic reconstruction, as one is incomplete and the other represents a hybrid between the Reγ and the Reδ sequences. Bb, *Berylmys bowersi;* Rn, *Rattus norvegicus;* Rr, *Rattus rattus;* Re, *Rattus exulans;* Rf, *Rattus fuscipes;* Rt, *Rattus tiomanicus.*

Results

DNA Sequence Analyses

Figure 1 presents the alignment of complete derived amino acid sequences of the mature protein (including C-terminal halves of signal peptides) of 23 RNases obtained by PCR amplification of genomic DNA of three *Mus*, one *Berylmys*, one *Sundamys*, one *Niviventer*, one *Leopoldamys*, *and* five *Rattus* species and of an incomplete sequence from *R. exulans* (Reγ), which could not be completed. Table 1

lists the EMBL gene bank accession numbers received upon deposit of the DNA sequences.

Phylogenetic analysis of the sequences of mature RNases did not result in clear paralogous groupings of orthologous sequences (see below). Nevertheless, we tried to make some order by proposing a nomenclature based on characteristic signatures, notably of insertions in the sequences: sequences with designation α (or ancestral) do not exhibit insertions relative to those observed in the genus *Mus*. In sequences with the designations β and γ there is a duplication of the first three amino acid residues of the mature sequence (Beintema and Gruber 1967; MacDonald et al. 1982). Within this group the γ sequences have several typical signatures such as the consensus sequence EEGPFPS at positions 15–21 (Fig. 1). The sequences with designation δ have an insertion of one residue between site 67 and site 68, probably as a result of duplication of the codon at position 67, and share a number of other signatures, such as the consensus sequence SWATVKAICSQRQM at positions 50–63. The latter sequences are also much more basic than the others with a net charge difference of about 10 units. The earlier investigated RNase from rat pancreas (Beintema and Gruber 1967) has the designation β according to this nomenclature.

Southern Blot

Genomic DNA was cleaved with several restriction enzymes, which will not cleave in the coding sequences of known murid rodent RNase 1 sequences. Figure 2 presents a Southern blot of digests with several restriction enzymes of genomic DNA of *R. norvegicus*. In agreement with the result presented by Rosenberg et al. (1995), two bands are observed in the digest with *Pst*I (Fig. 2, lane 3), but in other lanes up to four bands are visible. Southern blots of genomic DNA of *M. musculus* and *R. exulans* cleaved with restriction enzyme *Eco*RI and hybridized with a probe of the RNase gene of *M. saxicola* showed one band for the former species (as expected) and four for the latter (results not shown).

Expression of R. norvegicus *RNase* δ

The specificity of amplification products with primers for RNase δ were tested on genomic DNA of *R. norvegicus*, and a single band of the expected molecular weight, and with the expected restriction pattern, was obtained. The reverse-transcribed amplified cDNA from testis, spleen, and liver gave a strong smear from about 700 to 124 bp, probably due to the abundance in these tissues of mRNA species with some sequence similarity to the primers used. Hybridization with ^{32}P-labeled cDNA ruled out the possibility that these smears represent mRNA coding for RNases and allowed us to identify mRNA coding for RNase δ in spleen tissues (Fig. 3A) but not in pancreas, brain, kidney, and testis. These results were confirmed by restriction analysis with two enzymes: expected bands of 179 bp with *Bam*HI and of 122 bp with *Hpa*II were observed (Fig. 3B).

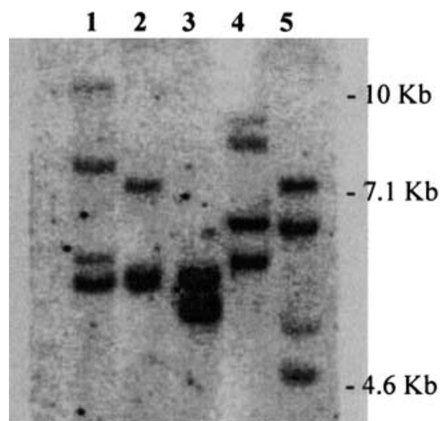

Fig. 2. Southern blot analyses of genomic DNA of *R. norvegicus* after hybridization with a probe encoding the complete gene of *R. norvegicus* RNase β. (Identical results were obtained using probes coding for RNases γ and δ of this species). Restriction enzymes used: lane 1, *Eco*RI; lane 2, *Pst*I; lane 3, *Eco*RI + *Pst*I; lane 4, *Hind*III; lane 5, *Nco*I.

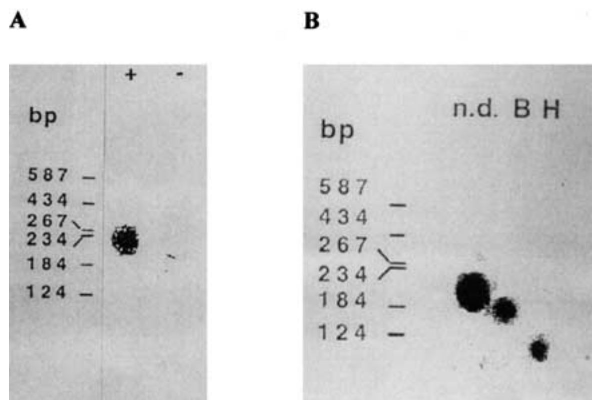

Fig. 3. Expression of *R. norvegicus* RNase δ in spleen. **A** Amplified products obtained from spleen RNA by RT-PCR reactions carried out in the presence (+) or in the absene (−) of reverse transcriptase. **B** Restriction analysis of the amplified product shown in A. n.d., not digested; B, *Bam*HI; H, *Hpa*II.

Isolation and N-Terminal Sequences of RNases

Pancreatic tissue of 10 animals were used for the isolation of RNase from *R. rattus*. After HPLC one peak with RNase activity was observed. On

SDS-PAGE the protein showed one band with the mobility of *R. norvegicus* β. The results of the N-terminal sequence analysis (28 residues) are also presented in Fig. 1. Pancreatic tissue from six animals was used for the isolation of RNases from *R. exulans*. After HPLC two equally high peaks with RNase activity were observed, one peak at the position where the pancreatic enzyme from *R. rattus* elutes (Re protein α) and the second one about 2.5 min later (Re protein x). On SDS-PAGE each protein showed one band with a mobility similar to those of *R. norvegicus*. RNase β and bovine pancreatic RNase A, respectively. The N-terminal sequences of the two RNases (25 and 24 residues, respectively) differ and are also presented in Fig. 1.

Very little RNase activity could be detected in a homogenate of spleen tissue from *R. norvegicus*. After HPLC fractions with some enzymatic activity were detected in the region where pancreatic RNases generally elute. On SDS-PAGE faint bands were visible at the position of *R. norvegicus* RNase β but not at a lower positions, where very basic proteins with the sequence characteristics of RNases δ are expected.

Many pancreatic RNases are glycoproteins (Beintema et al. 1988). In rat pancreatic RNase (Rnβ) no recognition sites for N-glycosylation occur, while only a minor component (less than 10%) of mouse pancreatic RNase is glycosylated, although this sequence contains two sites (Lenstra et al. 1979). Several of the other sequences presented in Fig. 1 also contain recognition sites for N-glycosylation. The sequence of Rrβ has an –N–M–T– sequence at positions 34–36, but the isolated protein has the same mobility on SDS-PAGE as Rnβ, suggesting that the enzyme is not glycosylated. Furthermore, the Re prot X sequence has a nonglycosylated sequence, –N–S–S–, at positions 20–22 (Fig. 1). These observations indicate that little or no glycosylation occurs in pancreatic RNases in Murinae, independent of the occurrence of recognition sites for N-glycosylation.

Species Characteristics

Below we summarize data obtained for each of the investigated species.

R. norvegicus. Three sequences were found by PCR amplification (Fig. 1). Southern blot analysis showed four bands, of which one is fainter than the other three. It is possible that a fourth gene deviates quite a bit in sequence from the other three and was not picked up during PCR amplification. The RNase β gene is the only gene expressed in pancreas. Expression of cDNA encoding the basic RNase δ was demonstrated in rat spleen (Fig. 3), but no protein with the expected properties could be isolated from this tissue. Grassi et al. (1992) demonstrated the synthesis of RNase 1 in rat brain by reaction with an antibody against pancreatic rat RNase. But as at that time the presence of several RNases 1 genes in this species was still unknown, the identity of this brain RNase is uncertain.

R. rattus. Initially only two RNase 1 sequences were found in genomic DNA of this species: RNase γ and RNase δ (Fig. 1). In the RNase γ sequence five residues are deleted in a structurally essential part of the molecule. This indicates that this sequence likely codes for a pseudogene.

Initially no orthologous sequence of the expressed RNase β in *R. norvegicus* was found in *R. rattus*. Therefore we isolated the pancreatic enzyme from the latter species and determined its N-terminal sequence, which clearly showed that it is an RNase β-type protein (Fig. 1). At last we succeeded in finding the sequence coding for this RNase in the genome of *R. rattus* by using a forward primer coding for residues identified by the protein sequence analysis. The derived protein sequence differs from the N-terminal protein sequence in having a serine at position 15 instead of a threonine, which probably represents a polymorphism in the RNase β gene (Fig. 1).

R. exulans. PCR amplifications were performed on two DNA preparations, one originating from the island of Sumatra and the other from Java. The two preparations yielded identical sequences for *R. exulans* RNase β and δ genes (Fig. 1). From the DNA originating from Java three other gene sequences were obtained: one coding for RNase α, the second coding for a typical RNase γ sequence (only the N-terminal half could be determined), and the third coding for a hybrid sequence with the RNase β sequence at positions 1–30 and the RNase δ sequence at positions 31–124. Isolation and determination of N-terminal sequences of two pancreatic RNases from animals collected on Java yielded one sequence that is identical to the one derived from the RNase α gene (not RNase β, which is the expressed gene in the two other *Rattus* species investigated). The other one has not yet been found as a DNA sequence and is too short to assign to one of the RNase 1-type sequences as defined above (Fig. 1). It starts at residue 2 but may have been shortened at the N terminus by proteolytic degradation. All together there may be at least five genes coding for RNases 1 in the *R. exulans* genome, of which several were not found during PCR amplification of DNA from the two investigated sources. However, Southern blot analysis did not indicate a higher number of RNase 1 genes in *R. exulans* than in *R. norvegicus*. It cannot be excluded that the two pancreatic RNases identified in this species are products of two alleles at the same locus which differ

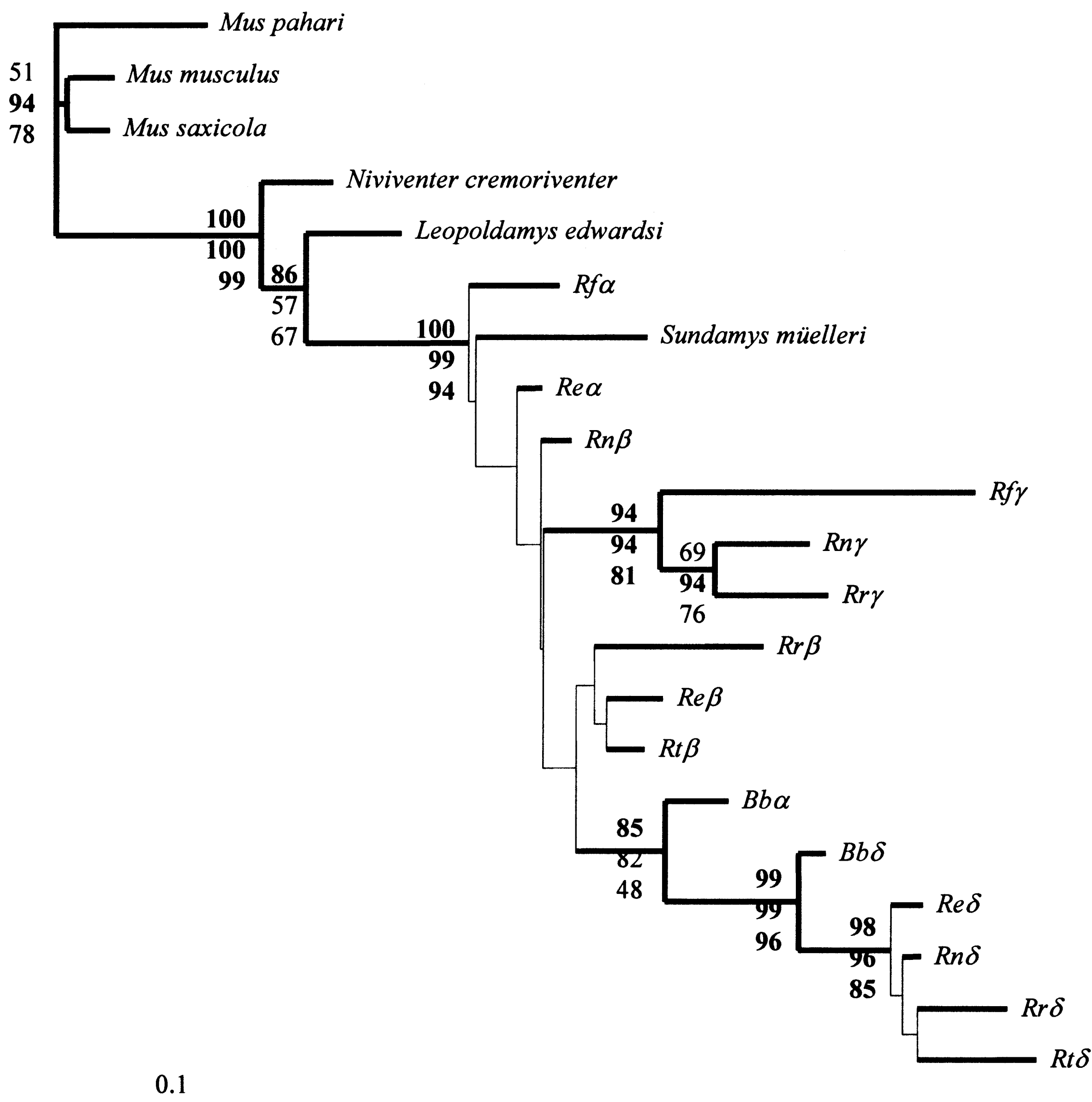

Fig. 4. Maximum likelihood tree based on nucleic acid sequences. Bootstrap values along ancestral segments are indicated for maximum likelihood (top number), GTR distance (middle number), and maximum parsimony (bottom number). *Bold face* branches indicate well-supported nodes, that is, nodes with at least one support value >80.

substantially in sequence. Thus the genomic organization of RNase 1 duplicates in the genus *Rattus* may be more complex than indicated by results from PCR amplification of only the coding sequences.

R. tiomanicus. Two RNase 1 sequences were found by PCR amplification: RNase β and RNase δ.

R. fuscipes. Only two RNase 1 genes were found in this species: one "ancestral" RNase α gene and a second gene which groups with the RNase γ genes in phylogenetic trees (Fig. 4). The latter gene indeed has the sequence EEGPYQS, similar to the consensus EEGPFPS at positions 15–21, but also the sequence SWATVKAI at positions 50–57, which is more similar to the RNase δ sequences, suggesting that this sequence represents a hybrid. The C-terminal residue of its signal peptide sequence is a valine, which may indicate that the RNase γ gene of *R. fuscipes* is not expressed. The genera *Berylmys* and *Sundamys* are considered by Verneau et al. (1998) to be close relatives of the taxon *Rattus stricto sensu*. In the genome of *Berylmys bowersi* two RNase 1 genes were demonstrated: one RNase δ gene and one "ancestral" RNase α gene, which, on phylogenetic analysis, groups with the subtree of *Rattus* RNase δ genes, although the typical RNase δ signatures are not yet present in this sequence. Only one RNase 1 gene was demonstrated in the genomes of *Sundamys müelleri*, *Leopoldamys edwardsi, and Niviventer cremoriventer.* These species may have diverged before the occurrence of gene duplications in the *Rattus* ancestor, but

phylogenetic analyses of the sequences did not support this for *Sundamys* (see below). There are unpaired cysteine residues at position 85 in the *Sundamys* sequence and at position 77 in that of *Leopoldamys* (Fig. 1), which may have a negative influence on the stability of the encoded proteins. This also might indicate that these genes are not expressed. But no other RNase 1 genes were identified in these two species.

So, summarizing this overview on the presence of RNase 1 genes in the representatives of the genus *Rattus*, we conclude that for most cases of duplicated RNase genes in this taxon, the fate of the genes is unknown concerning expression and the localization and development stages of expressed genes.

Phylogenetic Analyses

The complete nucleotide and amino acid sequences listed in Fig. 1 were used for phylogenetic analysis, except for the *R. exulans*, sequence Reγ/δ, which represents a hybrid between the N terminus of RNase Reγ and the C terminus of RNase Reδ.

To locate homoplasy, we searched for evidence of saturation by applying a method recently developed by Hassanin et al. (1988a, b). These terms (data not shown) are not significantly different for third codon position changes, therefore we did not treat them differently in phylogenetic analyses.

The maximum likelihood procedures were conducted using PAUP. The nucleotide composition of each sequence was compared by chi-square tests to the average composition of the data matrix, and none of the sequences failed the test at a significance level of 5%. Sequential analyses were computed to find the best values for the parameters involved in the rate matrix [substitution rates of 0.9309 (AC), 1.77 (AG), 0.9392 (AT), 0.6818 (CG), and 1.576 (CT)] and the γ shape (α = 0.442258; eight categories). The maximum likelihood tree of the 21 RNase sequences has a −ln likelihood value of 2579.94. Bootstrap (100 replications) yields a weak support for most ancestral segments. The relevant supports are indicated on the tree in Fig. 4.

Distances were calculated using the GTR method as implanted in PAUP, with "objective = me." Robustness (1000 bootstrap replicates) values for the most supported segments are indicated on the tree in Fig. 4.

The maximum parsimony reconstruction by heuristic search using PAUP (option: addseq = random, swap = TBR, and enforce = no) yielded three most-parsimonious trees. When all variable sites are included, these trees are 412 steps long (TS + TV + indels) respectively, with a consistency index (excluding uninformative characters) of 0.51 and a retention index of 0.65 bootstrap (1000 replicates; options, addseq = random and swap = NNI) yields weak support for most ancestral segments, and the relevant values are indicated on the tree in Fig. 4.

Discussion

After finding only one RNase 1 gene in earlier investigated murid taxa (Dubois et al. 1999), it was rather a surprise to discover several sequences coding for this enzyme in *R. norvegicus*. Preliminary results from our laboratories and those observed in a figure of Rosenberg et al. (1995) already suggested the presence of duplicated genes in this species. PCR amplification and sequence analysis of RNase 1 genes in representatives of the genus *Rattus* indeed demonstrated the presence of multiple RNase 1 sequences in this taxon. But contrary to the situation observed in ruminants, where an ancestral RNase 1 gene duplicated twice, resulting in three paralogous subfamilies within each subfamily and generally one orthologous sequence in each investigated extant species (Breukelman et al. 2001), such a clear phylogeny cannot be derived for the RNase 1 genes in the genus *Rattus* (Fig. 4). The multiplicity of RNase 1 sequences in this genus originates most likely from gene duplications, but the presence of alleles at a single gene locus cannot be ruled out.

General Topology of the Trees

Trees derived using several methods show few well-supported ancestral nodes (Fig. 4) and limited congruency (data not shown). The divergence between the RNases 1 from the three *Mus* species and those from the other investigated species is well supported. In the line to the genus *Rattus*, there is an unresolved divergence of the enzymes from *Niviventer* and *Leopoldamys* [classified by Verneau et al. (1998), with other genera, in the taxon *Rattus latto sensu*], species in which only one RNase 1 could be identified. The next node is again well supported and represents the ancestor of all RNases 1 so far identified in the genera *Sundamys, Berylmys*, and *Rattus*. Nevertheless, descending lines from this node are unresolved. Only nodes in the cluster of *Berylmys* and the *Rattus* δ sequences and in that of the three *Rattus* γ sequences are well supported.

The multiplicity of RNase 1 genes in this study should be accounted for by gene duplications which occurred in ancestors of the investigated species from the genera *Rattus* and *Berylmys*, but, it is not possible to locate these duplications in the reconstructed trees. This is caused not only by the unresolved nodes of the tree, but also by the fact that not all sequence types

(α, β, δ, γ) could be identified in each of the investigated species. The reasons are multiple.

1. As discussed above, we did not obtain all the RNase 1 sequences of *Rattus rattus* and *Rattus exulans* by PCR.
2. We cannot exclude the possibility that RNases 1 with different sequences observed in one species are products of two alleles from the same gene locus.
3. Hybrid genes were found, indicating that gene conversion could also have occurred over time.
4. Zhang et al. (2000) have explained their findings on the multiplicity of RNase 2 genes in rodents (mouse, rats, gerbils, and hamster) by evolution by the birth-and-death process of genes (Nei et al. 1997). This mechanism can also explain our findings on RNases 1 of rats. Our results are similar to those obtained by Zhang et al. (2000) for RNases 2 in species belonging to the genus *Mus*, although, in our study, we found less evidence for pseudogenes.

The γ and δ RNases 1 sequences are rather well resolved in the phylogenetic tree (Fig. 4), but the α and β types form an unresolved cluster. These two types of sequences differ in the presence of a duplicated N-terminal sequence of nine nucleotides. No α-type sequences were found in three of the investigated *Rattus* species, while with the PCR primers used, it would have been easier to find such sequences than the more diverging ones of the three other types, and only in *R. exulans* are both α-type and β-type sequences found. Thus the duplication of the N-terminal sequence may have occurred more than once during the evolution of RNases in rats and α-type and β-type sequences may be both orthologues and paralogues of each other.

As already mentioned, we know little about the expression and biological role of the additional RNase 1 sequences identified in species belonging to the genus *Rattus*. The ratio of nonsynonymous-to-synonymous substitutions should be lower than 1 when negative selection is predominant. Values of about 1–2 indicate neutral selection, while values higher than 2 indicate that positive selection occurs. In the cases of our RNase 1 genes, the ratios of nonsynonymous-to-synonymous substitutions for intraspecies and intra-"gene type" comparisons (K_a/K_s) (Table 2) are generally lower than 1 and indicate that negative selection is predominant in the evolution of RNase 1 genes in rats, despite the probability of the γ-type sequences to be pseudogenes (see above).

The δ-type genes deserve more attention. Although expression of the messenger RNA was demonstrated in spleen tissue of *Rattus norvegicus*, the protein product could not be identified. The gene, found in five other species, does not demonstrate pseudogene features but encodes a much more basic protein than the other *Rattus* RNase 1 sequences (about 10 charge units), as shown in Table 3. RNases 1 with a high excess of positive charges generally show a high activity on double-stranded RNA (Libonati and Sorrentino 2001), which may be important as a defense against pathogens. In mammalian taxa in which the RNase 1 gene is duplicated, the

Table 2. Comparison of the intraspecies, and intra-"gene type," rates of nonsynonymous (K_a) versus synonymous (K_s) substitutions

	K_a/K_s
Rnβ–Rnγ	1.19
Rnβ–Rnδ	0.63
Rnγ–Rnδ	0.62
Reα–Reβ	0.58
Reα–Reδ	0.75
Reβ–Reδ	0.76
Rrβ–Rrγ	0.64
Rrβ–Rrδ	0.61
Rrγ–Rrδ	0.66
Rfα–Rfγ	0.70
Rtβ–Rtδ	1.01
Bbα–Bbδ	0.81
Bbα–Reα	0.47
Bbα–Rfα	0.28
Reα–Rfα	0.24
Rnβ–Reβ	0.32
Rnβ–Rrβ	0.53
Rnβ–Rtβ	0.85
Reβ–Rrβ	0.64
Reβ–Rtβ	0.36
Rrβ–Rtβ	0.59
Rnγ–Rrγ	0.42
Rnγ–Rfγ	0.59
Rrγ–Rfγ	0.43
Bbδ–Rnδ	0.54
Bbδ–Rrδ	1.03
Bbδ–Reδ	0.46
Bbδ–Rtδ	0.89
Rnδ–Rrδ	0.61
Rnδ–Reδ	0.12
Rnδ–Rtδ	0.79
Rrδ–Reδ	0.45
Rrδ–Rtδ	0.66
Reδ–Rtδ	0.72

Table 3. Computed net charge [(R + K + H) − (D + E)] of the various RNase groups identified[a]

Group	Charge
Mus (3 species)	9.3 ± 1.2
Sundamys, Rfα, Bbα, Reα	7.5 ± 1.3
Rnβ, Rrβ, Reβ, Rtβ	8.3 ± 1.0
Rfγ, Rnγ, Rrγ	7.0 ± 4.5
Bbδ, Rnδ, Reδ, Rrδ, Rtδ	18.6 ± 2.6

[a] Histidine (H) contributes only partially to the positive charge at neutral pH values. There are four to six histidines in each of the sequences.

Table 4. Evolutionary rates[a]

	Rate	Ratio
From rat/mouse ancestor (12.2 Myr)		
3 *Mus* species	6.0 ± 1.2	(1.0)
Ancestor of *Niviventer* + *Leopoldamys* + *Rattus* (5.4–5.7 Myr)	5.7	1.0
Niviventer and *Leopoldamys*	8.4 ± 1.1	1.4 ± 0.2
Sundamys and *Rattus* (α and β sequences)	16.0 ± 2.4	2.7 ± 0.4
Rattus and *Berylmys* (δ sequences)	25.0 ± 2.5	4.2 ± 0.4
From *Niviventer* + *Leopoldamys* + *Rattus* ancestor (5.4–5.7 Myr)		
Niviventer and *Leopoldamys*	8.5 ± 2.5	1.4 ± 0.4
Sundamys and *Rattus* (α and β sequences)	24.0 ± 6.0	4.0 ± 1.0
Rattus and *Berylmys* (δ sequences)	45.0 ± 6.0	7.5 ± 1.0

[a] Substitutions per site per 10^3 Myr.

gene expressed in the pancreas generally has a digestive function and is the less basic one, while the other, more basic genes are expressed elsewhere (Kleineidam 1999a; Zhao et al. 2001; Breukelman et al. 2001).

Distances and Rates

The three *Mus*, *Leopoldamys*, and *Niviventer* sequences were already included in the analyses of RNase sequences in our previous paper (Dubois et al. 1999). In that analysis, we could use the paleontological record and calibrate the mouse/rat divergence of 12–14 million years (Myr) versus the K_s values. Mice and *Leopoldamys* + *Niviventer*. differ by 0.31 synonymous substitutions per site. These values give respective rates of 0.013–0.011 substitution per site per Myr, depending on the calibration date (12 and 14 Myr), for the supposed split between the ancestors of mice and *Leopoldamys* + *Niviventer*. The quartet-puzzling likelihood tree presented by Dubois et al. (1999) shows that the RNases 1 of the murid species investigated do not differ significantly in evolutionary rates and allowed us to estimate other ancestral dating by extrapolation.

For deriving evolutionary rates in separate groups of the duplicated genes, the maximum likelihood tree presented in Fig. 4 was used. To position the root of this tree, an additional analysis was performed with several additional RNases 1 of other murid species (Dubois et al. 1999). We used 12.2 Myr for the divergence date of the ancestor of mouse and rat and 5.4–5.7 Mgr for that of *Niviventer* and *Leopoldamys* relative to the genera *Rattus*, *Berylmys*, and *Sundamys* (Verneau et al. 1998; Ruedas and Kirsch, 1997).

Table 4 presents rates calculated for four groups of sequences. Although the γ sequences do not show deviating K_a/K_s values (Table 2), and do form a nice monophyletic group of three sequences in Fig. 4, we did not include them in rate calculations. This group includes a rather deviating sequence from *R. fuscipes*, which might be a hybrid, and only two other sequences, of which one (*R. rattus*) shows possible pseudogene features.

The data in Table 4 show that evolutionary rates were similar in the three *Mus* species and in the ancestral lineage of the rats. After the divergence between *Niviventer* + *Leopoldamys* and the other rats, the rate increased about 40% in the former lineages (in which no gene duplications were observed) and four- to eight-fold in the latter ones. These increased rates may be related to the RNase 1 gene duplications which occurred in the genus *Rattus* and related genera, leading to separate lineages with new, still unknown, properties, and/or other characteristics, of the gene products. The highest evolutionary rates are observed in the δ sequences.

The evolutionary rates in RNase 1 genes of 5–50 substitutions per sites per 10^3 Myr are much higher than those observed in other mammalian taxa. In Cetartiodactyla (including ruminants) rates of 1–4 substitutions per site per 10^3 Myr were observed (Breukelman et al. 2001). An increase in evolutionary rate at the protein level of RNases 1 in rat and mouse, relative to other mammalian RNases, and a significant difference in rate in pancreatic RNases from *Mus musculus* versus *Rattus norvegicus* was observed before (Beintema et al. 1988). However, the rate difference between mouse and rat was not related to gene duplications in the genus *Rattus* as observed in the present work. Also, in Cetartiodactyls (Breukelman et al. 2001) and primates (Zhang et al. 2002a), increased RNase 1 evolutionary rates occur in lineages with gene duplications (ancestral ruminants, tylopods, leaf-eating monkey), but not in taxa in which the gene is not duplicated (pig, hippopotamus, cetaceans, other primates).

Conclusion

The multiplicity of RNases in mammalian taxa is intriguing. In the majority of investigated mammalian species, only one RNase 1 gene is found, which may be expressed at a high level (pig, mouse) or at a

low level (human) as pancreatic enzymes. However, in ancestors of several taxa (ruminants, tylopods, rats, leaf-eating monkey) gene duplications have occurred, which allowed specialization of the duplicates, with generally only one gene product expressed at a high level in the pancreas. These gene duplications are accompanied by high evolutionary rates. RNases 2, of which several are eosinophil associated, have three or four representatives in human (Strydom 1998; Zhang et al. 2002b) but many in four investigated rodent taxa, not only in rat, but also in mice species, gerbil, and hamster (Zhang et al. 2000). The total genomes of human, mouse, and rat are determined or under investigation. It will be interesting to learn from the latter studies if the difference in multiplicity of RNase 1 genes between mouse and rat is an isolated feature or a more general characteristic of the two species.

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