REVIEWS

Duplication and Divergence of 2 Distinct Pancreatic Ribonuclease Genes in Leaf-Eating African and Asian Colobine Monkeys

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Unique among primates, the colobine monkeys have adapted to a predominantly leaf-eating diet by evolving a foregut that utilizes bacterial fermentation to breakdown and absorb nutrients from such a food source. It has been hypothesized that pancreatic ribonuclease (pRNase) has been recruited to perform a role as a digestive enzyme in foregut fermenters, such as artiodactyl ruminants and the colobines. We present molecular analyses of 23 *pRNase* gene sequences generated from 8 primate taxa, including 2 African and 2 Asian colobine species. The *pRNase* gene is single copy in all noncolobine primate species assayed but has duplicated more than once in both the African and Asian colobine monkeys. Phylogenetic reconstructions show that the *pRNase*-coding and noncoding regions are under different evolutionary constraints, with high levels of concerted evolution among gene duplicates occurring predominantly in the noncoding regions. Our data suggest that 2 functionally distinct pRNases have been selected for in the colobine monkeys, with one group adapting to the role of a digestive enzyme by evolving at an increased rate with loss of positive charge, namely arginine residues. Conclusions relating our data to general hypotheses of evolution following gene duplication are discussed.

Introduction

Although conservation of orthologous gene sequences between divergent species suggests that there is an important, conserved function of its protein product (negative selection), rapid evolution of orthologous or paralogous sequences on certain lineages can suggest selection of an equally important, novel function in adaptation to a new environment (positive selection). The case for positive Darwinian selection becomes stronger when distantly related species share that new environment and convergently share similar changes in the orthologous sequence, whereas more closely related lineages share neither. A classic example of this is seen when comparing lysozyme *c* (stomach lysozyme) between foregut-fermenting and non–foregut-fermenting mammals (Stewart and Wilson 1987). Foregut fermentation, as a means of utilizing otherwise indigestible plant parts, has evolved independently at least twice during the radiation of placental mammals, once in the artiodactyl ruminants, such as cow, and later in the colobine monkeys, such as langur (Bauchop and Martucci 1968). In both cases, lysozyme has been recruited to function as an enzyme in the true stomach to degrade the cell walls of bacteria coming from the foregut (Dobson et al. 1984) allowing their contents to be hydrolyzed by other digestive enzymes (Beintema et al. 1977; Vonk and Western 1984). This recruitment has involved increased expression of lysozyme in the stomach, as well as key amino acid replacements in the protein that result in greater stability and functionality with respect to the pH, proteases, and bacterial fermentation products present in the guts of foregut fermenters (Dobson et al. 1984; Stewart and Wilson 1987; Stewart et al. 1987).

Another enzyme that appears to have been recruited to a new function in foregut-fermenting mammals is pancreatic ribonuclease (pRNase) (Barnard 1969). RNase 1, more commonly known as pRNase (due to its high levels in ruminant pancreas), appears to serve a role as a digestive enzyme, but its role in the guts of non–foregut-fermenting mammals is unknown. In the ruminants, bacterial RNAs must be digested in the small intestine to release the phosphorus and nitrogen necessary for the dietary needs of the host animal as well as recycling to the microbial fauna in the foregut (Barnard 1969). Artiodactyl ruminants have as much as 15–1,200 times the amount of pRNase (ug/g) in their pancreas relative to most other non–foregut-fermenting mammals (Barnard 1969; Beintema et al. 1973). Similarly, langur monkey has an increase of approximately 60-fold of pRNase relative to human (Beintema 1990). Through isolation and sequencing of the pRNase protein directly from langur pancreas, Beintema (1990) discovered that the langur enzyme has less positive charge, one less potential glycosylation site, and much less glycosylation relative to the human sequence. These findings supported the hypothesis that species with ruminant-like digestion would have less positive charge and less attached carbohydrate (Beintema et al. 1976; Libonati et al. 1976; Beintema et al. 1988) and suggested that pRNase had been recruited in colobine monkeys to perform a similar digestive function to that in artiodactyl ruminants (Beintema 1990).

This enzyme is a member of the RNase A superfamily, a group of homologous proteins that have been isolated from many vertebrate taxa including, mammals, birds, reptiles, and amphibians (Barnard 1969; Beintema et al. 1973). All presumably evolved from a common ancestral gene but have diverged and acquired novel functions. For example, RNases 2 and 3, also known as eosinophil-derived neurotoxin (EDN) and eosinophil cationic protein (ECP), are present in the large specific granules of eosinophilic leukocytes (Snyder and Gleich 1997) and are transcribed from genes that are thought to be derived from a single ancestral gene that duplicated on the Old World monkey lineage after the divergence of the Old World monkeys from the New World monkeys (Rosenberg et al. 1995). EDN has a high

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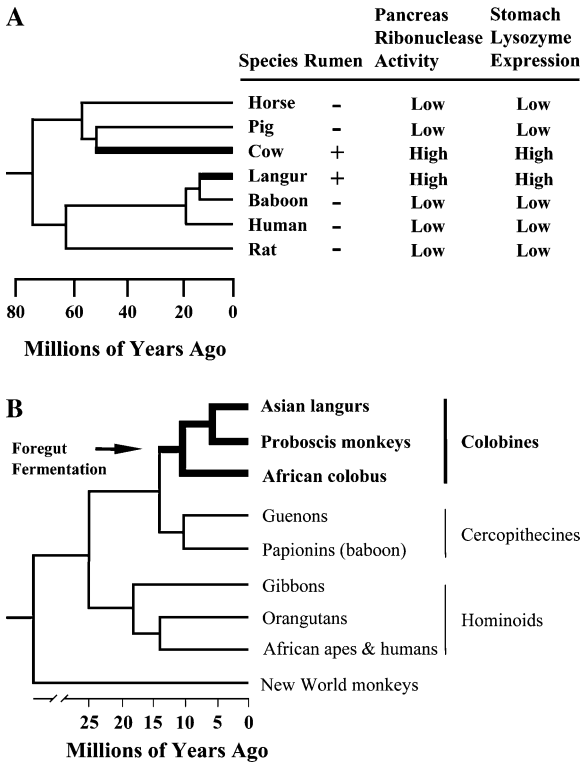


FIG. 1.—Mammalian foregut fermentation evolution and ribonuclease. (*A*) Phylogram of 8 mammalian lineages depicting independent evolution of foregut fermentation in artiodactyl ruminants (cow) and colobine monkeys (Langur). The presence or absence of a rumen (foregut) is indicated by a "+" or "−," respectively. (*B*) Phylogram of representative lineages of primates depicting the origin of foregut fermentation in the ancestral colobine lineage approximately 10–15 MYA. In both phylograms, thick, horizontal lines denote a foregut in that lineage.

RNase activity, nonphysiological neurotoxicity, and probably functions as an antiviral agent by destroying genomic RNA of retroviruses (Domachowske and Rosenberg 1997). In contrast, ECP has very low RNase activity but is toxic to various pathogens by making pores in their cellular membranes (Rosenberg and Dyer 1995). Similarly, bovine RNase 1 (pRNase) has duplicated twice in some artiodactyl ruminants to generate 2 novel proteins, bovine seminal and brain ribonucleases (D'Alessio et al. 1972; Suzuki et al. 1987; Watanabe et al. 1988; D'Alessio et al. 1991; Sasso et al. 1991; Breukelman et al. 1993). Interestingly, seminal ribonuclease forms a protein dimer and is not bound nor inhibited by ribonuclease inhibitor protein in this form (Murthy and Sirdeshmukh 1992), although the function of either the seminal or brain RNase is unknown. Another, more extreme, example of disparate function in this superfamily is the comparison of mammalian RNase 5 (angiogenin), which is involved in tumorogenesis, with amphibian onconase, which has specific antitumor activity. Clearly, duplication, divergence, and acquiring novel function is recurrent in the RNase A superfamily.

Evolution of foregut fermentation in artiodactyl ruminants probably occurred between 55 and 60 MYA. In these species, levels of stomach lysozyme and pRNase are much increased in comparison with their nonforegut relatives such as horse and pig (fig. 1*A*). Colobine monkeys have also evolved foregut fermentation and, similarly, have increased levels of these 2 enzymes in their guts when compared with the other primate groups (fig. 1). However, this adaptation in colobine monkeys has occurred relatively recently (ca. 15 MYA) and is thus a model system to detect positive selection in enzymes that have been recruited to serve a role in this system (fig. 1*B*) (Stewart and Wilson 1987; Stewart et al. 1987). There are 2 main groups of colobine monkeys (the African and the Asian), which diverged approximately 10–11 MYA. For this study, 2 species from each continent, representing deep lineages, were chosen for *pRNase* sequencing. The black-and-white colobus and red colobus species from Africa diverged approximately 8–9 MYA. Similarly, the langur and proboscis monkey species from Asia diverged at approximately that same time.

For several years, our lab has been tracing the evolutionary history of *pRNase* in the primates (Lee 1993; Auerbach 1997; Schienman et al. 2000), with emphasis on adaptive changes in protein sequence and/or gene expression that might have occurred on the colobine lineage with regards to foregut fermentation biology. In all other primates assayed, *pRNase* is a single gene, but in the colobines, we have discovered a complicated history of gene duplication and adaptation. In this study, we present molecular analysis of 23 *pRNase* sequences generated from 8 taxa; 2 homonoids, one cercopithecine, one New World monkey, and 4 colobines (2 from Africa and 2 from Asia, representing the most divergent lineages based on our phylogenetic studies of the colobines). Similar to stomach lysozyme and *pRNase* in artiodactyl ruminants, but unlike stomach lysozyme in most colobines, we have shown that *pRNase* has duplicated at least once in all colobines assayed. Our data suggest that there are divergent functions of the pRNase paralogues. There appears to be 2 main classes of sequences with respect to net positive charge (more specifically, arginine content) and to their rate of evolution. Phylogenetic reconstructions using either the *pRNase*-coding sequences or their associated noncoding sequences show that the coding and noncoding regions of a given gene have different evolutionary histories with respect to concerted evolution, providing further evidence for selection of 2 functionally distinct pRNases. The data from these 4 colobine species suggest that, similarly to artiodactyl ruminants, one group of *pRNase* sequences has evolved to adapt to a role as a major digestive enzyme in the colobine monkeys.

Materials and Methods

Tissues, DNA Sources, and DNA Isolation

Tissues for DNA extractions used in this study were from a variety of sources (animal's ID number or name included if applicable): orangutan (*Pongo pygmaeus*), YN93-312, liver, Yerkes Regional Primate Research Center, H. McClure; baboon (*Papio hamadryas*), 821222, liver, Washington Regional PRC; patas monkey (*Erythrocebus patas*), R229, testicle, New York University, T. Disotell; Guyanese squirrel monkey (*Saimiri sciureus sciureus*), 4530, liver, University of South Alabama, K. Gibson; black-and-white colobus (*Colobus guerza*), Flat-top, liver, Houston Zoological Gardens, B. Lester; red colobus (*Piliocolobus*

badius), liver, Louisiana State University, P. Marx and P. Telfer; Hanuman langur (*Semnopithicus entellus*), Coast, heart, University of California, Berkeley, P. Dolhinow; proboscis monkey (*Nasalis larvatus*), 861205, liver, Bronx Zoo, G. Amato. The tissues from all species, except red colobus, were from captive animals in the United States; red colobus tissue was imported by P. Marx under his CITES and other permits. The amount of red colobus tissue we obtained was insufficient for preparation of DNA for blot analysis. Human DNA was from an unidentified Caucasian male. Genomic DNA was isolated either using a phenol/chloroform extraction and ethanol precipitation protocol (Strauss 1998) or using a genomic DNA buffer set and tip-100s (Qiagen Inc., Valencia, CA, cat # 19060 and cat # 10243).

DNA Sequencing

In order to generate DNA sequence for the *pRNase* gene from a variety of primate species, the strategy of polymerase chain reaction (PCR) from genomic DNA isolated from individuals for these species using primers designed based on the human *pRNase* gene was employed. This project was initiated before the Human Genome was publicaly available; thus, to obtain the human DNA sequence upstream of the *pRNase* gene, a Human GenomeWalker Kit (Clontech, Mountain View, CA, cat # K1803-1) was used according to the manufacturer's instructions. To obtain these sequences from the other species that have a single copy of the *pRNase* gene, an upstream oligo primer, RH43 (5′-GCTGAAACAATCTGAATACAGAGC-3′), was designed and used with RH27 or RH28 to produce amplicons of approximately 2.5 kb; these amplicons were sequenced directly. To obtain gene-specific sequences for direct sequencing from the colobine species, oligo primer, RH43, was used with downstream, gene-specific oligo primers for each unique *pRNase* sequence that was generated.

PCR amplicons were generated from 1 μg of genomic DNA of a single individual using the oligonucleotide primers JS1 (5′-GATGAGCCATTTGAGCAC-3′) or JS2 (5′-GTTCTTTTTCTAAGAGGGCTC-3′) and RH28 (5′-GCCAGAAACAGTACCTAG-3′) or RH27 (5′-GCTTCAGAAATCAGGTGTTTGCA-3′) in a 50-μl reaction containing 1× PCR Buffer II (50 mM KCl, 10 mM Tris-HCl, pH 8.3), 3 or 4 mM $MgCl_2$, 200 μM deoxynucleoside triphosphates, 0.2 μM each primer, and 1–2 units of *Taq* Polymerase (Roche Diagnostics, Branchburg, NJ). Cycling conditions were 95 °C for 1 min, 60 °C for 1 min, then 72 °C for 1 min, and 20 s for 30 cycles. Amplicons were sequenced directly using primers JS1, JS2, MA2, NewN, NewC, RH27, and/or RH28 and a BigDye Terminator DNA Sequencing Kit (Version 1) according to the manufacturer's instructions (Applied BioSystems, Foster City, CA, cat # 4303149). For sequencing from species that generated multiple gene sequences, the PCR amplicons were cloned using a TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA, cat # K4500-01), and plasmid DNA was isolated using a QIAprep Spin Miniprep Kit (Qiagen Inc., cat # 27106). Sequence was generated from 20 to 30 clones and analyzed by creating a multiple sequence alignment using the software SeqEd version 1.0.3 (Applied Biosystems). Based on the clone sequences from a given individual, primers were designed with 3′ ends specific to individual genes or alleles using the differences observed between gene sequences. These gene/allele-specific primers were used to generate amplicons from genomic DNA and to directly sequence these amplicons to determine if sequences that were rare among the clones were real or PCR artifacts. The sequences of all oligo primers used for PCR and/or complete double-stranded coverage sequencing, and for which gene sequences they were designed, are available on request. The 23-*pRNase* sequences generated and analyzed for this study can be found in the GenBank database by the accession numbers DQ494857–DQ494879.

Genomic Blot Analyses

Purified genomic DNAs were quantitated by comparison with a known amount of human genomic DNA (Clontech) after electrophoresis in an ethidium bromide–stained 0.7% agarose gel. Approximately 10 μg of DNA was digested with restriction enzymes *Kpn*I, and/or *Pst*I according to manufacturer's instructions (Fermentas, Hanover, MD), and electrophoresed in a 0.55% agarose gel at 3.5 V/cm for 20 h. The DNA in the gel was blotted onto a Zeta-Probe GT blotting membrane (Bio-Rad Laboratories, Hercules, CA). The membrane was hybridized with either a 5′- or 3′-pRNase probe (see below) and was washed according to the manufacturer's instructions (with HCl depurination).

For the 5′-pRNase probe, the primers JS1 and JS11 were used to PCR amplify a 569-bp fragment of the *pRNase* gene (see fig. 2*A*) from black-and-white colobus (see below). Similarly, for the 3′-pRNase probe, the primers RH24 and RH27 were used to amplify a 323-bp fragment (see fig. 2*A*). These fragments were gel purified, diluted 1,000-fold, and reamplified twice. Each fragment was labeled with [α-^{32}P] deoxycytidine triphosphate and deoxyadenosine triphosphate using a High Prime DNA Labeling Kit (Roche Diagnostics).

Sequence Alignments and Phylogenetic Analyses

DNA sequences were aligned using ClustalX freeware (Thompson et al. 1997) and then imported into GeneDoc software (Nicholas 1997) for adjustments of the alignment, printing, and exportation to other software programs. Phylogenetic trees of the *pRNase* nucleotide-coding region were generated with PAUP* versions 4.0 beta8-10 (Swofford 1998). See figure legends for details. To reconstruct the placement of amino acid replacements on specific lineages and branches of a specific phylogram, the *pRNase* DNA sequence alignment was imported into MacClade 4 (Maddison DR and Maddison WP 2000) and translated using a PROTPARS matrix (Felsenstein 1993). Amino acid replacements were mapped onto the tree topology presented in figure 6. Ambiguous replacements were mapped by averaging over all equally parsimonious reconstructions.

Results and Discussion

Determination of Gene Copy Number by Genomic Blot Hybridization

As an alternative method to determine *pRNase* gene copy number in the colobines relative to noncolobine

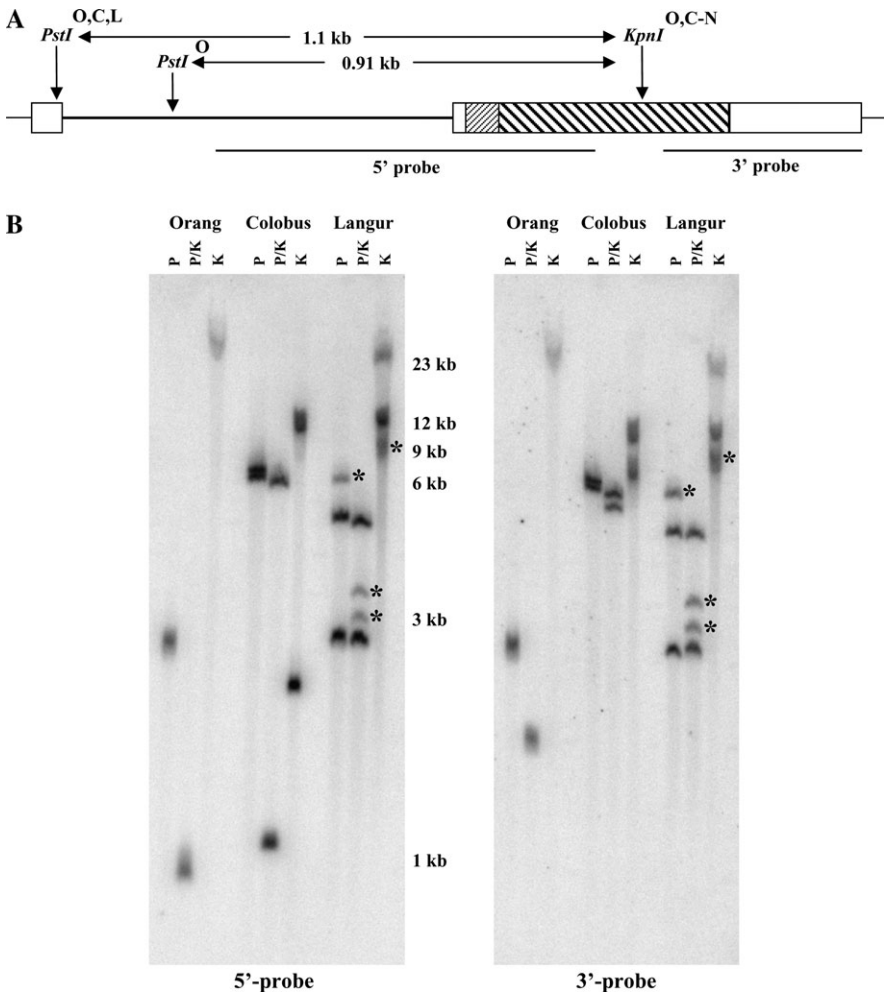


FIG. 2.—*pRNase* gene structure and genomic DNA blot analysis. (*A*) Diagram of the mammalian *pRNase* gene structure. Lines and rectangles represent DNA of the *pRNase* gene and surrounding DNA. Thin lines on the far left and right represent the adjacent DNA to the locus. The thickest black line denotes the 702- to 709-bp intron. The open rectangles denote the 5′- and 3′ UTRs of the gene. The rectangle filled with thin diagonal lines denotes the coding region for the signal peptide, and the rectangle filled with thick diagonal lines denotes the coding region of the mature protein. Downward arrows show the approximate location of the restriction enzyme cut sites listed directly above. The *Pst*I cut site located in the first exon is present in all *pRNase* genes sequenced in this study. The *Pst*I cut site located in the intron is present only in the human, orangutan, and baboon sequence generated in this study. The *Kpn*I cut site located in the coding region of the second exon is present only in the human, orangutan, baboon, *B&WCol-N*, *RedCol-N*, *Prosboscis*-ψ gene sequences. The superscripts of the restriction enzymes denote which species presented in the blot below have that site (O = orangutan, C = black-and-white colobus, C-N = *B&WCol-N* gene only, L = langur). The approximate distance in kilobase pairs from the *Pst*I to the *Kpn*I cut sites are shown. The 2 middle thick lines below the gene denote the DNA regions covered by each hybridization probe. The drawing is approximately to scale. (*B*) Duplicate genomic DNA blots from the same orangutan (Orang), black-and-white colobus (Colobus), and Hanuman langur (Langur) individuals used for *pRNase* gene sequencing. Letters at the top of each lane denote the restriction enzymes, P (*Pst*I) and/or K (*Kpn*I), used in digestion of the genomic DNA. The numbers with the kilobase designation following them represent the expected distance of migration for a DNA fragment of that size (based on standard molecular weight ladders not seen in blot). An asterisk (*) denotes that the hybridizing DNA fragment just to the left of it is produced from the intronless, retroposed pseudogene.

primates, several genomic blots were generated and hybridized with probes specific to various regions of the gene (fig. 2 and data not shown). Figure 2*A* presents a schematic of the primate *pRNase* gene structure, which consists of 2 exons separated by an intron ranging from 702 to 709 bp (depending on species and allele or paralogue). The total gene length, from transcriptional start site to the polyadenylation signal, consists of approximately 1500 bp. The 5′ untranslated region (UTR) is split between exons 1 and 2. The second exon also contains the entire coding region for the signal peptide and the mature protein, as well as the 3′ UTR. Genomic blots were created from DNAs isolated from the same individuals used to generate the *pRNase* gene sequences for the various species. Prior to electrophoresis and blotting, the DNA had been digested with 2 enzymes in single and double enzyme digestion combinations. See figure 2*A* for the approximate location of the cutting sites for the restriction enzymes *Kpn*I and *Pst*I within the *pRNase* gene sequences. Several identical blotted membranes were made and then hybridized with either a probe specific for the 5′-coding and intron portion of the gene ("5′ probe," in fig. 2), the 3′-coding portion ("3′probe"), or the intron portion only ("intron probe," data not shown). Figure 2*B* shows the blot and hybridization results for the orangutan, black-and-white colobus, and Hanuman langur individuals using the 5′ probe (left) and the 3′ probe (right).

The *pRNase* genomic blots from orangutan (fig. 2*B*), as well as those from human and baboon (data not shown), show a single hybridizing fragment for both the 5′- and 3′ probes for DNA digested with these enzymes. This confirms our PCR sequencing results and previous blot experiments using human and mouse genomic DNAs done by others (Breukelman et al. 1993) that suggest there is a single gene in most mammals, with the notable exception of the artiodactyl ruminants. The presence of a processed pseudogene in the Asian colobines is suggested by the blot data because there are fragments that hybridize to the 5′- and 3′ probes but not the intron probe (* in fig. 2*B*; plus data not shown). The blot data for the digestions of these colobine individuals are consistent with the minimum number of paralogous *pRNase* genes as determined by PCR sequencing, with the presence of 2 hybridizing fragments in the black-and-white colobus and 3 in the Hanuman langur. The *Kpn*I/*Pst*I double digestion of the langur DNA shows 4 hybridized fragments. The 2 middle-sized fragments do not hybridize to the intron probe and most likely represent the 2 pseudogene alleles, *Langur-ψa* and ψb (see gene name designations in next section). For these 2 alleles, the first *Kpn*I site located downstream of the gene's 3′ end must be a slightly different base pair distance from the upstream *Pst*I site. Proboscis monkey genomic DNA had a pattern of hybridization similar to that of langur (not shown). Not enough genomic DNA was available for preparation of blots for the red colobus individual, so a comparison of blot hybridization patterns between red colobus and the other colobine species could not be made.

Sequencing of Pancreatic Ribonuclease Genes

To determine *pRNase* gene sequences as well as gene copy number, one primer pair, JS1 and RH27, was used to generate PCR products for direct sequencing from DNA of a single individual for all species tested. Direct sequencing was performed with these primers, as well as with primers JS2 and MA2. Direct sequencing from the human, orangutan, and baboon PCR products produced a single, clean gene sequence (data not shown). However, the 4 colobine species (black-and-white colobus, red colobus, Hanuman langur, and proboscis monkey) all produced "dirty" sequence; that is, more than one nucleotide peak was observed at multiple base positions throughout the sequencing electropherograms, which is suggestive of more than one allele or *pRNase* gene sequence being present in that individual.

To determine each unique *pRNase* gene sequence, PCR products from each of these 4 species were cloned and between 20 and 30 clones were sequenced from each. Additionally, a second primer pair, NewN and NewC, was used to generate PCR products that were subsequently cloned and sequenced. When analyzing the aligned sequence data from all the clones (data not shown), a point mutation contained in one clone only was considered to be an error generated either by the *Taq* DNA polymerase (Roche) during the PCR or the bacterial cloning process and not considered a real gene or allele sequence. In addition, there existed sequences in singular clones that appeared to be a mosaic or hybrid of 2 of the other unique sequences represented by multiple clones. There are at least 2 ways that mosaic or hybrid sequences could be generated as artifacts during PCR when multiple homologous gene sequences are amplified by a single set of primers. One way is through crossover PCR (Meyerhans et al. 1990). A second way is through mismatch annealing of a complete Watson strand of one gene sequence with a complete Crick strand of another, followed by unbiased mismatch repair in the *Escherichia coli* host on transformation. To determine which cloned sequences might be such PCR artifacts, specific primers were designed to regions of closely spaced nucleotide differences in the sequences represented most frequently among the initial clones. PCR products were then generated again from genomic DNA using these specific primers and directly sequenced. If multiple products were present in direct sequencing, then these products were cloned and sequenced. This process was repeated until single "clean" gene sequence could be obtained directly from PCR products generated from genomic DNA.

From the above procedures, 3 unique *pRNase* gene sequences were produced from a single black-and-white colobus individual and 5 unique sequences each were produced from single individuals of red colobus, Hanuman langur, and proboscis monkey. The letter and number designations of each sequence name were decided based on several criteria. First, the amino acid that is present at 3 specific positions in the translated pRNase sequences of this study can define 2 mutually exclusive groups (positions 1, 6, and 39 of the mature protein). For one of these groups, 2 of these 3 amino acid residues match the human sequence that is known to be expressed in relatively low or moderate levels in numerous human tissues, including pancreas (numerous = N type). Second is the knowledge that one of the langur gene sequences, which is part of the second group defined above (i.e., does not match the human sequence when translated), corresponds to the single ribonuclease protein sequenced from an individual langur pancreas (Beintema 1990). This along with our reverse transcriptase–PCR experiments on the black-and-white colobus sequences (data not shown) suggest that this second group of colobine sequences are the gene sequences expressed predominantly in colobine pancreas (predominantly pancreatic = P type). Third is the fact that 2 of the gene sequences in langur and one in proboscis monkey are processed, retroposed pseudogenes (pseudogene = ψ type, see below). Lastly is the assumption of a simple model of gene duplication of *pRNase* in the colobine species (See "Evolutionary Tree Inference section" and fig. 3). The number of distinct gene sequences generated from single individuals and the genomic blot hybridization results suggest a minimum of 2 *pRNase* genes in black-and-white colobus and a minimum of 3 *pRNase* genes in the other 3 colobine species studied here. The 3 gene sequences for black-and-white colobus were thus named *B&WCol-Pa*, *B&WCol-Pb*, and *B&WCol-N*. The 5 gene sequences of red colobus were named *RedCol-P1a*, *RedCol-P1b*, *RedCol-P2a*, *RedCol-P2b*, and *RedCol-N*. The 2 Asian species (langur and proboscis) share a gene duplication event not found in the African colobus monkeys. As mentioned above, langur and proboscis monkey share unique gene sequences in that these sequences appear to be processed, retroposed pseudogenes. They lack the intronic sequence found in other

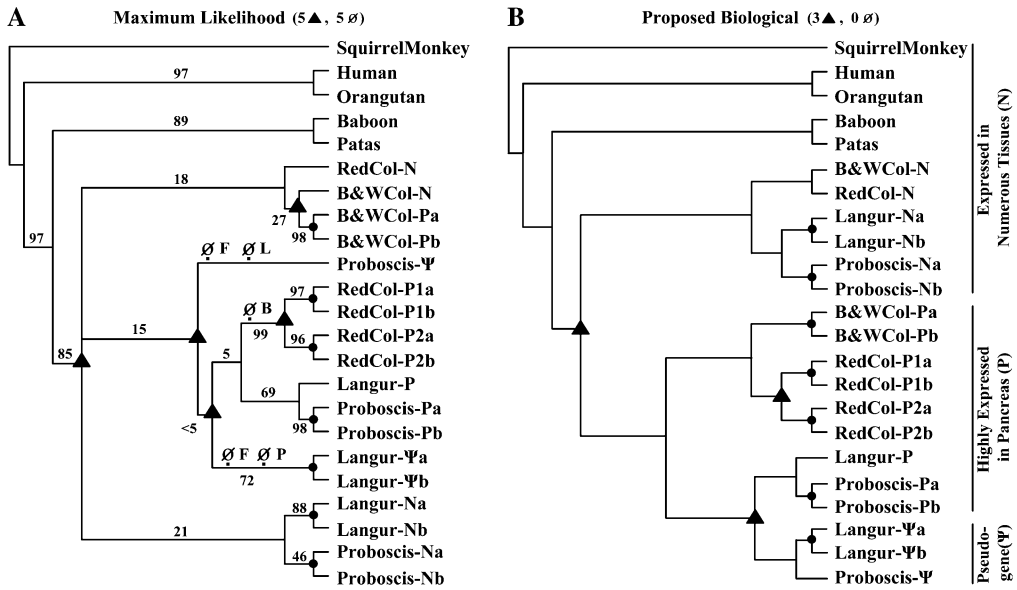


FIG. 3.—Evolutionary hypotheses for the primate *pRNase* gene sequences. Presented are 2 different cladograms depicting the relationships between the gene sequences presented in this study. The presence of a required gene duplication or a required gene deletion are denoted by the symbols (▲) and (Ø), respectively. The letter immediately following the gene deletion symbol denotes for which species it has been deleted (B = black-and-white colobus, L = Hanuman langur, P = proboscis monkey, and F = both African colobines). The number of required gene duplications and deletions required for each tree is listed in parentheses directly above that tree. Sequences that we believe are an allelic pair is denoted by (●). The proposed biological tree is based on the known phylogenetic relationships between the species groups and reasons discussed in the results section. The maximum likelihood cladogram was generated in PAUP* 4.0b8-10 (Swofford) using the General Time Reversible + G + I model with estimation of all parameters, heuristic search, starting trees via stepwise additions (10 replicates) with random seed; 100 bootstrap replicates with fast heuristic search, parameters estimated, and starting trees via stepwise addition with random addition sequence. For parameters not mentioned here, the default settings were used. The squirrel monkey *pRNase* sequence was used as the outgroup. The number directly above or below each lineage branch is the bootstrap support for that clade.

pRNase genes, have the remnants of a polyA tail, and are flanked by direct repeats. The 5 gene sequences of Hanuman langur and proboscis monkey were thus named *Langur-P*, *Langur-Na*, *Langur-Nb*, *Langur-ψa*, and *Langur-ψb* and *Proboscis-Pa*, *Proboscis-Pb*, *Proboscis-Na*, *Proboscis-Nb*, and *Proboscis-ψ*, respectively.

Evolutionary Tree Inferences of the Primate *pRNase* Gene Sequences

The species chosen for this study have a well-established phylogeny with the black-and-white colobus and red colobus species belonging to an African colobine clade, and the Hanuman langur and proboscis monkey species belong to an Asian colobine clade. All 4 species represent deep lineages in their respective clades. This known species tree allowed us to subject the *pRNase* sequences to phylogeny-based analyses to help infer the orthologous and paralogous relationships between the genes. Under ideal circumstances, orthologous gene trees should recreate the species tree. Ideal circumstances include higher levels of phylogenetic signal than noise (e.g., homoplasy) and lack of gene conversion or unequal crossover exchange between paralogues. The expectation would be the following order of relatedness of the colobine gene sequences. Allelic sequences should be the most similar, followed by orthologues within the African or Asian colobines, then orthologues between the African and Asian colobines, and finally paralogues that are the result of gene duplication that occurred before the species divergence.

Two trees depicting inferred phylogenetic relationships between the gene sequences are presented in figure 3. The maximum likelihood tree was generated by using approaches available in PAUP*, see figure legend (Swofford 1998). The second tree represents our "proposed biological" tree based on the known phylogenetic relationships of the colobines and the reasoning used to name the different gene sequences discussed earlier. Trees were also generated using parsimony and distance methods (data not shown). The computationally derived trees have different topologies relative to each other, as well as to the proposed biological tree. The discrepancies between these trees become less of a concern when considering the fact that the supporting bootstrap values for many of the clades are quite low, in many cases well below 50%, suggesting that the ideal circumstances discussed above are not being met. Possible reasons for this will be discussed below.

To test if any of these trees were significantly different from each other with respect to the sequence data, a Shimodaira–Hasegawa test (one tailed) with 1,000 bootstrap replicates under full optimization was performed on 6 tree topologies. In addition to the 2 tree topologies shown in figure 3, trees generated by parsimony, distance, distance using a maximum likelihood to generate DNA distances, and Bayesian maximum likelihood methods (not shown) were also tested. Parameters for the base frequencies, substitution rate matrix, proportion of invariable sites, and gamma shape of the frequency of substitution at variable sites were estimated for each tree. In testing the null hypothesis that the trees are not significantly different statistically,

none of the trees were found to be significantly better than the others. The tree topology with the best log-likelihood score was the one generated by maximum likelihood (fig. 3*A*) using full estimation of the parameters listed above. The differences in log-likelihood scores from this tree ranged from 1.01 ($P = 0.723$) for the Bayesian maximum likelihood tree to 7.62 ($P = 0.283$) for the distance tree using maximum likelihood for DNA distance (significance when $P < 0.05$). The proposed biological tree had a difference in log-likelihood of 4.91 ($P = 0.461$) from the best tree and thus cannot be ruled out by this test.

For any of the computationally derived trees to be a true representation of the evolutionary history of these colobine *pRNase* gene sequences, a specific number of gene duplications and deletions are required at nodes throughout each tree (e.g., fig. 3*A*). The phylogeny generated by parsimony requires that 5 independent duplications and 5 independent deletions of the genes from the various genomes would have had to occur in the colobine lineage. Likewise, the distance- and maximum likelihood–based phylogenies require 7 and 5 gene duplications, respectively and 10 and 5 gene deletions, respectively. In contrast, the proposed biological phylogeny requires only 3 gene duplications and no gene deletion events. Thus, the proposed biological tree is the most parsimonious with respect to gene duplications and deletions and is consistent with the genomic blots. Lack of high bootstrap support could be in part due to the short time intervals of some nodes (2–4 Myr). Other reasons include gene conversion, concerted evolution, and selection on coding regions.

Nonindependent Evolution of Colobine *pRNase* Gene Duplicates

There are several possible reasons for these low support values and the different tree topologies generated. First, gene conversion could have occurred between *pRNase* sequences making a gene sequence in one species appear as a mosaic of paralogous and orthologous sequences relative to another species' sequence. Supporting evidence for such gene conversion occurs when examining the *B&WCol-Pa* allele and *B&WCol-N* gene sequence; these genes share a single nucleotide deletion 5 bp before the normal stop codon that is not shared with the *B&WCol-Pb* allele. This substitution creates a premature stop codon and a resulting protein that would be 2 amino acids shorter than the other primate ribonucleases. If the *Pa* and *N* sequences from this individual are not alleles of the same genetic locus, it is very unlikely that they would have independently deleted the same critical nucleotide. But, by pairwise nucleotide difference, the *Pa* and *Pb* gene sequences are 3-fold more similar to each other than to the *N* sequence (0.9% vs. 2.8–3.2%, respectively), suggesting that the *Pa* and *Pb* sequences are most likely alleles (or the result of a more recent duplication event), and if so, the *Pa* and *N* sequences cannot be allelic. Alternatively, unequal crossover or exchange could be occurring if the gene duplicates are linked relatively closely on a chromosome. Unequal alignment of sister or homologous chromatids during meiosis, followed by a crossover event and fixation, could delete and reduplicate different gene sequences in the different species. This could create the situation where certain *pRNase* orthologues may no longer exist in every colobine species. Of course, these 2 possibilities are not mutually exclusive and could have both occurred in each species.

If a gene duplication event occurs before speciation and the duplicates are evolving independently and at relatively equal rates, then the orthologues between species should be more similar at the nucleotide level than paralogues within a species. Additionally, if multiple species share a duplicated gene, it suggests that the duplication event preceded the speciation event. Contrary to this, if biased gene conversion and/or unequal crossover events are occurring between the paralogues within species, then these duplicates are not evolving independently. In the extreme case of high levels of recombination, the duplicates within a species are more similar to each other than to their orthologues in the other species. This phenomenon is known as "concerted evolution" (Zimmer et al. 1980; Coen et al. 1982). In our data, the level of pairwise nucleotide divergence between apparent paralogous coding sequences (within one species) is similar to that observed between some of the apparent orthologous coding sequences (e.g., *RedCol-P2a* to *RedCol-N*, 5.3% compared with *RedCol-P2a* to *B&WCol-Pb*, also 5.3%). This suggests that the duplicates within a species are not evolving independently and the possibility that relatively high levels of recombination might be occurring between paralogous *pRNase* genes. One caveat to this possibility is that positive selection, causing rapid evolution of the pancreatic-type coding sequences, could be influencing these levels of divergence, that is, the duplicates are not evolving at relatively equal rates.

To further test this hypothesis on sequences that should have little or no selection acting on them, 1,744 bp of noncoding contiguous DNA, consisting of upstream 5′ untranslated, intronic, and 3′ UTRs, were sequenced for 13 of the *pRNase* genes and subjected to phylogenetic analyses. Figure 4 presents phylograms obtained from maximum likelihood analysis performed on the noncoding and coding sequences for the 13 taxa. In support of the idea that concerted evolution is occurring between the *pRNase* duplicates, the noncoding sequences of duplicates within a species are more closely related to each other than to their orthologues, with high bootstrap support. Shimodaira–Hasegawa tests were performed, as before, with the coding and, separately, the noncoding sequence data and these 2 tree topologies, as well as the proposed biological topology, for the 13 sequences only. The noncoding sequences were consistent only with the topology generated for them in maximum likelihood ($P < 0.0005$ for the other 2 topologies). For the coding sequences, none of the 3 topologies were significantly better than the others (proposed biological topology, $P = 0.46$; noncoding topology, $P = 0.16$), but clearly, the coding data are more consistent with the former than the latter. To test if these results were simply due to the disparity in the length of sequences analyzed (1,744 bp of noncoding vs. 468 bp of coding), the 1,744 bp of noncoding sequence was divided into smaller subsections and reanalyzed as above (data not shown). The 1,744 bp of noncoding sequence was divided up in several different ways before analysis. First, the noncoding sequence was divided into the first 540 bp, the second 540 bp, and the final 664 bp. Second, just the 884 bp of intron and 3′UTR were analyzed. Lastly, this

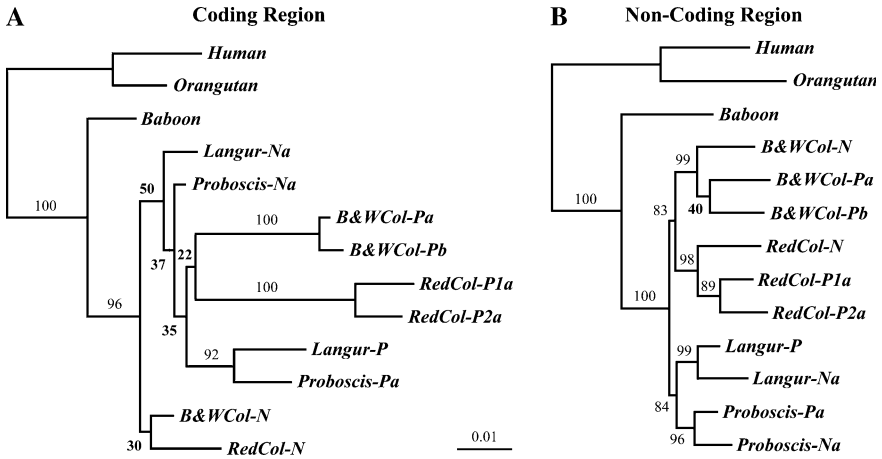


FIG. 4.—Phylogram comparison between *pRNase*-coding and noncoding sequences. Presented are phylograms depicting the relationships between the coding (*A*) and noncoding (*B*) regions for 13 of the *pRNase* genes sequenced in this study. The phylograms were generated by maximum likelihood in PAUP* version 4.0beta10 (Swofford). Parameters were estimated using a General Time Reversible (GTR) + G + I model. A heuristic search was performed with starting trees obtained by random stepwise addition (10 replicates) with a random tree as a starting point. The numbers just above the lineage branches are the bootstrap support for that lineage based on 1,000 replicates under the GTR + G + I model. The 468 characters of the coding sequences and the 1,744 characters of the noncoding sequences were resampled in each replicate and starting trees were obtained by stepwise addition (10 replicates) with a random starting point. All other settings used were default. Human and orangutan were set as the outgroup species. Branch lengths for each lineage are proportional to each other with the branch length scale bar shown equal to 0.01 substitutions per site.

884 bp was divided into 2 approximately equal halves and analyzed. Although there was somewhat lower bootstrap support for some of the clades, neither were there significant differences in the best tree topologies nor were any of the subdivided noncoding data consistent with the proposed biological or coding sequence tree topology. These results strongly suggest that the intron and other noncoding sequences adjacent to the *pRNase*-coding sequence are being highly homogenized between duplicates within each of the 4 colobine species; thus, the *pRNase* gene duplicates are not evolving totally independently.

This nonindependent evolution of apparent paralogous sequences within each of these species could be due to biased gene conversion or the occurrence of independent duplications and deletions after speciation. Discerning between these 2 mechanisms is difficult, because both could be occurring to some degree. However, if either mechanism was predominant, different predictions would be made with respect to shared amino acid replacements between paralogues within and orthologues between each species. Independent duplications and deletions through unequal exchange occur after speciation; thus, this scenario would predict that paralogues within a species would share neutral amino acid replacements not seen in the proteins of the other species. Conversely, if the duplication event occurred before speciation and biased gene conversion was the predominant mechanism of nonindependent evolution, then there should be a significant number of shared amino acid replacements between apparent orthologues that arose before the speciation events.

Amino Acid Replacements and Selection of Colobine *pRNase*

This can be tested with our data by generating phylogenetic reconstructions of the most parsimonious amino acid replacements with respect to specific lineages or branches in a tree relating the sequences. We performed this test on both the proposed biological tree configuration (see fig. 3*B*) and a tree configuration that can be best explained by postspeciation duplications and deletions (see fig. 4*B*) using the software package MacClade 4 (Maddison DR and Maddison WP 2000) and a PROTPARS matrix (Felsenstein 1993) to translate the gene sequences (data not shown). The results show that for the recent duplication and deletion tree configuration, the majority of amino acid replacements map to the terminal branches of the P-type sequences with few, if any, replacements being shared uniquely by paralogues within a species. This would require many independent parallel changes in the P-type sequences. On the other hand, when the replacements are mapped onto the proposed biological configuration, the amino acid replacements are more evenly distributed throughout the branches leading from the putative, original duplication event before speciation to the terminal branches of the P-type sequences. Additionally, the tree configuration representing independent duplications and deletions requires a tree length of 92 amino acid replacements, whereas the proposed biological tree configuration requires only 83 replacements. These results further support the proposed biological tree as the true representation of the evolutionary history of the *pRNase* gene sequences and strongly suggest that biased gene conversion is the predominant mechanism for the nonindependent evolution of paralogues within each species.

Also of note—and consistent with the hypothesis of intergenic recombination—is the fact that the branch lengths of the noncoding sequences are relatively equal to each other, that is, these regions are evolving at similar rates. In comparison, the branch lengths for the coding sequences are not equal. From the point of the first duplication, the P-labeled (P-type) DNA sequences are evolving 2.83 (*Langur-P/Langur-Na*) to 5.97 (*B&WCol-Pb/B&WCol-N*)

times faster than the N-labeled (N-type) sequences within the species. To compare branch lengths between coding and the adjacent noncoding sequence for each gene, the coding sequences were mapped onto the tree topology obtained from the 13 noncoding sequences (data not shown). Interestingly, when this is done and the branch lengths compared, the P-type coding sequences are evolving 1.71–3.44 times faster than their corresponding, adjacent noncoding sequences, whereas the N-type coding sequences are evolving slightly slower than the corresponding noncoding sequences (0.42–0.93). If it is assumed that the majority of each noncoding sequence is evolving at the neutral rate, then the P-type sequences are evolving 2–3 times faster than the neutral rate, suggesting the possibility that they have been positively selected (provided that the majority of change is not just at synonymous sites), against a background of strong homogenization due to intergenic recombination.

To more rigorously test for selection on these genes, rates of nonsynonymous and synonymous substitution (abbreviated dN for number of nonsynonymous changes per number of nonsynonymous sites and dS for number of synonymous changes per number of synonymous sites in the DNA sequence) were first calculated using pairwise comparisons performed in the FENS program (APJ de Koning and CB Stewart, program available from the authors). A $dN/dS < 1$ implies negative or purifying selection, $dN/dS = 1$ implies neutral or no selection, and $dN/dS > 1$ implies positive or adaptive selection. Although all P-type sequences had a $dN/dS > 1$, few were found to be significant at less than the 5% level (using the statistical *z*-tests performed by FENS), and significance level was dependent on the pairwise comparison and/or the dN/dS method used. Several of the N-type sequence comparisons had $dN/dS > 1$ as well, but these were always less than the P-type sequences in the same species. For example, using Ina's method (Ina 1995), *Proboscis-Na* and *-Nb* had a dN/dS of 1.66 and 1.49 relative to 2.15 and 2.41 for *Proboscis-Pa* and *-Pb*, respectively. The number of nonsynonymous substitutions for the P-type sequences (ranging from 17–23, $\bar{x} = 20.4$) was always between 2–2.5 times higher than that of the N-type sequences (ranging from 8 to 10, $\bar{x} = 9$), but the synonymous rate for the P-types was higher as well (ranging from 3–5, $\bar{x} = 4$ compared with 2–6, $\bar{x} = 3$).

In the previous analysis, individual pairs of sequences are compared and statistically analyzed as if each pair is independent of the others, when in reality they are not. To determine and compare dN/dS ratios in a simultaneous manner for a large group of sequences from many species, an alternative approach was performed using lineage-specific likelihood ratio tests for positive selection against the null hypothesis of $dN/dS = 1$ (Yang 1998). Using this method with the nucleotide sequences and the tree topology shown in figure 6, 2 of the 3 P-type lineages were significant with respect to positive selection (*RedCol-P1* and *-P2* lineage, $P = 0.015$ and the *Langur-P*, *Proboscis-Pa*, and *-Pb* lineage, $P = 0.088$). The *B&WCol-Pa* and *-Pb* lineage showed no significance ($P = 0.548$). This lineage did have a relatively high number of nonsynonymous substitutions (7.3) but also had 1.8 synonymous substitutions, whereas the other 2 lineages mentioned above had zero. These analyses suggest that positive selection is occurring on at least some of the P-type sequences at the nucleotide level due to pressure for adaptive change at the protein sequence level in response to a new environment or function. We next examined whether there are specific amino acid residue changes shared uniquely by the P-type proteins, suggesting that those residues are important with respect to such adaptive change.

Loss of Arginines in the Highly Expressed *pRNase* Genes

To visualize the differences at the protein level between the DNA sequences generated in this study and which amino acid residue positions might have functional importance, the DNA sequences were translated to protein sequences and aligned (fig. 5). The inferred ancestral primate protein sequence is presented at the top and the bovine pRNase sequence at the bottom for comparison. The 8 cysteines, which are well known to stabilize the three-dimensional structure of ribonuclease through disulfide bridges (Klink et al. 2000), as well as the active site lysine and 2 histidine residues, are conserved among almost all of the sequences. The one exception is the Langur-ψa sequence, which would have a threonine substituted for the active site lysine-41, if it were actually transcribed and translated; this provides further evidence that this gene is, as it otherwise appears to be, a retroposed processed pseudogene. RNase A enzymes are known to bind 3 adjacent bases of a ribonucleotide chain in the active site cleft (Cuchillo et al. 1997). In addition to the cysteine and active site residues, there are 13 other residues thought to help stabilize the binding of the ribonucleotide chain by interacting with the 3 nitrogenous bases as well as the 3 phosphate groups (see Nogues et al. 1998 for review). The majority of these residues are also conserved in the sequences presented here, but there are some exceptions. The Langur-Na sequence has a glycine-10 for arginine-10 substitution. This is one of the 2 residues thought to stabilize binding of the substrate at the phosphate group linking the second and third bound bases of the RNA molecule being cleaved (Richardson et al. 1990; Boix et al. 1994) and allows the enzyme to have endonuclease as opposed to exonuclease activity (Moussaoui et al. 1996). Lysine-66, thought to stabilize the binding through the 5′ phosphate of the first bound base (Matthew and Richards 1982; Brunger et al. 1985), has been substituted by glutamic acid in the B&WCol-Pa sequence. Glutamic acid-111, thought to be one of the 3 residues involved with stabilizing the second bound base, has been conservatively substituted by aspartic acid in the B&WCol-Pa and -Pb sequences. Site-directed mutagenesis and kinetic studies showed no effect when Glu-111 was mutated, but Glu-111 appears to be involved in catalysis when CpG is used as a substrate (Tarragona-Fiol et al. 1993). Lysine-1 is thought to interact with the third bound base; however, this residue position is an arginine in the New World monkey, Cercopithecine, and Colobine N-type sequences presented in this study. The Colobine P-type sequences have a lysine, serine, or glycine at this residue, suggesting the possibility that simply the lack of an arginine residue is of functional importance here for the pancreatic-expressed ribonucleases.

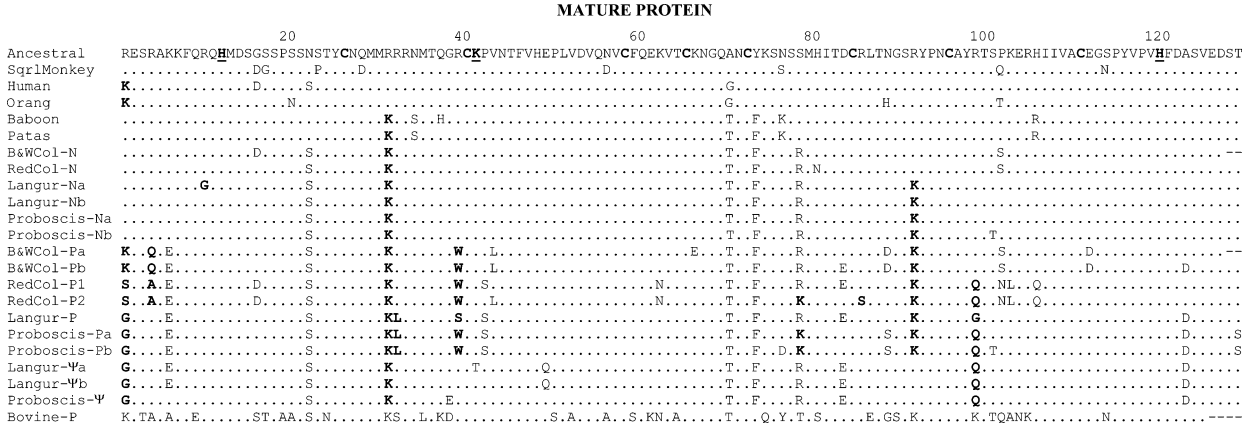


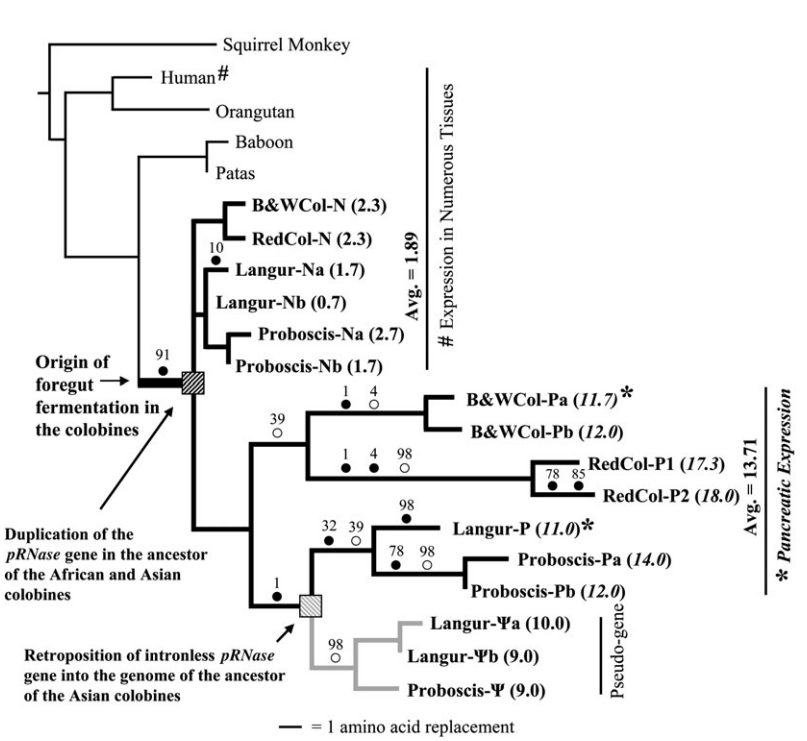
FIG. 5.—Alignment of the mature pRNase amino acid sequence. Presented is an alignment of the mature protein sequences translated from the coding region of the *pRNase* genes sequenced in this study, as well as the ancestral primate sequence inferred from the data. The bovine pRNase sequence is also included for comparison. A dot (.) indicates that the residue is identical to the residue of the primate ancestral sequence presented at the top of the alignment. A dash (-) indicates that no residue is at that position for that sequence. In the inferred ancestral sequence, the amino acid residues shared by all RNase A family members, the 8 cysteines involved in disulfide bridges (bold), and the 3 active site residues involved in cleavage (bold & underlined) are highlighted. In all other sequences, amino acid residues in bold indicate those that evolved from an arginine. Directly above the sequence alignment is the residue position numbering starting with the first residue of the mature protein as the first position.

Overall, there is an apparent trend of substitution in the Colobine P-type protein sequences to eliminate arginine residues. Ten such sites have been highlighted by bold characters in figure 5, with 7 of them exclusive to some or all of the Colobine P-type relative to the N-type proteins. Nine of these sites are arginines in the inferred ancestral primate sequence. One of these sites is an apparent gain of an arginine in the Colobine lineage with a subsequent substitution to Lysine in some of the Colobine P-type sequences. Interestingly, for 7 of these 10 positions, an arginine is not present at the corresponding residue in the bovine pRNase. This suggests a possible selective pressure to eliminate arginines that are not essential to the protein's function in foregut-fermenting animals. A similar trend has been noted for Colobine stomach lysozymes (Stewart and Wilson 1987).

Figure 6 presents a summary of our working hypothesis of *pRNase* gene duplicates in foregut fermenting, colobine monkeys. We believe this phylogeny best represents the true evolutionary history of the colobine pRNase sequences. Our model proposes that soon after the origin of foregut fermentation in the ancestral colobine lineage, the single *pRNase* gene duplicated, presumably, under selective pressure for more gene product to better able the animal to digest copious amounts of bacterial RNA and thus absorb and recycle the nitrogen and phosphorus fixed into the bacteria during foregut fermentation (Barnard 1969). One of the duplicates began to evolve changes in the protein-coding region, notably loss of arginine residues relative to lysines. Concomitantly, changes possibly occurred in the regulatory sequences of this duplicate to enhance its expression in pancreas. Evidence for this change in expression level is taken from the fact that (Beintema 1990) was able to generate a single, clean P-type protein sequence from Hanuman langur pancreas tissue. At least one other duplication event occurred in the ancestor of the Asian colobine lineage, with the retroposition of a P-type sequence, suggesting that this gene is, or at least was, also expressed in germ line cells.

The rate of evolution at the protein sequence level of the P-type relative to N-type sequences is approximately 7 times faster, suggesting positive selection in the P-type sequences. The average number of replacements averaged over all equally parsimonious reconstructions for the P-type sequences ranges from 11.0 (Langur-P) to 18.0 (RedCol-P2), with the average being 13.7. In comparison, the average number of replacements for N-type sequences is 1.89. A significant proportion of these changes appear to be loss of arginines along the P-type lineages. In figure 6, independent losses of arginine residues have been mapped onto the protein sequence lineages. The type of mutation at the nucleotide level responsible for that arginine loss is also denoted, with respect to the CpG mutational mechanism. A methylated CpG dinucleotide that becomes deaminated will be repaired to TpG or CpA, which is estimated to have a frequency 10 times that of the neutral point mutation rate. Four of the 6 codons for arginine contain a CpG dinucleotide, and thus, the loss of arginines could simply be loss of that particular amino acid at neutral positions in the protein due to the elevated CpG dinucleotide mutation rate. Given that only 6 of the 17 independent arginine losses along colobine lineages appear to be due to CpG dinucleotide instability, we argue that the data strongly support the hypothesis of selection for arginine loss.

Three of the arginine (R) residues that changed along colobine lineages (R39, R91, and R98) have several equally parsimonious placements on the tree at the amino acid level. However, analysis of the DNA sequences for these amino acid positions reveals that, for 2 of the cases, one assignment is actually more parsimonious than the others. For example, R39 has 2 arrangements on the tree, each requiring just 2 independent changes. The arrangement where R39 was lost on the lineage leading to all of the P-type and ψ-type sequences requires one loss due to mutation at a CpG dinucleotide and a subsequent regain of the arginine along the lineage leading to the pseudogenes, via a non-CpG



(+)	(-)	Arg	R/K	PI	pH7.0
18	14	11	1.57	8.31	4.51
18	12	10	1.25	8.65	6.50
18	11	10	1.25	8.82	7.67
20	11	11	1.22	9.15	9.50
20	11	11	1.22	9.15	9.34
19	12	11	1.38	8.83	7.50
19	11	11	1.38	9.00	8.34
18	11	9	1.00	8.82	7.50
19	11	10	1.11	8.98	8.50
18	12	10	1.25	8.67	6.51
19	11	10	1.11	8.98	8.50
15	**14**	**7**	**0.88**	**7.61**	**1.51**
16	**14**	**7**	**0.78**	**7.90**	**2.51**
12	**13**	**6**	**1.00**	**6.70**	**−0.66**
11	**13**	**4**	**0.57**	**6.31**	**−1.66**
14	**13**	**6**	**0.75**	**7.61**	**1.51**
14	**13**	**5**	**0.56**	**7.61**	**1.51**
14	**14**	**5**	**0.56**	**7.22**	**0.51**
15	12	9	1.50	8.13	3.51
16	12	9	1.29	8.32	4.51
17	13	9	1.13	8.31	4.51

FIG. 6.—Summary of our working hypothesis of *pRNase* evolution in the colobine species group. The configuration of the phylogenetic tree shown is the one that is most likely to represent the true relationships between the *pRNase* gene duplicates found in the foregut fermenting, colobine monkeys. The branch lengths reflect the average number of amino acid replacements averaged over all equally parsimonious reconstructions. In parentheses, immediately to the right of each colobine protein sequence name, is the average number of residue replacements mapped to that sequence. Just to the right of this are the average of all P-type and all N-type colobine sequences. The sequence empirically shown to be expressed in numerous tissues is denoted by a pound sign (#). The sequences empirically shown to have relatively high expression (ribonuclease activity) in pancreas are denoted by an asterisk (*). The square filled with black diagonal lines indicates that at least one gene duplication event occurred before the African/Asian colobine split. The square filled with gray diagonal lines reflects the duplication event via retroposition of *pRNase* mRNA back into the genome specific to the Asian colobines. Circles (● and ○), with numbers directly above, denote independent losses of arginine residues on that lineage at that residue position relative to the start of the mature protein sequence. The open circles denote that the loss was due to a CpG mutation. The total number of postively charged arginines and lysines (+), total number of negatively charged glutamic and aspartic acids (−), total number of arginines (Arg), arginine versus lysine ratio (R/K), isoelectric point (PI), and the overall charge of the protein at a pH of 7.0 (pH 7.0) appears in a table to the right of each species protein sequence.

point mutation. The other, more likely, arrangement requires that R39 was lost independently twice, once along the lineage leading to the African colobine P-type sequences and once on the lineage leading to the Asian colobine P-type sequences. This arrangement is considered more likely here because both events would be due to a mutation at a CpG site. Similarly, the assignments chosen here for R98 (which requires a minimum of 4 changes) is for 4 independent losses, 3 being due to mutations at CpG sites. The mutation for the Langur-P sequence is a CGG codon mutating to a GGG codon. This mutation is at a CpG dinucleotide site but is not due to the mutational mechanism responsible for the elevated CpG mutation rate. The alternative would require 2 losses due to a CpG site and a regain with subsequent non-CpG loss in the Langur-P sequence. R91 has 3 equally parsimonious arrangements, with none of the possible mutations involving CpG sites, so in this case, one loss in the ancestor before duplication followed by 2 independent regains of the arginine in the ψ-type lineage and in the African colobine N-type sequences is shown in the figure. This was chosen over 2 losses/one regain or 3 losses because it is more conservative with respect to the total number of independent arginine losses. Interestingly, under this scenario, R91 is regained in the African N-type lineage and the Asian ψ-type lineage which we propose are not under selection for arginine loss. For comparison, there are only 2 independent losses that occur in the N-type sequences, the R91 loss that predates the first duplication event and R10 for the Langur-Na sequence. If considered from the opposite perspective, namely arginine gain, the same overall pattern is seen. One arginine gain that predates the duplication event, R78, and R91 being independently regained in the African colobine N-type and Asian colobine ψ-type sequences. So, in summary, these data strongly suggest that colobine pRNase P-type sequences are selectively losing arginines (and regaining arginines at a lower rate), relative to colobine N-type sequences.

Conclusions

In this study, we present data showing multiple *pRNase* gene sequences, and their corresponding translated protein sequences, for 2 African (black-and-white and red colobus) and 2 Asian (Hanuman langur and proboscis monkey) colobine species. Both sequencing of PCR-amplified DNA and genomic blot analyses are consistent with the following number and type of *pRNase* duplication events in the colobine clade. These data show that at least one duplication of *pRNase* occurred in the lineage leading to the modern day

African and Asian colobine monkeys. Both Asian colobines as well as red colobus show evidence of a second *pRNase* duplication event. In the Asian colobines, this duplication is due to retroposition of a transcribed copy of the gene and was most likely "dead-on-arrival," suggesting this duplicate has had little, if any, impact on the evolution of functional *pRNase* duplicates. In contrast, the additional duplication in red colobus is of the entire gene, similar to the original duplication event, and thus likely due to segmental duplication or unequal crossover. Lineage-specific likelihood ratio tests for positive selection and episodic evolution suggest such selection on some of the P-type DNA sequences (Yang 1998). We report the rapid evolution at the protein sequence level of the P-type duplicate relative to the N-type duplicate (13.71 vs. 1.89 average changes, respectively), despite high levels of homogenizing gene conversion between noncoding regions of the duplicates. A pattern of selective loss of arginine residues from the P-type duplicate is apparent from these data. Independent losses of several of the arginine residues in multiple colobine species suggest positive selection for that loss.

Beintema (1990) showed that the langur pancreas had 50–60 times the amount of pRNase relative to human (Beintema et al. 1984). The cow, also a foregut fermenter, has higher levels of pRNase in the gut as well. Although artiodactyl ruminants have 3 duplicate pancreatic-like *RNaseA* genes, only one of these genes codes for a pancreatic RNase. In correspondence with this, one of the initial impetuses for this work was to find hypothetical positive selection events at nucleotide sites in the promoter of a single colobine *pRNase* gene that would be the cause of increased transcription and thus increased levels of protein in the pancreas of these species. We generated 1700 bp of DNA sequence upstream of the translational start site for 13 of the 23 gene sequences in hopes of finding altered promoter elements that could be responsible for such expression differences, such as a consensus pancreatic transcription factor-1–binding site. This site is found in many genes expressed in the pancreas and usually occurs within 500 bp upstream of the 5′ transcriptional start site (Samuelson et al. 1991; Kruse et al. 1995; Rose and MacDonald 1997). No pancreatic promoter elements specific to the colobine sequences were found by visual examination or by TRANSFAC (Windgender et al. 2000). What we did find was a pattern of gene duplication and divergence for the colobine *pRNase*-coding region despite high levels of homogenization between paralogues by gene conversion.

Mosaic concerted evolution (Wen and Irwin 1999) appears to be playing a significant role in the evolution of *pRNase* gene duplicates. There is an apparently high level of homogenization occurring in the 1,744 bp of noncoding sequence immediately adjacent to the 468 bp of coding sequence. This level of homogenization appears to extend into the coding region, but only to a certain degree, suggesting that the complete homogenization of the entire coding sequence is being prevented by selection for 2 functionally distinct pRNase proteins in these species. This supports the case for positive selection of a novel pRNase protein in the past because it would be expected that any new variant might be quickly overwritten. It also supports negative selection on both duplicates once the episodic events had created a reasonably well-adapted pRNase duplicate to act as a digestive enzyme in foregut fermenters, because these 2 distinct duplicates are being maintained. Although independent duplications and deletions occurring in each species separately cannot be ruled out, we favor gene conversion events between *pRNase* duplicates that existed prior to speciation as the predominant mechanism of this concerted evolution. The reasons we favor this hypothesis are the number of independent duplications and deletions required for the alternate hypothesis, apparent paralogous *pRNase* sequences sharing the same single nucleotide deletion in the coding region, and the results obtained from amino acid replacement reconstructions mapped onto the phylogenetic trees depicting both scenarios. In addition, this pattern is not without precedence. The gene duplicates of both the human red and green opsin (Zhao et al. 1998) and the *RHCE* and *RHD* (Innan 2003) genes appear to be undergoing a similar pattern of evolution. In both cases, high levels of apparent gene conversion are keeping the respective paralogues homogenized while divergence has occurred for a small region within each gene (and corresponding protein) that is critical for the functional difference of the 2 distinct types.

Following up on our earlier report (Schienman et al. 2000), Zhang et al. (2002) sequenced 2 of the *pRNase* genes from a douc langur (an Asian colobine), which they name, RNASE1B and RNASE1, respectively. Our results strongly confirm these findings and expand them to additional Asian species and to the African colobines as well. The authors report that analysis of the douc langur sequences suggest a duplication event of approximately 4 MYA. Due to the accepted divergence time of African and Asian colobines (Delson 1994), our results would strongly suggest that the timing of the duplication event is prior to the African/Asian colobine split and is more on the order of 10–12 MYA. The more likely scenario, consistent with both results, is that homogenization by intergenic recombination between *pRNase* duplicates is the cause of a more recent estimate of the event that created the douc langur *pRNase* duplicates.

These authors also report that the douc langur RNASE1B (P-type) has 6 times the ribonuclease activity relative to RNASE1 (N-type) at pH 6.3. This pH optimum for RNASE1B is within the unusual pH range of 6–7 (for a primate) of the colobine monkey small intestine (Kay and Davies 1994) and suggests that this duplicate has been positively selected to function in this intestinal environment. They state that this change in optimal pH for RNASE1B function is the result of the loss of the overall positive charge of the protein at the amino acid level. Our work confirms this but also extends this result by showing that this change in net charge of the P-type proteins is predominantly due to the selective loss of positively charged arginine residues. There are at least 2 possible reasons why a loss of arginine residues might be favored in an enzyme that functions in the environment of the digestive tract of a foregut-fermenting animal (Stewart and Wilson 1987). First is the fact that arginyl bonds are more sensitive than lysyl bonds to hydrolysis by pancreatic trypsin (Vonk and Western 1984; Craik et al. 1985). Second is the fact that arginine can be irreversibly modified by bacterial fermentation products such as

diacetyl (Davies and Neuberger 1969; Kaiser et al. 1985). In the case of chicken LZM, this modification alters or "kills" the enzyme activity. In foregut-fermenting animals, not only will pancreatic trypsin be present in the small intestine but also bacterial fermentation products will be as well. Additionally, in such animals, there is a need to digest large amounts of bacterial RNA in order to be able to recycle sufficient levels of nitrogen and phosphorus sequestered by the foregut microbes (Barnard 1969). From these facts, it follows that the loss of arginine residues in a protein required to have high activity and/or stability in the gut environment might be selected for. This hypothesis is strengthened by the fact that a similar pattern of arginine loss is seen in an unrelated enzyme, stomach lysozyme, also recruited to function in the unique digestive tract environment of foregut fermenters (Stewart and Wilson 1987). To test the hypothesis that the P-type protein has evolved as a digestive enzyme by being more stable in the small intestine of a colobine or ruminant, in vitro experiments with P- and N-type purified proteins (including site-directed mutagenic variants) incubated with biologically relevant concentrations of diacetyl and pancreatic trypsin could be assayed for level and longevity of their RNase activity.

Gene duplication has long been thought by evolutionary biologists to be the source of novel gene function. One of the first hypotheses to describe what happens to paralogues after the duplication event was put forth by Ohno (1970). His hypothesis stated that after gene duplication, one gene copy would be entirely redundant and thus freed from all selective constraint. This copy would accumulate mutations at a neutral rate and in most cases become a nonfunctional pseudogene. Occasionally, these accumulated neutral mutations could eventually resurrect the gene with a new function. We believe our data to be another example that do not support this hypothesis. The biological scenario of foregut fermentation development suggests that the original gene duplicates were beneficial from the beginning via a protein dosage effect. This is in support of data seen by Hughes MK and Hughes AL (1993) and Kondrashov et al. (2002). The former showed that duplicate genes of the tetraploid frog *Xenopus laevis* were not freed from functional constraint but were under strong purifying selection. The latter used data from a number of bacterial, archaeal, and eukaryotic genomes to compare the mode and strength of selection acting on recently duplicated genes. They hypothesized that gene duplications that persist are beneficial from their time of origin, due primarily to a protein dosage effect in response to variable environmental conditions. If the stability and activity of the original pRNase protein in the colobine lineage were curtailed as described above, then it follows that 2 gene copies enhance the ability to digest RNA in the small intestine. Also, in contrast to Ohno's hypothesis, our data support that one duplicate was not evolving neutrally due to redundancy but under strong positive selection despite high levels of homogenization by gene conversion similar to the situation seen in the human red and green opsin and *RHCE* and *RHD* genes (Zhao et al. 1998; Innan 2003). Our data may also be in support of a subfunctionalization model (see Hughes 2005 for a short review), where the original protein had 2 functions and the duplicates each evolved to specialize in only one of the functions. The expression of human pRNase in many tissues other than the gut along with data published by Sorrentino et al. (2003) and Zhang (2003) suggest that the original protein in colobines had a second function of high activity toward double-stranded RNA (not seen in bovine pRNase), perhaps acting as an agent against viruses with a dsRNA genome. Given that this activity seems to be dependent on noncatalytic positive charges on the exposed surface of the protein (Sorrentino et al. 2003), the retention of this activity in both duplicates of the colobine lineage would be selected against by the unique environment of the colobine small intestine.

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