Molecular Evolution of Pancreatic-Type Ribonucleases[1,2]

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Amino acid sequences of 39 mammalian ribonucleases have been used to construct trees by the maximum parsimony procedure. These trees are in fairly good agreement with the biological classification of the species involved. In the branching order of the six investigated eutherian mammalian orders, the edentates diverge first, followed, probably, by the primates. No definite conclusions can be drawn about the order of divergence of the perissodactyls, the rodents, and the group consisting of artiodactyls plus cetaceans. Nucleic acid sequences of part of the messenger RNAs of rat pancreatic and bovine seminal ribonuclease were compared. Both messengers have a second stop codon at position 129, which is in agreement with the addition of four residues at the C-terminus in several other ribonucleases. Turtle pancreatic ribonuclease and human angiogenin differ from each other and from the mammalian ribonucleases at 55%–70% of the amino acid positions; they share a number of structural features. Mammalian nonsecretory ribonucleases are homologous to the pancreatic ribonucleases in sequence regions where the active-site histidine residues are located.

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Introduction

Pancreatic ribonucleases form a group of homologous proteins found in considerable quantities in the pancreas of a number of mammalian taxa. This group has also been found in a few reptiles (Barnard 1969; Beintema et al. 1973). The ribonuclease content varies greatly in different species. Large quantities are found in ruminants and species that have ruminant-like digestion and in a number of species with cecal digestion. Barnard (1969) proposed that an elevated level of pancreatic ribonuclease occurs in response to a need to digest large amounts of ribonucleic acid derived from the microflora of the stomach of ruminants. This explanation agrees with the elevated level of stomach lysozyme in several ruminants and species that have a ruminant-like digestion (Dobson et al. 1984).

In previous reviews (Beintema et al. 1977; Beintema and Lenstra 1982) we discussed the relation between molecular evolution and functional properties of ribonuclease as derived from amino acid sequences obtained from mammalian species. Since then, additional sequence information has been collected and used to modify earlier constructed phylogenetic trees as presented below.

The field of molecular evolutionary studies of this group of ribonucleases has broadened, however, as the first sequences of nucleic acids coding for ribonucleases

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2. This paper was presented to the Fourth International Theriological Congress, Edmonton, Alberta, Canada, August 13–20, 1985.

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are becoming available. Also, recent studies of distantly related ribonucleases and other proteins, both from other vertebrate classes and from other mammalian tissues than pancreas, have contributed new information, which will be summarized in this review.

Trees

Amino acid sequences of ribonucleases from 41 species (40 mammals and one turtle) are presented in figure 1. The amino acid sequence of bovine seminal ribonuclease was found to be homologous to that of pancreatic ribonuclease and is, therefore, also included in this comparison. Evolutionary trees of the 39 complete mammalian sequences given in figure 1 were derived with the maximum parsimony procedure (Fitch 1971). In previous investigations (Beintema et al. 1977; Beintema and Lenstra 1982) two trees were derived: a most parsimonious one and a tree that combines current biological opinion with evidence from ribonuclease sequences (the "biological tree"). Both trees differed little within mammalian orders but deviated much from each other in regions where distantly related taxa are connected. Both trees were used as a starting point for the present investigation, in which we initiated a search for a new most parsimonious tree by interchanging neighboring branches (local branch swapping). The previous most parsimonious tree was found to yield a tree that required more nucleotide substitutions than that obtained from the former "biological" tree. The reason is that a number of ribonuclease sequences have been corrected since our previous studies. A one-position shift of a short stretch of residues in horse ribonuclease has an especially strong influence on the number of substitutions in trees derived by the maximum parsimony procedure. This observation strengthened our confidence in the new "biological" tree as being the best approximation of the evolutionary history of the ribonuclease gene in mammals.

In total, 141 trees have been investigated with the branch-swapping procedure. Sixteen most parsimonious trees requiring 495 nucleotide substitutions were found. Nine trees require 496 substitutions, and 14 trees require 497. Since many parallel and back substitutions occur in the molecular evolution of ribonuclease (Beintema et al. 1977) and since many unnoticed substitutions presumably have occurred in the long branches of the tree where distantly related taxa are connected, trees that do not differ very much from the most parsimonious ones should be considered as being equally likely representations of the evolutionary history of the ribonuclease gene, especially if they are not refuted by other biological data.

Figure 2 shows one of the trees that require 496 substitutions—that is, one more than the most parsimonious ones. In our opinion, this tree is the most probable representation of the history of the ribonuclease gene at this moment. It differs from the most parsimonious tree by grouping together the Odoicoileinae (reindeer, moose, and roe deer) separate from the Cervinae (red deer and fallow deer) instead of separating reindeer from the other four deer sequences (see below; fig. 3).

The high number of most parsimonious trees is caused in part by the presence of four internodal branches with zero substitutions in the tree. In such cases, the two nodes can be fused to create a trichotomy, indicating an unknown branching order.

Figure 3 summarizes a number of alternative topologies occurring in trees requiring 495–497 nucleotide substitutions. The first topology represents one of the most parsimonious trees mentioned above, the one with the reindeer separated from the other four deer sequences. The other topologies presented in figure 3 are modifications of this most parsimonious one.

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                                                1         2          3         4         5         6         7         8         9        10        11        12
                                      0    5    0    5    0     5    0    5    0    5    0    5    0    5    0    5    0    5    0    5    0    5    0    5    0    5
OX                                1 ---KETAAAKFERQHMDSSTSAASSS-NYCNQMMKSRNLTKDRCKPVNTFVHESLADVQAVCSQKNVACKNGQTNCYQSYSTMSITDCRETGSSKYPNCAYKTTQANKHIIVACEGNPYVPVHFDASV---
WATER BUFFALO, SWAMP TYPE         2            Q         S               SM S
WATER BUFFALO, RIVER TYPE         3            Q         S                M S                       E                                                          Y
ELAND                             4                      S               DM                                                                   z
NILGAI                            5                      S               SM QN                                                           T    K
GNU                               6      S               S                  Q            P                                                A   K
TOPI                              7      S               S                  Q                                                                 K
IMPALA                            8      S               S                  QS                                                                K
THOMSON'S GAZELLE                 9      S     z         S                  Q                          Q                                      Q
GOAT                             10      S               S                  Q                                                                 E
PRONGHORN                        11                I  NP SV                 QG                                                                K                Y
GIRAFFE                          12      S         I     SV           T     Q                                        A          N        Q    E                Y
REINDEER                         13      S           P P S            Q  D  Q                     F          S     N A H               V      E
ROE DEER                         14      S           P P S            Q     Q                     F    I     S     N A H      S N      V      E
MOOSE                            15      S           P A SI           Q     Q                     F          S     N A H      S N D    V      E
RED DEER                         16      S           P   S            Q  KM Q                     F          S     N A H      S N      V  A   E
FALLOW DEER                      17      S           P M S            Q  KM Q                     F          S     N A H      S N      V  A   E
OX (from seminal plasma)         18      S            GN PS        L  CC KM QGK               K      K T           K   R                     VE       G K S
DROMEDARY                        19    S    E         YS SS N          R EM DGW       I    E         S T        H  ST  H                  ASNLK    I
BACTRIAN CAMEL                   20    S    E         YS SS N          R EM DGW       I    E         S T        H  ST  H                  ASNLQ    I
HIPPOPOTAMUS                     21         E  Q     T S LSND         VR  M Q             E   K        T           N   H                   S LQ         D      Y
PIG                              22      SP K  Q     PDS SSN       L  SR  M QG                      I  N           N   H     Q            AS EQ           P
LESSER RORQUAL                   23    R SP M  Q      GN PGNNP        MR KM QG             E  K        L    R    E N   H     Q             S KE                  N
HORSE                            24      SP M         GSTSSSNP T       R  M QGW          P      I L   IT     S     S S H     L SG        Q S KER                    EVST
RAT                              25 GESR SS D  K     TEGPSK  P T       RQGM  GS          P E    I   GQ T    RN  HK S  LR     LK        D T  DSQ    I  D
MOUSE                            26    R S  Q  Q     PDG SIN P T       R DM NGS          P          E  T   RKS   K S ALH    HLK N      D     YQ                   T
HAMSTER                          27      S  M         TVATS  P T       R  M QGY               H     E       KS   K H ALH     LK NA     D Q S LQ Q         F
MUSKRAT                          28       S Q         TG SS  P T       R EM QGY          P          E  T    NS   K R ALH     LK N      D Q S LQ QV      S F
PORCUPINE                        29      SS M          G PS N      E  RR  M Q            P    R   F                N L H     V       D S RMS LERS V     S           GPST
CAPYBARA                         30    A SS M  Q   V  EG SS NA     E  VR KM Q            P        F    P             S H     V SN  F D S R    Q S V      L          EPST
GUINEA-PIG A                     31    A SS M      V  GG SS NA     E   K EM              P  E       R  S             S H  E  L SG  F   S R S  Q S       K        N
GUINEA-PIG B                     32    A SS M  Q     PEG PSN       V  IR  M QG                    F    L             R R     V S   F   S RMS  Q S       D           EPST
CUIS                             33    A SS M  Q      DGHPDTNT     E  VR SM QG           P EA          P           H S R     V S       S RM   Q S       T S       T
CHINCHILLA                       34      SS M  Q       G PSTNA     E   G  M QGY          P        F    P     S     N N H     L SN      S R SRE  G
CASIRAGUA                        35      SS K  Q   I   G PSTNP     A      M QE           P        F    P     S   E T N H     L SN  F D L R S EE S                   AASA
COYPU                            36    S SS K         RG PSTNP     E      M QG           P        F    L           N N H     V SN D    S R S EE S V                 AASA
MAN                              37      SR K  Q      DS PS    T      RR  M QG           P V   N  F EK T     G   K N S H     L NG R      R SPKER        S           EDST
TWO-TOED SLOTH                   38         M  Q      GS LS    D   K   V  M QES            Q      F E  T     Q  H  R N H     Q SG      L Q SNM R   I                EDST
THREE-TOED SLOTH                 39      SS    Q      DS xxxxx xxxxK      M QES  A       P T      L E  T  b zzb Hxxxxx H     Q SG T    L    NKxxxxxxxxxxxxx       T
RED KANGAROO                     40    -  P E  Q     TEH T         L   A DM SG    L   I  PKSV D   H E  T    R    K N RL   N  Q  A      Q E SNL  Q       Q-        Y
WALLABY                          41    -    E  Q z  bTEH T     b  bL   A EM S         I  PKSV b   zzEb T         K N RL   N  Q  A b  b z z SbLQ Q       Q-        Y
SNAPPING TURTLE                  42    -  RYE  L   V YPK S PD RT      QR GM SPV  FT     A A SITT  GSGGTPASGD---LRD NASFAL T  LQ G QT   P NADASTQR RI  V G--L   Y K I
                                                1         2          3         4         5         6         7         8         9        10        11        12
                                      0    5    0    5    0     5    0    5    0    5    0    5    0    5    0    5    0    5    0    5    0    5    0    5    0    5
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Although our ribonuclease data give no information on several unsettled questions in mammalian phylogeny, there are fairly strong indications for the following points:

1. Generally, the topology of the tree presented in figure 2 is in satisfactory agreement with traditional biological classification within the mammalian orders. Likely positions of taxa about which there is conflicting biological opinion are as follows: (a) the positioning of giraffe with pronghorn and not with deer, (b) the positioning of hippopotamus with ruminants and camel and not with pig, (c) the positioning of African porcupine with the South American caviomorphs.

2. Of the six investigated eutherian mammalian orders, the edentates (sloth) diverge first, probably followed by the primates (man). However, no definite conclusions can be drawn about the divergence of the perissodactyls (horse), the rodents, and the group consisting of artiodactyls and cetaceans (whale).

3. Two gene duplications have occurred in the evolutionary history of ribonuclease as depicted in figure 2—a gene duplication, after the divergence of the camels, in the ancestor of the remaining ruminants that leads to a separate ribonuclease that is expressed in bovine seminal vesicles and a gene duplication in the ancestor of guinea pig and capybara that leads to two pancreatic ribonucleases in guinea pig. Only one of these is actually found in capybara pancreas; the other is presumed to be silent or lost. In a previous study (Beintema and Neuteboom 1983) we positioned this gene duplication in the ancestor of guinea pig, capybara, and cuis; however, in the present study we found that a tree with this topology requires one additional substitution (fig. 3).

Nucleic Acids Coding for Ribonucleases

MacDonald et al. (1982) published the sequence of the mRNA coding for rat ribonuclease. This RNA is 783 nucleotides long, with a poly(A) tail of approximately

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FIG. 1.—The amino acid sequences of 41 pancreatic ribonucleases and bovine seminal ribonuclease in the IUB one-letter code (z = Glx; b = Asx). Only differences from the bovine pancreatic sequence are shown. Residues are numbered according to homology with the bovine enzyme. In the sequences, deletions are indicated by a dash (—) and unidentified residues by x. Many residues in peptides from the ribonucleases of bovidae and pronghorn have been positioned according to homology with the bovine enzyme; a similar procedure has been used with the ribonucleases of deer species (with reference to red deer) and bactrian camel (with reference to dromedary). Bison is identical to ox; sheep is identical to goat. Heterogeneities and residues in other (minor) components are as follows: roe deer, 64: A; dromedary, 103: Q; hippopotamus, 37: K; horse, 23: S; porcupine, 98: G; guinea-pig B, 64: P; chinchilla, 32: D. References: man (Beintema et al. 1984, 1985*a*), capybara and cuis (Beintema and Neuteboom 1983), correction rat (MacDonald et al. 1982; Beintema 1983), correction bovine seminal plasma (Krietsch et al. 1983), corrections horse, dromedary, and bactrian camel (Beintema 1985), turtle (Beintema et al. 1985*b*). Other references may be found in Beintema et al. (1977), Lenstra et al. (1977), Beintema and Lenstra (1982). The scientific binomens for the animals listed in fig. 1 are as follows: ox, *Bos taurus;* water buffalo (swamp and river types), *Bubalus bubalis;* eland, *Taurotragus oryx;* nilgai, *Boselaphus tragocamelus;* gnu, *Connochaetes taurinus;* topi, *Damaliscus korrigum;* impala, *Aepyceros melampus;* Thomson's gazelle, *Gazella thomsoni;* goat, *Capra hircus;* pronghorn, *Antilocapra americana;* giraffe, *Giraffa camelopardalis;* reindeer, *Rangifer tarandus;* roe deer, *Capreolus capreolus;* moose, *Alces alces;* red deer, *Cervus elaphus;* fallow deer, *Dama dama;* dromedary, *Camelus dromedarius;* Bactrian camel, *Camelus bactrianus;* hippopotamus, *Hippopotamus amphibius;* pig, *Sus scrofa;* lesser rorqual, *Balaenoptera acutoro;* horse, *Equus caballus;* rat, *Rattus rattus;* mouse, *Mus musculus;* hamster, *Mesocricetus auratus;* muskrat, *Ondatra zibethica;* porcupine, *Hystrix cristata;* capybara, *Hydrochoerus hydrochoeris;* guinea pig A and B, *Cavia porcellus;* cuis, *Galea musteloides;* chinchilla, *Chinchilla brevicaudata;* casiragua, *Proechimys guairae;* coypu, *Myocastor coypus;* man, *Homo sapiens;* two-toed sloth, *Choloepus hoffmanni;* three-toed sloth, *Bradypus infuscatus;* red kangaroo, *Macropus rufus;* wallaby, *Macropus rufogriseus;* snapping turtle, *Chelydra serpentina.*

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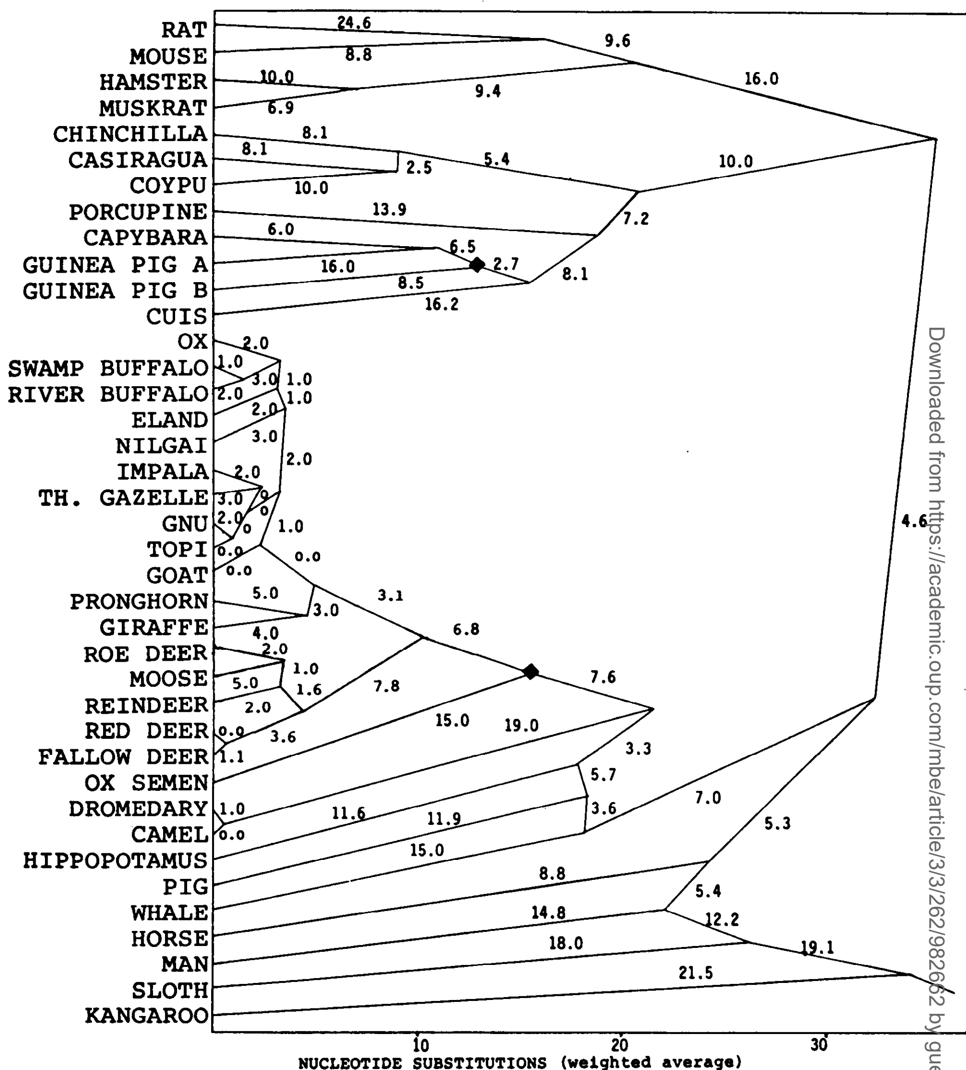

FIG. 2.—Parsimonious tree of mammalian ribonucleases that requires 496 nucleotide substitutions. The number on each leg is the minimum number of nucleotide substitutions required to account for the descent from the ancestor to its immediate descendant in the tree. Fractions result from averaging over more than one parsimonious solution. The nodes are placed at a height equal to the weighted average number of nucleotide substitutions between the node and its descendant sequences. Sequences are given in fig. 1. ◆ = Gene duplication resulting in two paralogous gene products.

140 nucleotides. The coding part includes a signal peptide of 25 amino acid residues. In vitro translation of bovine pancreatic (Haugen and Heath 1979) and seminal ribonuclease (Furia et al. 1983) indicated signal peptide lengths of the same size in both proteins, in contrast to pig and horse pancreatic ribonuclease, which are synthesized as precursors with extra peptides approximately 60 amino acid residues in length (Carsana et al. 1985). The mRNAs of bovine seminal ribonuclease (Palmieri et al. 1985) and pig ribonuclease (Carsana et al. 1985) have a length of approximately 950 nucleotides, similar to that of the ribonuclease found in rat.

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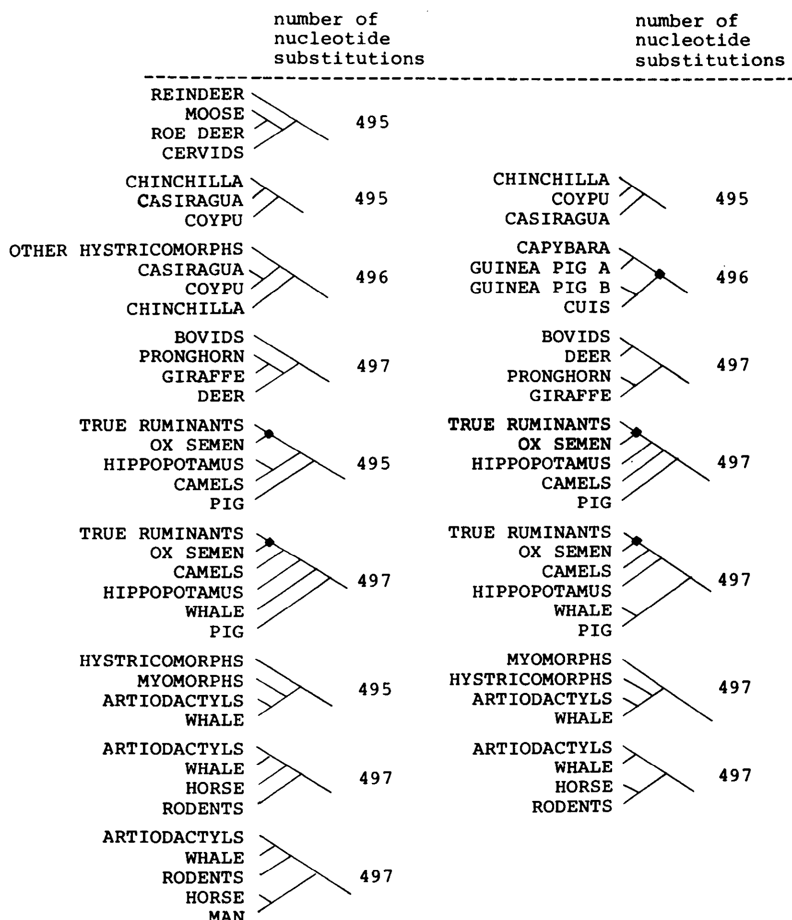

FIG. 3.—Several alternative topologies of ribonuclease trees requiring 495–497 nucleotide substitutions. The upper left subtopology shows one of the most parsimonious trees (495 substitutions) when the tree shown in fig. 2 is modified to this grouping of the deer sequences. The other topologies in this figure are further modifications of this topology. ♦ = gene duplication.

Recently, approximately two-thirds of the nucleotide sequence of the mRNA of bovine seminal ribonuclease has been determined (Palmieri et al. 1985). Like the messenger of rat ribonuclease, this one has a noncoding region approximately 400 nucleotides long. Figure 4 compares part of the coding and 3′-noncoding regions of the mRNA of bovine seminal ribonuclease with the homologous part of rat ribonuclease mRNA (there is no easily recognizable similarity in the remainder of the 3′-noncoding region). The coding parts of the two sequence fragments differ at 23 amino acid positions (30%) and at 51 nucleotide positions (22%). There are 15 silent substitutions at nonreplacement sites and eight at replacement sites.

Most pancreatic ribonucleases have their C-terminal residues at position 124. Accordingly, rat pancreatic and bovine seminal ribonuclease have a stop codon at position 125. However, ribonucleases from several representatives of four different mammalian orders have four additional residues with rather similar sequences at the C-terminus (fig. 5). Interestingly, both ribonuclease messenger RNAs code for similar

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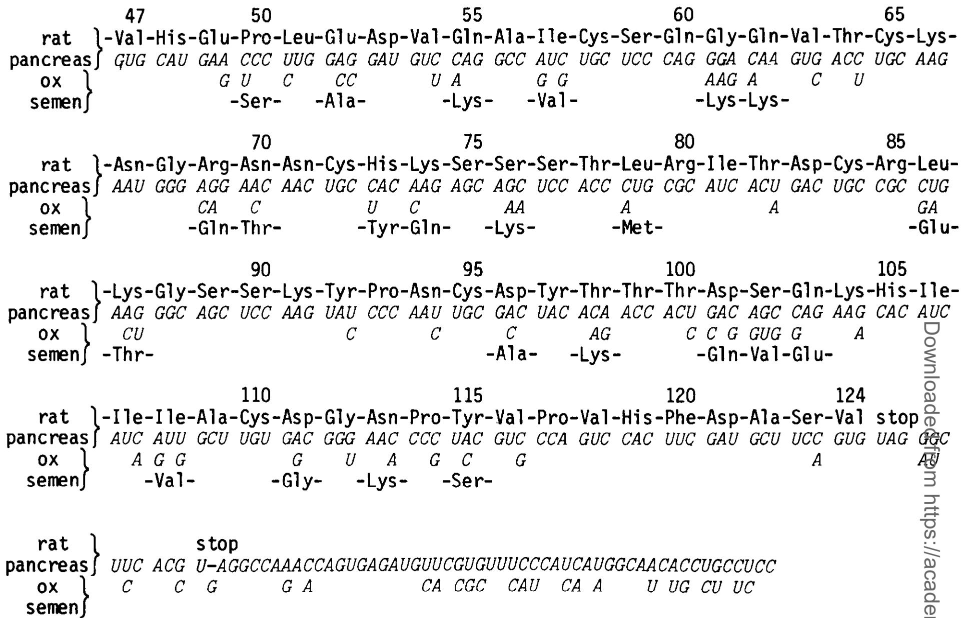

FIG. 4.—Part of the mRNA sequences and coded amino acids of rat pancreatic ribonuclease and bovine seminal ribonuclease. Only the differences from the rat sequence are shown for the bovine seminal sequence. A dash (—) = deletion.

sequences at positions 126–128 and possess a second stop codon—although a different one—at position 129. Evidently, this part of the sequence has been rather well conserved during the evolution of the mammals, even when it formed part of the 3′-noncoding sequence of the messenger.

		124 125 126 127 128 129
ARTIODACTYLS	ALL SPECIES INVESTIGATED	-VAL
CETACEANS	WHALE	-VAL
PERISSODACTYLS	HORSE	-VAL-GLU-VAL-SER-THR
EDENTATES	TWO-TOED SLOTH	-VAL-GLU-ASP-SER-THR
	THREE-TOED SLOTH	-VAL
MYOMORPH RODENTS	ALL SPECIES INVESTIGATED	-VAL
HYSTRICOMORPH RODENTS	COYPU, CASIRAGUA	-VAL-ALA-ALA-SER-ALA
	CHINCHILLA	-VAL
	GUINEA PIG A, CUIS	-VAL
	GUINEA PIG B, CAPYBARA	-VAL-GLU-PRO-SER-THR
	AFRICAN PORCUPINE	-VAL-GLY-PRO-SER-THR
PRIMATES	MAN (PANCREAS)	-VAL-GLU-ASP-SER
	,, (URINE, SEMEN)	-VAL-GLU-ASP-SER-THR
MARSUPIALS	KANGAROO, WALLABY	-VAL
REPTILES	TURTLE	-VAL
MESSENGER RNA:		
	RAT RNASE MESSENGER RNA	-GUG-UAG-GGC-UUC-ACG-UAG- (-VAL-STOP-GLY-PHE-THR-STOP-)
	BOVINE SEMINAL RNASE MESSENGER RNA	-GUG-UAG-AUC-UCC-ACC-UGA- (-VAL-STOP-ILE-SER-THR-STOP-)

FIG. 5.—Additions at the C-terminus of mammalian ribonucleases.

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Three-dimensional Structure and Properties of Ribonucleases

Figure 6 shows the main-chain conformation of bovine pancreatic ribonuclease (Richards and Wyckoff 1973; Borkakoti et al. 1982; Wlodawer and Sjölin 1983). Internal parts of the molecule and the active-site cleft (on the left side of the molecule) contain many unvaried residues while the other surface parts are more varied.

In a previous review, Beintema and Lenstra (1982) summarized the correlations between structural variations in mammalian ribonucleases, formation of a stable secondary and tertiary structure, and other properties, such as enzymic activity on RNA, on small-molecular-weight substrates and on double-stranded RNA, and interaction with protein inhibitors. These will not be repeated here.

The most striking differences between pancreatic ribonucleases concern the presence or absence of covalently attached carbohydrate. These differences may be related to the digestive system and the diet of the species involved. We found that species with cecal digestion—such as pig, horse, and most hystricomorph rodents—produce ribonucleases with large carbohydrate moieties attached to several positions at the surface of the molecule (20%–30% increases in molecular weight have been found). Therefore, we have suggested that the presence of carbohydrate protects ribonuclease from absorption in the gut, causing it to be transported to the large intestine, where it should hydrolyze the ribonucleic acid derived from the cecal microflora (Beintema et al. 1976; Beintema and Lenstra 1982).

Carbohydrate has been found to be attached to asparagine residues at positions 21, 22, 34, 62, 76, and 88 (fig. 6). Only asparagine residues in Asn-X-Ser/Thr sequences have been found to act as attachment sites, where X may be any residue except proline. However, not all Asn-X-Ser/Thr sequences possess carbohydrate chains. Other features of the ribonuclease molecule and/or the effectiveness of the carbohydrate-attaching system apparently influence the glycosylation process. Figure 7 summarizes the occurrences of Asn-X-Ser/Thr sequences at the six carbohydrate attachment sites in ribonucleases and the glycosylation characteristics of these sites. Interestingly, human pancreatic ribonuclease has carbohydrate attached to only one site on the molecule, whereas a ribonuclease isolated from human urine, which has an identical amino acid sequence, is extensively glycosylated at three sites (Beintema et al. 1985*a*).

The Ribonuclease Superfamily: Distantly Related Relatives

Ribonucleases from different mammalian orders differ from each other at ~30% of the amino acid positions. No other homologous but more-deviating sequences were known until the primary structure of pancreatic ribonuclease of snapping turtle was determined (Beintema et al. 1985*a*). The latter differs from the mammalian ribonucleases at ~60% of its amino acid positions. Using the correction for superimposed changes according to Dayhoff et al. (1972), we found evolutionary distances between turtle and mammalian ribonucleases to be three times greater than those among placental mammalian ribonucleases. Since turtle and mammals diverged ~300 Myr ago and the placental mammals diverged ~90 Myr ago, the evolutionary distances do not indicate significantly different evolutionary rates. Within the mammals, on the contrary, considerably different evolutionary rates of ribonuclease have been found (Beintema and Lenstra 1982).

The discovery of another member of the ribonuclease superfamily has been reported by Strydom et al. (1985). They sequenced a protein, called angiogenin, that stimulates blood vessel formation. This protein was found to be homologous with the

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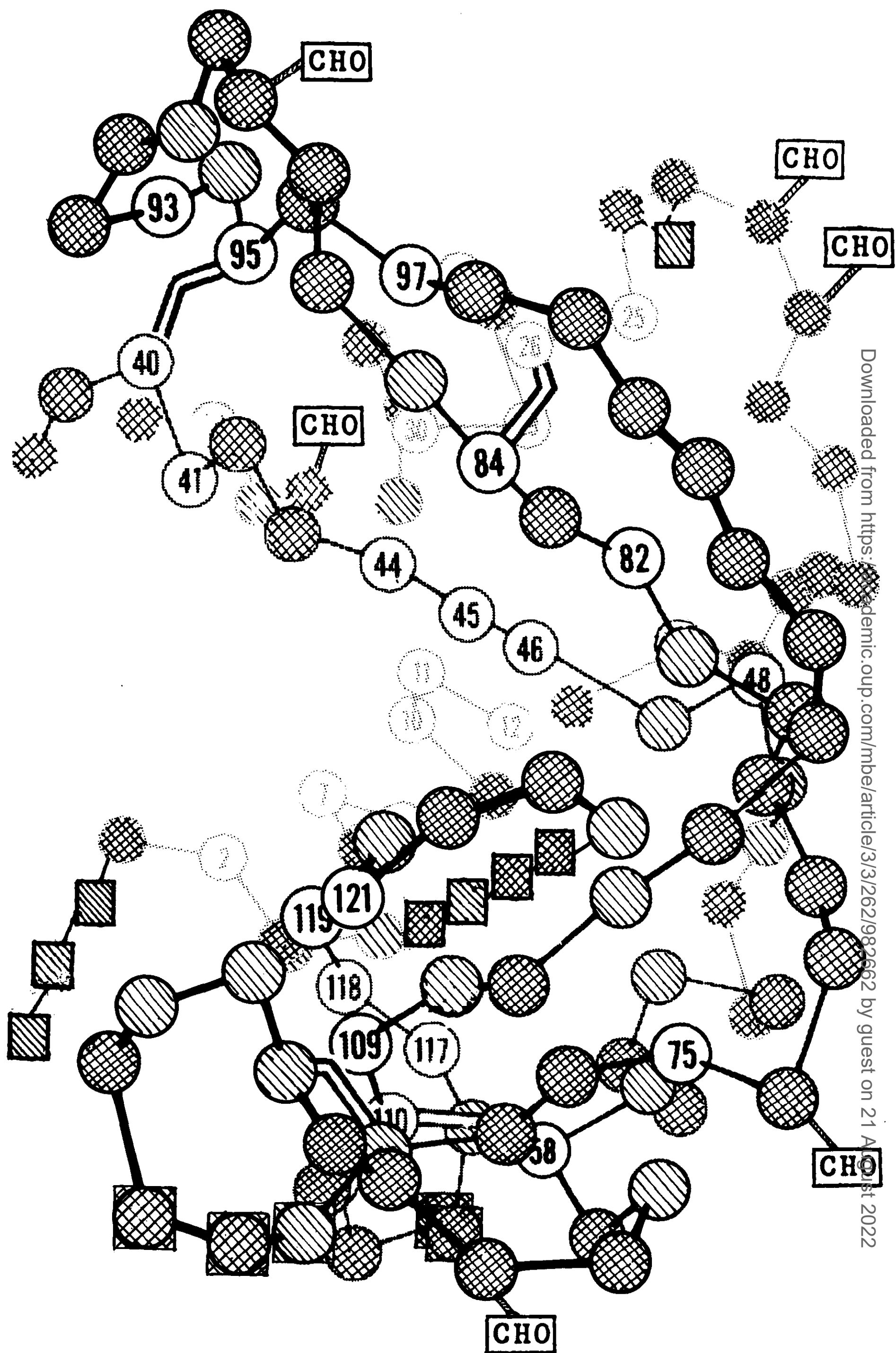

FIG. 6.—Three-dimensional structure of bovine pancreatic ribonuclease. Amino acid residues at unvaried positions are indicated by open circles, varied positions by cross-hatched circles, and positions with only two different residues by single-hatched circles. □ = positions involving additions and deletions; CHO = positions where covalently linked carbohydrate is found in several ribonucleases. (Figure derived from Dickerson and Geis [1969].)

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sequence	glycosylated	part of the molecules glycosylated	carbohydrate-free
34 36			
-Asn-Leu-Thr-	giraffe moose	6 bovids, pronghorn, roe deer	
-Asn-Met-Thr	water buffalo (river type), hippopotamus (with Gln-37), pig, horse, 6 rodents, sloth		hippopotamus (with Lys-37)
	man (urine)	man (pancreas)	
21 23			
-Asn-Ser-Ser-	pig, guinea-pig B		
-Asn-Asp-Ser-			hippopotamus
22 24			
-Asn-Ser-Thr-	horse		
-Asn-Pro-Thr-			horse
62 64			
-Asn-Ile-Thr-	horse		
-Asn-Val-Thr-			hippopotamus, mouse, hamster, sloth, kangaroo
-Asn-Val-Ser-			guinea-pig A
76 78			
-Asn-Ser-Thr-	pig	whale	hippopotamus
-Asn-Ser-Ser-	man (urine)		man (pancreas)
88 90			
-Asn-Gly-Ser-	man (urine)		man (pancreas)

FIG. 7.—Carbohydrate attachment sites in ribonucleases.

pancreatic ribonucleases, although no enzymic activity on any of a number of ribonuclease substrates was found (Strydom et al. 1985). In figure 8 the amino acid sequence of angiogenin is compared with those of human and turtle pancreatic ribonucleases. It differs from the amino acid sequences of mammalian ribonucleases at 65%–70% of the positions and from that of turtle ribonuclease at 57%. Structural features that turtle ribonuclease and human angiogenin have in common are the deletions in the external loops near residues 69 and 115 and the absence of the disulfide bond linking residues 65 and 72. There are 27 unvaried residues in all investigated ribonucleases and human angiogenin (fig. 8). Four of these occur in the N-terminal part of the molecule (the S-peptide part), 15 in the upper part of the molecule in the orientation of figure 6, and only eight in the lower part. Several residues near the active-site histidine residue 119, and the disulfide bridge 58–110, are the only conserved residues in this part of the molecule, where the deletions in turtle ribonuclease and human angiogenin mentioned above are located as well.

The difference between human angiogenin and mammalian and turtle ribonucleases indicates that a gene duplication leading to separate genes for ribonuclease and angiogenin may have occurred ~300 Myr ago, when reptiles and mammals diverged, and that turtle ribonuclease diverged somewhat less from the ancestral sequence than did the other members of the superfamily.

The nucleotide sequence of the angiogenin gene has been determined (Kurachi et al. 1985). The coding region—consisting of a signal peptide of 22 or 24 residues and the mature protein—and the 3′-noncoding sequence are not interrupted by introns. As mentioned earlier, several ribonucleases have four additional amino acid residues at the C-terminus, in agreement with the occurrence of a second stop codon at position 129 in the messenger RNAs of rat pancreatic and bovine seminal ribonucleases (fig.

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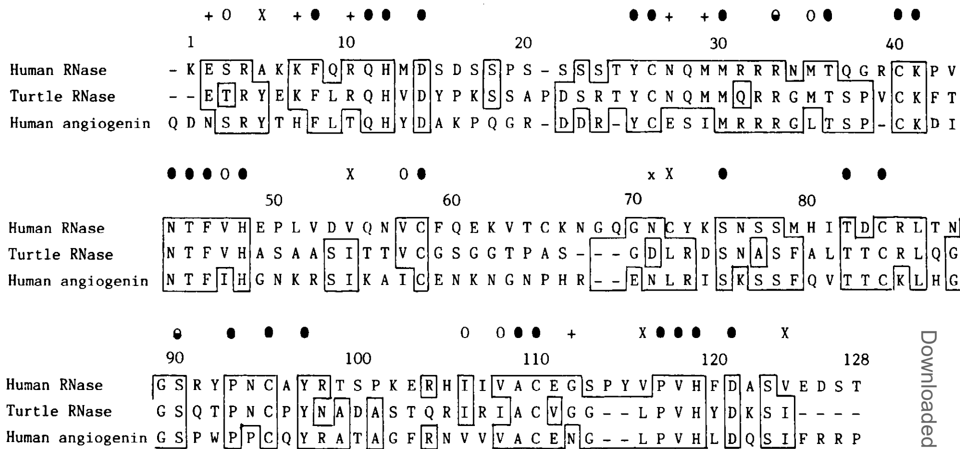

FIG. 8.—Comparison of the amino acid sequences of human pancreatic ribonuclease, turtle pancreatic ribonuclease, and human angiogenin. A dash (—) = deletion. Identical residues in two or three of the sequences are enclosed in blocks. The deletion at positions 67–69 in turtle ribonuclease is slightly differently placed than it is in fig. 1 in order to match the two glycines in this figure. ● = identical in all investigated ribonucleases and in human angiogenin; + = identical in all investigated ribonucleases; × = identical in all investigated mammalian ribonucleases and in human angiogenin; X = identical in turtle ribonuclease and human angiogenin but different and unvaried in all investigated mammalian ribonucleases; ⊖ = identical in all investigated ribonucleases and in human angiogenin, except for one mammalian sequence; ○ = positions with two different residues. All other positions have at least three different residues.

5). Human angiogenin as well has four additional residues at positions 125–128. However, the stop codon at position 129 differs from those found in the messengers of rat pancreatic and bovine seminal ribonuclease at this position.

In mammalian tissues other than pancreas, ribonucleases have been found that are similar to the pancreatic ribonucleases in several but not all respects. Several groups have studied ribonucleases isolated from human urine (Cranston et al. 1980; Iwama et al. 1981; Sugiyama et al. 1981). The main component is identical to the pancreatic enzyme, except for (1) the presence of an additional residue at position 128 and (2) glycosylation characteristics (Beintema et al. 1985*a*). Currently we are investigating the amino acid sequence of another component, called nonsecretory ribonuclease by Bardon et al. (1976), ribonuclease U_S by Iwama et al. (1981), and band D by Sugiyama et al. (1981). The N-terminal and C-terminal regions of this sequence are evidently homologous to the regions with the active-site histidines in pancreatic ribonucleases (fig. 9). However, we have not yet found similarities in other parts of the molecule.

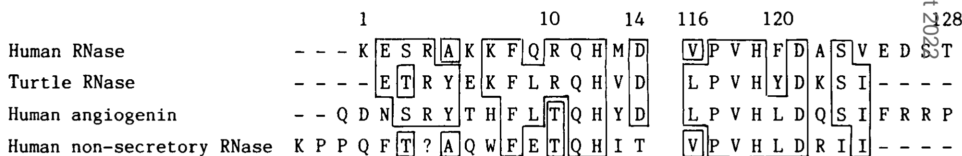

FIG. 9.—Comparison of the N-terminal and C-terminal parts (including the ribonuclease active-site histidine residues) of the sequences presented in fig. 8 and of human nonsecretory ribonuclease. (Its N-terminal sequence has been determined by Dr. J. Hofsteenge, Friedrich Miescher Institut, Basel; similar sequences have been obtained by Cranston et al. [1980], Niwata et al. [1985], and D. G. Glitz [in a ribonuclease isolated from human liver; personal communication].) A dash (—) = deletion; ? = unidentified residue. Identical residues in two or more of the sequences are enclosed in blocks.

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Niwata et al. (1985) isolated a ribonuclease from bovine kidneys and determined its N-terminal sequence, which was found to differ from the nonsecretory ribonuclease from human urine at more than half of the positions. This indicates a higher evolutionary rate than that observed for the pancreatic ribonucleases, if the kidney and urine proteins are indeed orthologous gene products.

There are several similarities in enzymic properties between the mammalian nonsecretory ribonucleases and turtle ribonuclease. Both exhibit (1) a similar activity on RNA but a much lower rate of hydrolysis of the cyclic substrate cytidine 2′,3′-phosphate than the mammalian pancreatic ribonucleases (D. Meinsma, unpublished data) and (2) a much weaker binding to the affinity matrix agarose-APUP (agarose 5′-(p-aminophenyl)-uridine 2′(3′)-phosphate) used for the isolation of pancreatic-type ribonucleases (Iwama et al. 1981; Beintema et al. 1985*b;* Niwata et al. 1985).

Several features of the sequences presented in figure 9 are as follows:

1. Phe-8, Gln-11, His-12, Pro-117, Val-118, His-119, and Asp-121 are unvaried.
2. Position 3 is always occupied by Ser or Thr; position 5 by Ala or Tyr; position 116 by Val or Leu; and position 124 by Val or Ile.
3. The ion bridge between Glu-2 and Arg-10, which is important for the stabilization of the N-terminal S-peptide helix, is absent in human angiogenin and in human nonsecretory ribonuclease.
4. Asp-14, which is a helix-stop signal (Kim and Baldwin 1984), is replaced in human nonsecretory ribonuclease.
5. Position 120 is occupied by the nonaromatic residue leucine in human angiogenin and nonsecretory ribonuclease. In a study of semisynthetic derivatives of bovine ribonuclease, Lin et al. (1972) found that the replacement of an aromatic residue at position 120 by leucine lowers the enzymic activity tenfold.

No firm conclusions can be drawn from these observations as long as no complete amino acid sequence of a nonsecretory ribonuclease has been determined and no X-ray structure is known of any of the distant relatives of the mammalian pancreatic ribonucleases. Likewise, no explanation can be given for the absence of ribonuclease activity in human angiogenin.

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