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Molecular Cloning and Sequencing of cDNA Encoding Plasma Countertrypin, a Member of Mammalian Fetuin Family, from the Mongolian Gerbil, *Meriones unguiculatus*[1]

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Complementary DNA clones coding for countertrypin were isolated from a liver cDNA library of the Mongolian gerbil, and sequenced. They contained one open reading frame encoding 348 amino acid residues, which were assigned to consist of an 18-residue signal peptide and a 330-residue mature protein. The amino acid sequence was about 74% identical with mouse countertrypin and rat fetuin, 60% with bovine fetuin, and 55% with human α_2HS glycoprotein, indicating that this protein belongs to the mammalian fetuin family. The members of this family are known to consist of three domains, *i.e.*, two tandemly arranged cystatin domains (D1 and D2) and an unrelated domain (D3) located at the C-terminal region. When compared with the other members of this family, D3, especially its N-terminal half, varies greatly with deletion or insertion as well as nucleotide substitutions even among three rodent species, *i.e.*, gerbil, rat, and mouse. The sequence comparison also suggests that the conformation of human α_2HS glycoprotein differs greatly from that of other members of this family. A molecular phylogenetic tree of 7 members, constructed on the basis of the synonymous substitution rate of D1 and D2, shows that the gerbil gene diverged prior to the separation of mouse and rat.

Key words: countertrypin, fetuin homologue, molecular evolution, Mongolian gerbil.

Fetuin was discovered in 1944 as a protein present in abundance in bovine fetal plasma (*1*). Sixteen years later, Heremans (*2*) and Schmid and Burgi (*3*) independently isolated a glycoprotein from human plasma, and named it α_2-Z-globulin and Ba-α_2-glycoprotein, respectively. In the following year, Schultze *et al.* (*4*) showed the identity of these two α_2-globulins, and proposed to rename the protein α_2-HS mucoid according to the initials of the original discoverers. This protein, later called α_2-HS glycoprotein (*5*), as well as bovine fetuin, has been most extensively studied among plasma proteins (*5, 6*). However, the incorrect concept persisted for about 3 decades that the human counterpart of bovine fetuin is α-fetoprotein (*7*). During these years, numerous papers claiming specific functions for fetuin and HSG appeared (*5*); these include promotion of cell adhesion, suppression of B and T lymphocyte activities, stimulation of lipogenesis, modulation of brain cortical plate formation, regulation of bone formation and desorption, stimulation of opsonization, and thyroxine binding. None of these activities, however, was fully substantiated, and some conflicting and contradictory results, such as both stimulation and inhibition of lymphocyte activity, were also reported. Furthermore, most of the activities were studied in only one species, reflecting our ignorance of the relationship between fetuin and HSG. In 1988, Elzanowski *et al.* (*8*) found that (i) HSG consists of two tandemly arranged cystatin domains (D1 and D2) and a unrelated domain (D3) located C-terminally; (ii) the closest relative of D1 is kininogen I; and (iii) bovine fetuin whose partial amino acid sequence was then available, is closely related to human HSG. Two years later, Dziegielewska *et al.* (*9*) determined the complete cDNA sequence of bovine fetuin, and showed that the deduced amino acid sequence is 64% identical with that of human HSG. It is now clear that fetuin's counterpart in human plasma is not α-fetoprotein, but HSG and that most mammals contain fetuin homologues[2] not only in plasma but also within the

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Abbreviations: D1, domain 1; D2, domain 2; D3, domain 3; HSG, α_2-HS glycoprotein; NJ, neighbor-joining; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; UPG, unweighted pair-group.

[2] Many proteins with amino acid sequence similarity to human HSG have been reported to date and various names have been coined for these proteins. These include human, rat (*6*), and mouse HSG (*46*), rat 59-kDa bone sialic acid-containing protein (BSP) (*47*), rat insulin receptor tyrosine kinase inhibitor or phosphoprotein 63 (PP63) (*48*), mouse countertrypin (*14*), and rabbit hemonectin (*49*). However, the differences in amino acid sequence among these proteins were relatively large; for example, mouse countertrypin differs from human HSG by about 43% in their amino acid sequences and is shorter than it by 22 residues. Thus, it is not yet clear whether these proteins are orthologous or whether they are paralogous proteins of common ancestral origin. Accordingly, it may be appropriate to call them by the names proposed until further information is available about their structure and function. In this paper, the term "fetuin homologues" is used when it is necessary to refer to these proteins collectively.

cells of bones and brain (*5*).

While studying plasma proteinase inhibitors of experimental animals (*10-13*), we found a new mouse serum trypsin inhibitor whose inhibitory spectrum did not correspond to that of any of the well characterized trypsin inhibitors in human or other animals, and we tentatively named it countertrypin (*13, 14*). Purification and amino acid sequence analysis of countertrypin revealed that a total of 93 amino acid residues could be aligned with those of human HSG and bovine fetuin (*14*). Although they contain two cystatin-like domains, they have no inhibitory activity against cysteine proteinases (*5*). We found, however, that D2 has a stretch of amino acid residues showing similarity to the reactive site region of Kunitz-type trypsin inhibitors. Since the corresponding stretch occurs in both human HSG and bovine fetuin, we analyzed the trypsin-inhibiting activity of these proteins (*14*). The results indicated that both proteins, which had been freshly prepared without the use of ethanol, inhibited trypsin, but that the commercially available preparations had no inhibitory activity. Furthermore, recombinant countertrypins whose putative P1 site lysine was replaced by alanine, phenylalanine, or glutamic acid lost the trypsin-inhibitory activity (*15*). Since this region has been conserved in all members so far studied (*15*), it is likely that they share the trypsin-inhibitory activity, and that this activity is relatively unstable and lost readily during preparation or storage. It is also possible that this inhibitory activity underlies the above-mentioned diverse biological activities of fetuin homologues.

The fetuin homologues have recently shown to emerge in the first layer of cells forming the cortical plate during the development of brain neocortex in humans (*16*), cattle (*17*), sheep (*18*), pigs (*19*), tammar wallabies (*20*), and rats (*21*). This indicates, for the first time, a common role of the fetuin homologues in all mammals so far studied. However, the above animals except the rat are not suited for the study of neurogenesis. Recently, the Mongolian gerbil, *Meriones unguiculatus*, has become popular in brain research, since it is highly susceptible to cerebral ischemia induced by unilateral ligation of a common carotid artery (*22*), and since it is predisposed to spontaneous epileptic fits (*23*). Dam *et al.* (*24*) succeeded in increasing the susceptibility to seizures from 60 to 97% by inbreeding. It is known that the gerbils bred today in laboratories are all descended from a few animals caught in 1936 in the basin of the Amur River in Eastern Mongolia (*25*). Thus, it is possible that one or a few of their ancestors had some abnormality in brain development, and that some genes responsible for this trait became enriched by the founder effect, a similar mechanism to that underlying the high incidence of some hereditary diseases in restricted areas. For instance, human Northern epilepsy syndrome is highly restricted to Finland, possibly due to the recent mutations enriched in the regionally isolated population (*26*). Thus, laboratory gerbils may be suitable for elucidation of the susceptibility to seizures as well as neurogenesis in general. As an initial approach to this issue, cDNA encoding countertrypin of Mongolian gerbil was isolated and sequenced, and the results were compared with those for fetuin homologues from other species.

MATERIALS AND METHODS

Materials—Restriction endonucleases, DNA-modifying enzymes, cDNA Synthesis Kit, and DNA Sequencing Kit were purchased from the same source as described previously (*14, 15, 27*). Gerbil countertrypin was prepared essentially as described previously for mouse countertrypin (*14*). Mouse countertrypin and its polyclonal antibody were prepared as described previously (*14*).

N-Terminal Amino Acid Sequence Analysis—Sequence analysis of the final preparation was carried out by the Edman degradation using an Applied Biosystems model 477A gas phase sequencer, essentially as described previously (*14*).

Construction of cDNA Library, Screening, and DNA Sequencing—The experimental details were essentially the same as those described previously for the gerbil α_1-antiproteinase (*27*). Briefly, a cDNA library of Mongolian gerbil liver was constructed in λgt11 using the cDNA synthesis kit, and screened as described by Sambrook *et al.* (*28*) with antiserum against mouse countertrypin. The positive clones were isolated, and subcloned into M13mp18 and M13mp19 which were used for the construction of a series of deletion mutants containing various lengths of the inserts by the Cyclone system according to Dale *et al.* (*29*). Both strands were sequenced by the dideoxynucleotide chain termination method (*30*).

Phylogenetic Analysis—Synonymous and nonsynonymous substitutions per site for each pair were estimated by the method of Li (*31*). A phylogenetic tree was constructed by the UPG method of Li (*32*) or the NJ method of Saitou and Nei (*33*).

RESULTS AND DISCUSSION

N-Terminal Amino Acid Sequencing—Edman degradation of gerbil countertrypin was carried out twice and gave the following sequence (the values in parenthesis indicate the yield of each PTH-derivative in picomoles from one of the analyses): Ala(157)-Pro(79)-Gln(140)-Gly(133)-Thr-(107)-Gly(91)-Leu (118)-Gly(86)-Phe(103)-Arg(87)-Glu-(73)-Val(110)-Ala(85)-Cys-Asp(82)-Asp(81)-Pro(30)-Glu-(86)-Val(45)-Glu(40). Cysteine was identified as the pyridylethyl derivative in the third assay. The result shows 90% identity to the N-terminal sequence of mouse countertrypin (*14*). Two differences are conservative, *i.e.*, Leu^{12} and Ala^{19} in the mouse sequence are both replaced by Val in the gerbil sequence.

Isolation and Sequencing of cDNA Clones Encoding Countertrypin—Three positive clones were isolated by screening the gerbil liver cDNA library with anti-mouse countertrypin antibody. They were characterized by restriction enzyme analysis and partial sequencing essentially as described previously for gerbil and guinea pig α_1-antiproteinases (*12, 27*). All clones contained the same reading frame coding for 348 amino acids. As shown in Fig. 1, the longest clone consisted of 1429 nucleotides (nucleotide −88 to 1341). Residues 1-20 of the deduced amino acid sequence were identical with those determined by direct sequencing of the protein band, suggesting that the amino acid residues preceding this sequence form a signal peptide.

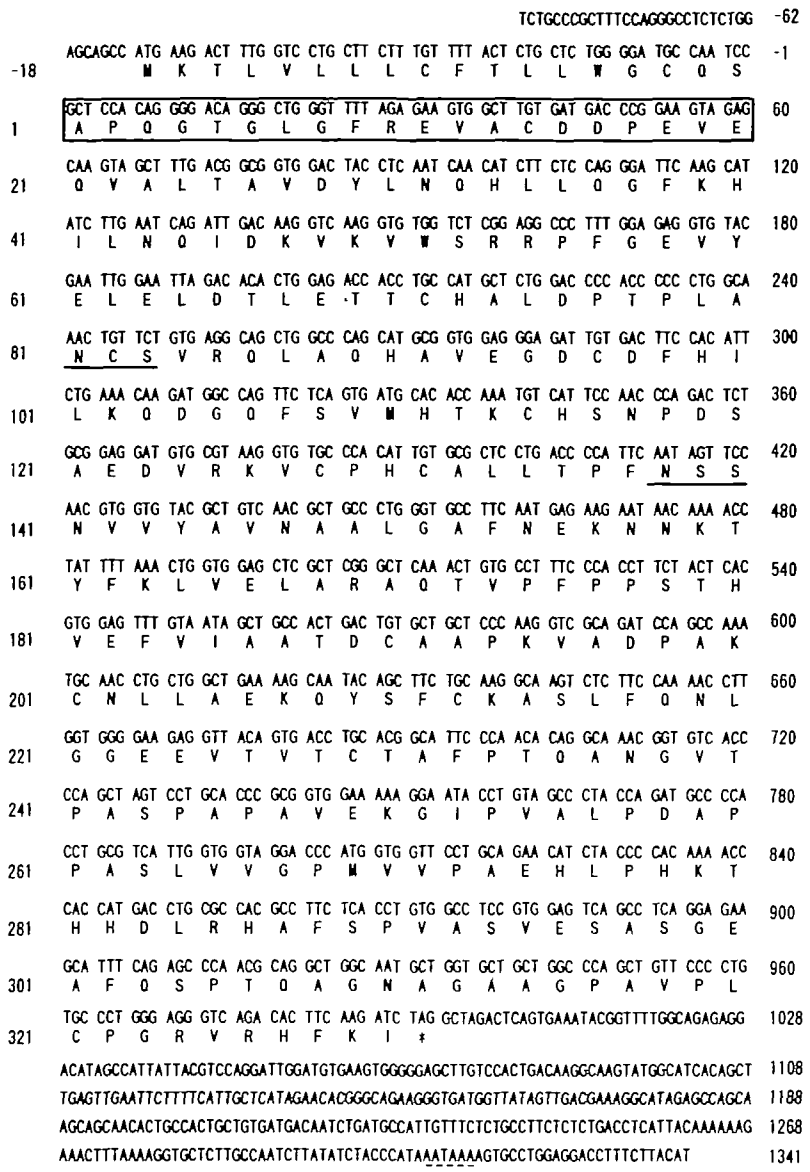

Fig. 1. **Nucleotide sequence of the cDNA and deduced amino acid sequence of gerbil countertrypin.** Nucleotide and predicted amino acid residues are numbered on the right and the left, respectively. Amino acid residues −18 to −1 comprise a putative signal peptide and the nucleotides encoding these amino acids are numbered negatively. Amino acid residues that have been determined by protein sequence analysis of purified countertrypin are boxed. Potential *N*-glycosylation sites are underlined. The broken underline and asterisk show the putative polyadenylation signal and stop codon limiting the open reading frame.

Sequence Comparison of the Fetuin Homologues—The deduced amino acid sequence of gerbil countertrypin is aligned with those of the six known homologous proteins in Fig. 2. A metalloproteinase inhibitor in Japanese habu snake venom is also included, because D1 and D2 of this protein show sequence similarity to the fetuin family (*34*). It is easily recognized that D3 of mammalian fetuin homologues, especially its N-terminal portion, D3A, varies to a large degree; for example, the gerbil D3A is 42 residues in length, while that of sheep fetuin is 55 residues. The reason for the large variation of this region is not clear at present. In humans, D3 is cleaved at two positions, *i.e.*, Leu^{295}-Leu^{296} and Arg^{336}-Thr^{337}, in the liver prior to secretion into blood, and a "connecting peptide" consisting of 40 amino acid residues is released (*5*). The resulting two polypeptides are linked by a disulfide bridge. These two cleavage sites are unique in the human protein, and the other members have different amino acid residues at the corresponding positions. Unlike human HSG, the major form of circulating bovine fetuin is a single polypeptide (*5*). These results suggest that most fetuin family members except HSG are secreted into blood without prior cleavage, although further work is needed to substantiate this hypothesis. Lebreton *et al.* (*35*) first noticed that purified HSG is fragile, readily breaking into several pieces. Later, it was found that HSG, as well as all other family members, including snake venom metalloproteinase inhibitor, contains a dibasic peptide, Arg (or His)^{128}-Lys (or Arg)^{129}, adjacent to the D1/D2 junction (Fig. 2d). All the family members, with the exception of human HSG and snake venom metalloproteinase inhibitor, contain another dibasic peptide, Arg^{55}-Arg^{56} in D1 (Fig. 2a). These dibasic peptides are thought to be cleaved by intracellular trypsin-like endopeptidase (*36*) and to be responsible for the posttranslational modification of some precursor proteins such as proinsulin. The same enzyme may hydrolyze physiologically irrelevant proteins during preparation or storage. These two dibasic sequences, *i.e.*, Arg^{55}-Arg^{56} and Arg^{128}-Lys^{129}, are conserved in the gerbil protein (Fig. 2). Thus, the gerbil protein seems to be similar to those of other rodents and

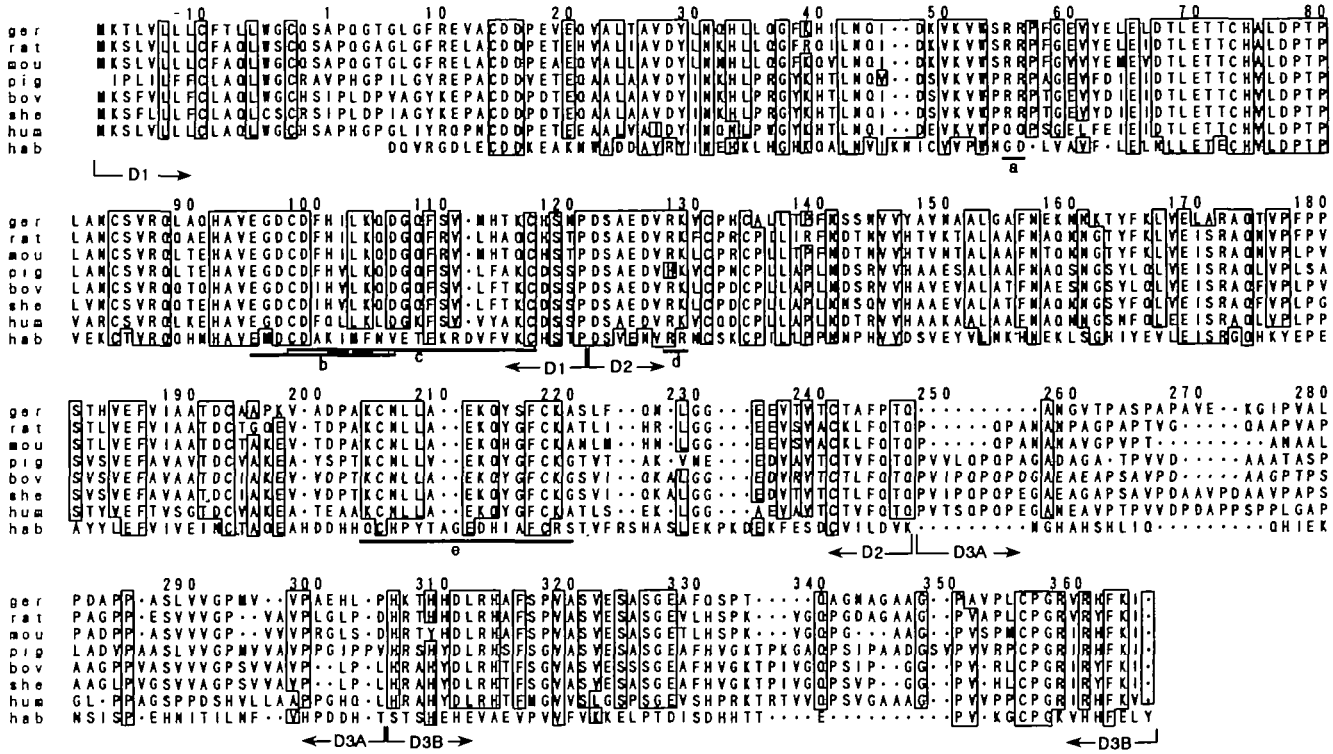

Fig. 2. **Comparison of amino acid sequences of the fetuin homologues.** The sources of sequences of the fetuin homologues are as follows: rat (*43*), mouse (*46*), pig (*37*), bovine (*9*), sheep (*37*), human (*50*), and habu (*34*). A minimum number of gaps, indicated by dots, has been introduced to maximize the alignment. The amino acid residues are numbered according to the putative longest sequence starting from the mature N-terminal amino acid, and indicated above the gerbil sequence. The signal peptides are numbered negatively. This arbitrary numbering system is specific for this multiple alignment and does not match any of the discrete numberings otherwise used for individual species, including the gerbil sequence shown in Fig. 1. The residues identical among six or seven species are boxed, and interdomain junctions are indicated by arrows below the sequences. The various putative motifs are indicated by lines below the sequences (a, dibasic sequence; b, EF-hand motif-like sequence, c, trypsin-inhibitory region; d, dibasic sequence). Abbreviations: ger, gerbil; mou, mouse; bov, bovine; she, sheep; hum, human; hab, habu.

artiodactyls in terms of proteolytic susceptibility. This finding further supports the notion that human HSG is unique among the fetuin homologues. Dziegielewska and Brown (*5*) pointed out that another potential cleavage site, Lys^{334}-Thr^{335}, occurs in human, pig, sheep, and bovine sequences. However, none of the rodent proteins contains this dipeptide sequence (Fig. 2), suggesting that the rodent fetuin homologues are somewhat different from other members in the stability or posttranslational processing of the mature protein.

In marked contrast to D3, the two cystatin-like domains, *i.e.*, D1 and D2, show a high degree of amino acid identity among all members, including snake venom metalloproteinase inhibitor (Fig. 2). This suggests that the functional and structural constraints imposed in D1 and D2 are greatly different from those of D3. Brown *et al.* (*37*) pointed out that D1 contains an EF-hand motif (*38*) (Fig. 2b), which is a likely candidate for the calcium-binding site of human HSG. Recently, Schinke *et al.* (*39*) showed that the sequence encompassing positions 53 to 81 which does not contain the above-mentioned EF-hand motif was responsible for the inhibition of apatite formation. These two stretches are highly conserved in all fetuin homologues including gerbil countertrypin (Fig. 2), and more work is needed to clarify the motif responsible for calcium binding. The gerbil protein contains the region responsible for the trypsin-inhibitory activity (Fig. 2e). This further suggests that the trypsin-inhibitory activity is shared by all the fetuin homologues. Recently, Demetriou *et al.* (*40*) showed that bovine fetuin contains a sequence which has similarity to a region of transforming growth factor-β receptor type II, and acts as an antagonist to the antiproliferative action of transforming growth factor-β in Mv1Lu epithelial cell culture. However, this is in conflict with the finding that in

TABLE I. **Numbers of substitutions per synonymous site (K_S) and nonsynonymous site (K_A) of the cystatin-like segment (D1+D2) among rodent fetuin family members as compared to the corresponding values (shown in italics) of orthodox type α-1-antiproteinase family members.** Values for K_S (upper diagonal) and K_A (lower diagonal), in substitutions per site, were determined by the method of Li (*31*); standard errors are given in parentheses. The data for rat (*42*) and mouse fetuins (*46*) were taken from the references indicated. The corresponding values for the orthodox α-1-antiproteinase were calculated from the sequences taken from the following sources; Mongolian gerbil (*27*), rat (*51*), and mouse (*52*).

	Gerbil	Rat	Mouse
Gerbil		*0.292 (0.049)*	*0.292 (0.048)*
		0.277 (0.039)	*0.302 (0.042)*
Rat	0.128 (0.017)		0.231 (0.041)
	0.152 (0.015)		*0.227 (0.033)*
Mouse	0.121 (0.016)	0.061 (0.011)	
	0.165 (0.016)	*0.122 (0.013)*	

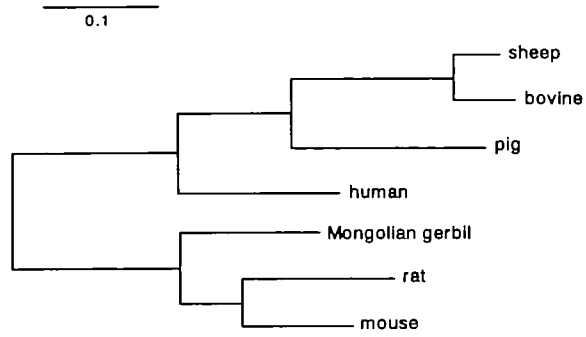

Fig. 3. **Phylogenetic tree of the fetuin homologues.** The branch lengths are drawn in proportion to the genetic distances, and the tree was constructed by the NJ method of Saitou and Nei (*33*). The scale indicates the genetic distance of 0.1.

the mouse, α-macroglobulin is the only plasma protein which inhibits the interaction between transforming growth factor-β and its receptor, although most fetuin homologues including the gerbil protein contain this sequence (Fig. 2b) (*41*). On the other hand, rat fetuin was shown to inhibit the insulin receptor tyrosine kinase activity while human HSG does not have the same activity when assayed under the same conditions (*42*). These results indicate that there are considerable species differences in the biological properties attributed to the fetuin homologues, and more data are needed to generalize the results obtained with human HSG or bovine fetuin.

Molecular Phylogenetic Tree of Fetuin Family—It is generally believed that the synonymous base substitutions within the coding regions of mRNAs are selectively neutral and accumulate at a constant rate over time, and that the number of synonymous substitutions between the two sequences (K_S) is a measure of the time since they shared the last common ancestor (*43*). However, there are genes which are exceptions to this generalization. For example, the bait region of mammalian α-macroglobulin genes evolved much faster than the directly flanking introns of both sides (*44*), and the above principle based on a constant evolutionary rate cannot be applied to these sequences. D3 of the fetuin homologues may be categorized into this exceptional group. It is practically impossible to calculate reliably the synonymous or nonsynonymous base substitutions of D3, because the unambiguous alignment of D3 is impossible (see Fig. 2). These results indicate that D3, especially D3A, has evolved under a strong selective pressure to change. In marked contrast, D1 and D2 have evolved in a manner similar to most genes. Since there was no statistically significant difference in the K_S and K_A values between D1 and D2 (data not shown), these values were calculated on the basis of an undivided cystatin-like segment. Table I shows the K_S and K_A values of the cystatin-like segment among the three rodent species. For comparison, the K_S and K_A values of α_1-antiproteinase of orthodox type (*27, 45*) were also calculated. The results indicate that the synonymous substitutions of the cystatin-like segment accumulated at a rate similar to that of the α_1-antiproteinase gene. One salient feature, however, is the finding that the K_A value between mouse and rat fetuin homologues is significantly smaller ($p<0.001$) than those of other pairs, whereas the corresponding K_S value is not significantly different from those of other pairs. This suggests that the amino acid sequence of the cystatin-like segment has been more strictly conserved during evolution of the family Muridae than the family Gerbillidae, whereas there has been no such difference in the selective pressure on the α_1-antiproteinase gene. The biological or functional significance of this difference in the selective pressure is not clear at present. A molecular phylogenetic tree of 7 fetuin members was constructed using the K_S values and the NJ method (Fig. 3). The branching order in the UPG tree (*31*) was essentially the same as in the NJ tree, although the branch lengths were different (data not shown). In this tree, the Mongolian gerbil diverged earlier than the separation of mouse and rat. This is different from the molecular phylogeny of α_1-antiproteinase, in which mouse lineage diverged prior to the separation of gerbil and rat (*45*). However, the present phylogeny is consistent with the conventional taxonomy, in which the rat and mouse belong to the same subfamily Murinae of the family Muridae, while the gerbil belongs to the different family Gerbillidae. Although more data are necessary to draw any conclusion as to the evolution of Myomorpha, the result suggests that the molecular evolution of the fetuin homologues is complex, and that the selective pressure has been different from domain to domain, as well as from evolutionary lineage to lineage. Thus, the elucidation of these differences among the family members will lead to a better understanding of the physiological function of this protein family.

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