When comparing the related binding specificities of the 434 and the 16-3 repressors we see that the Asn residue at position 6 in the operator recognition helix of the 16-3 repressor poses some difficulties in applying the most recent model of 434 repressor–434 14-mer operator interaction[12,14,21] to 16-3. The corresponding side chain at position 6 in the 434 repressor is longer, a Gln which contacts the operator at base pairs 4 and 5 (see Fig. 3, 14-mer, base pairs 4 A/T and 5 T/A). In the case of 16-3 this Asn/Gln difference might create a wide gap between the recognition α-helix (at position 6) and the operator bases, placing the interacting atomic groups too far from each other. However, coordinated changes in the conformation of the operator with a slight modification of the angle of the recognition helix axis and some rotation of the Asn side chain may narrow the gap between this amino acid and the bases. (For 16-3 we suggest, for example, a sharper bending of the DNA axis with more local twist at operator base pairs 4 and 5, and a deeper submergence of the α_3-helix toward the carboxy terminus than those determined for 434[12].) According to recent findings[21–23] such conformational differences are conceivable.

We thank László Dorgai, Ferenc Olasz, Sankar Adhya, John Anderson and Marvin Smith for encouragement and helpful comments; Ildikó Hetényi and Erzsébet Horváth-Bukva for technical assistance; Tamás Lukacsovich for suggesting the use of *Smu*cI and pKK 223-3; Miklós Gaszner for help in screening recombinant plasmids; and Rene Malone for correcting the manuscript. This work was supported by grants from the Hungarian Academy of Sciences, from the Ministry of Culture and from the State Office of Technical Development.

Received 7 July; accepted 1 October 1987.

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Adaptive evolution in the stomach lysozymes of foregut fermenters

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The convergent evolution of a fermentative foregut in two groups of mammals offers an opportunity to study adaptive evolution at the protein level. The appearance of this mode of digestion has been accompanied by the recruitment of lysozyme as a bacteriolytic enzyme in the stomach both in the ruminants (for example the cow) and later in the colobine monkeys (for example the langur). The stomach lysozymes of these two groups share some physicochemical and catalytic properties that appear to adapt them for functioning in the stomach fluid[1,2]. To examine the basis for these shared properties, we sequenced langur stomach lysozyme and compared it to other lysozymes of known sequence. Tree analysis suggests that, after foregut fermentation arose in monkeys, the langur lysozyme gained sequence similarity to cow stomach lysozyme and evolved two times faster than the other primate lysozymes. This rapid evolution, coupled with functional and sequence convergence upon cow stomach lysozyme, could imply that positive darwinian selection has driven about 50% of the evolution of langur stomach lysozyme.

The majority of evolutionary changes revealed by comparative studies of proteins and nucleic acids appears to fit the neutral theory of molecular evolution; that is, they could have become fixed by random drift of selectively neutral or nearly neutral mutations rather than by positive darwinian selection[3]. For this reason, biochemical adaptation has been difficult to demonstrate convincingly at the sequence level. Important adaptive changes may involve only a few of all evolutionary events[4–7] and may thus be masked by the preponderance of neutral replacements[4].

The present study illustrates an approach that minimizes such masking. Here we examine two lineages along which the same new function has arisen independently and compare them with lineages that retain the old function. The old function of lysozyme, evident in many mammals, is to fight invading bacteria[8,9]. Thus, many mammals—humans and rats included—have moderate to high levels of lysozyme in secretions like tears and saliva, as well as in white blood cells and tissue macrophages[9]. The new function, evident in ruminants and colobine monkeys, is to digest bacteria that pass from the fermentative foregut into the true stomach[1].

The colobine monkey studied here is the hanuman langur (*Presbytis entellus*), which has a large fermentation chamber followed by a tubular stomach[10,11], the anterior lining of which contains high levels (about 1 mg per g of tissue) of lysozyme *c*[1,2]. Saliva, serum and most non-stomach tissues of the langur contain very low levels (about 0.02 mg per g or less) of lysozyme[2]. A similar distribution is seen in cows and other ruminants[1,12–14]. Thus, the major selection pressure on these cow and langur enzymes may be to act in the stomach fluid.

Langur and cow stomach lysozymes have two characteristics that are consistent with functioning in the stomach fluid, which is acidic and contains pepsin. At physiological ionic strengths, they are most active as catalysts at low *p*H, in contrast to most conventional lysozymes whose activity profiles are broader and more basic[1,2]. In addition, they are unusually resistant to breakdown by pepsin[1].

The amino-acid sequence of langur stomach lysozyme was determined and compared to cow and other mammalian lysozymes of the *c* class (Fig. 1). It is most similar in size (130 amino acids) and overall sequence to the other primate lysozymes, with 14 differences from baboon (an Old World monkey with a simple stomach[11]) and 18 differences from human lysozymes (Table 1).

If these mammalian lysozymes had evolved in a conventional, predominantly divergent manner[15], then we would expect the

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                10        20        30        40        50        60        70        80        90       100       110       120       130
                |         |         |         |         |         |         |         |         |         |         |         |         |
Langur  KIFERCELARTLKKLGLDGYKGVSLANWVCLAKWESGYNTEATNYNPGDESTDYGIFQINSRYWCNNGKTPGAVDACHISCSALLQNNIADAVACAKRVVSDPQGIRAWVAWRNHCQNKDVSQYVKGCGV
Baboon  .............R......R.I.............D...Q........Q...........H....D.......N......N....D..T.............................R......Q....
Human   .V...........R..M...R.I.....M...........R.....A..R...............D.......N...L.......D.............R.............R...R..R...Q....
Rat     .TY....F.....RN.MS..Y.....D.....QH..N...Q.R..D...Q...............D....R.KN..G.P......DD.TQ.IQ......R...........QR..K.R.L.G.IRN...
Cow     .V.........................L..T....S...K......SS.............KW...D....N...G..V...E.ME.D..K......KI..E-...T.....KS..RDH...S..E..TL
Horse   .V.SK....HK..AQEM..FG.Y.......M.EY..NF..R.F.GKNANG.S...L..L.NKW..KDN.-RSSSN..N.M..K..DE..D.DIS......R..K.MS..K..VK..KD..L.E.LAS.NL
```

Fig. 1 Amino-acid sequence of langur stomach lysozyme shown in the single letter code and compared to five other lysozyme *c* sequences from placental mammals. Only differences from the langur sequence are shown for the other sequences, with identities indicated by a dot. An amino-acid deletion is indicated by a dash (−). Sequences shown are from baboon (*Papio cynocephalus*), human (*Homo sapiens*), rat (*Rattus norvegicus*)[9], cow (*Bos taurus*)[9,32], and horse (*Equus caballus*)[33] lysozymes.

Methods. Lysozyme was purified from langur stomach by ion-exchange chromatography (CM52, Whatman) and gel filtration (Sephadex G-50), reduced with dithiothreitol, and the cysteines carboxymethylated with iodoacetamide[2]. Its amino-acid sequence was determined by automated Edman degradation[34] on an Applied Biosystems Model 470A gas-phase protein microsequencer. The phenylthiohydantoin amino acids were separated by high-performance liquid chromatography (HPLC; Beckman) on an IBM Cyano analytical column[35]. The first 60 residues were sequenced from the whole molecule. Residues 51 to 106 were identified from the 9-kilodalton fragment (residues 51 to 130) prepared by complete digestion of the molecule with glutamate-specific protease from *Staphylococcus aureus* strain V 8 (Pierce)[36]. Mild acid hydrolysis yielded a fragment bearing residues 103 to 130 from which the carboxy-terminal sequence was determined[2]. The lysozyme was chemically cleaved[37] between Asn 67 and Gly 68, and the two fragments separated by HPLC. The fragment starting from Gly 68 was sequenced past residue 103. To confirm[2] the sequence of the C-terminal region, this same fragment was chemically cleaved after the tryptophans[38], the resulting fragments separated by HPLC, and the fragment bearing residues 113 to 130 sequenced.

parsimony tree built from them to match the branching order of the species (Fig. 2, tree A). Surprisingly, however, the tree that places cow stomach lysozyme with the langur enzyme (Fig. 2, tree B) is as parsimonious as the biological tree. Based on experience with divergently evolving proteins, such as haemoglobins[16], we expected tree B to require at least five more amino-acid replacements than tree A. The unexpected association of cow and langur lysozymes led us to consider three possible evolutionary explanations—horizontal transfer, gene duplication and homoplasy.

Horizontal transfer. The stomach lysozyme gene could have been transferred between an ancestor of the langur and an ancestor of the cow. This mechanism has testable consequences. Recently transferred genes should be nearly identical in overall sequence; yet, the langur and cow lysozymes differ at 32 positions (Table 1). The logical direction for such a selective transfer would be from a ruminant to the ancestral colobine monkey, since foregut fermentation arose more recently along the Old World monkey lineage (about 15–20 million years ago[17,18]) than along the ruminant lineage (about 55 million years ago[1]). However, tree B (Fig. 2) suggests that if such a transfer did occur it was from the monkey to the ruminant because the cow lysozyme resides in the primate part of the tree. Furthermore, if the cow gene was indeed acquired from a monkey, it should branch with the primates even when the langur sequence is omitted (Table 2, alternative tree D). This is not the case. When the langur sequence is omitted, the most parsimonious tree (Table 2, biological tree C) places cow lysozyme near horse lysozyme as in tree A of Fig. 2. Thus, horizontal transfer is unlikely to be the explanation.

Gene duplication. Suppose, for example, that there are two lysozyme genes, *X* and *Y*, resulting from a duplication predating the divergence of primates and ruminants and that the langur and cow stomach lysozymes are the products of the *X* gene while the other mammalian lysozymes could be the products of the *Y* gene. If so, langur lysozyme should not only differ greatly in sequence from the other primate lysozymes but also should lie on a lineage that branched off before the *Y* lysozymes diverged from one another. Instead, langur lysozyme is most similar in overall sequence to baboon lysozyme (Table 1) and is also genealogically close to it in both trees A and B (Fig. 2). In addition, when the cow sequence is omitted, the most parsimonious tree for the five remaining lysozymes (Table 2, tree E) now has the topology of the biological tree. But, if the above

Table 1 Pairwise comparisons of lysozyme sequences

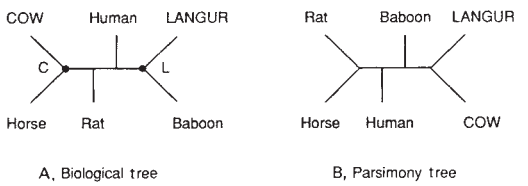
		Amino-acid differences					
	Species compared	La	Ba	Hu	Ra	Co	Ho
Uniquely shared residues	Langur	—	14	18	38	32	65
	Baboon	0	—	14	33	39	65
	Human	0	1	—	37	41	64
	Rat	0	1	0	—	55	64
	Cow	4	0	0	0	—	71
	Horse	0	0	0	0	1	—

For each pair of lysozymes in Fig. 1, the number of amino-acid differences appears above the diagonal; each deletion is counted as one difference. Below the diagonal is the number of positions at which each pair of lysozymes shares an amino acid that is found in no other vertebrate lysozyme of known sequence (excluding other ruminant and colobine stomach lysozymes). The additional lysozymes used here are complete sequences from echidna (H. McKenzie, personal communication), pig (J. Jollès and P. Jollès, personal communication), and numerous birds[9,28], as well as partial sequences from tortoise[9], dog[29], goat tears (J. Jollès and P. Jollès, personal communication), and mouse macrophages and intestines[30]. The number of uniquely shared residues between cow and langur is significantly greater than average (χ^2 test, $P > 0.95$). (The high divergence of horse lysozyme from these other mammalian lysozymes might be due either to accelerated evolution along the horse lineage or expression of a duplicate lysozyme gene[2].)

Table 2 Comparison of trees for lysozyme

Kind of tree	Minimum no. of amino-acid replacements	No. of wins
Six species		
A Biological	174	11
B Alternative	174	12
Langur omitted		
C Biological	163	13
D Alternative	169	8
Cow omitted		
E Biological	137	11
F Alternative	145	3

The term 'biological' refers to the tree for the species compared; 'alternative' refers to the other possible trees discussed in the text. Biological tree A is shown in Fig. 2; biological trees C and E would look like tree A if the langur (tree C) or cow (tree E) lineages were omitted. Trees C and E were the shortest ones found by the computer program PHYLIP[31] for the five sequences. Alternative tree D has the branching order that would result if the cow lineage were attached at node L (instead of node C) and the langur lineage omitted from tree A. Conversely, tree F would result if the langur lineage were attached at node C (instead of node L) and the cow lineage omitted from tree A. 'Number of wins' refers to the number of positions in the lysozyme sequence at which one tree requires fewer amino-acid replacements than the other to explain the sequence divergence.



A, Biological tree B, Parsimony tree

Fig. 2 Shortest, unrooted trees relating mammalian lysozyme sequences. Tree A is the biological tree for the six species, and tree B is an alternative tree for six lysozyme sequences.
Methods. Amino-acid parsimony trees were constructed with a computer program (PROTPARS in the package PHYLIP[31]) that can find the tree requiring the fewest amino-acid replacements. The program requires that any changes be consistent with the genetic code but does not count synonymous substitutions; a change between two amino acids which must pass through an intermediate state (a third amino acid) is counted as two replacements. Each deletion is here counted as one replacement. In this case, there were two shortest trees, each explaining the data with 174 amino-acid changes (see Table 2). This number includes derived replacements, which are the result of shared ancestry and therefore unite sequences according to relationship, as well as homoplasies (parallelisms and convergences), which are the result of independent evolutionary events and therefore do not imply relationship[21]. (Amino-acid parsimony trees were also built by the computer program PAUP[39], which allows any amino acid to change into another without regard for the genetic code. This program found trees that unite cow and langur to be the shortest[2]. The ruminant stomach and colobine monkey lysozymes were found to branch together even when other complete and partial sequences listed in Table 1 were included in the trees.) Nodes C and L are explained in Table 2.

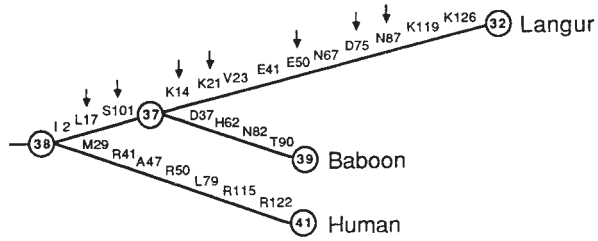


Fig. 3 Apportioning of amino-acid replacements on the lineages leading to primate lysozymes. Branch lengths are proportional to the number of replacements. The resulting amino acids are written in the single letter code followed by numbers indicating their positions in the sequence. The seven arrows point to amino acids which arose independently along the lineages leading to cow and langur lysozymes. (The two homoplastic replacements along the lineage leading to baboon and langur are consistent with the hypothesis[1,18] that the ancestral Old World monkey was a foregut fermenter.) There were no homoplastic replacements with the cow lineage along the baboon and human lineages after these two primate lineages branched from that leading to langur. In the circles are the numbers of amino-acid differences from cow stomach lysozyme (see Table 1). Since its time of common ancestry with the human and baboon lineages, the langur lysozyme lineage has gained sequence similarity to the cow enzyme: starting with 38 differences, this lineage has ended up with 32 differences from cow lysozyme. In contrast, the human and baboon lysozyme lineages diverged from the cow lysozyme lineage and have ended up with 41 and 39 differences, respectively, from this enzyme.
Methods. Amino-acid replacements were assigned along the lineages of the biological tree in a manner which requires the fewest amino-acid replacements and the fewest nucleotide substitutions. The additional sequences listed in the legend of Table 1, as well as partial sequences from other ruminant (J. Jollès and P. Jollès, personal communication) and colobine[2] stomach lysozymes, were used when necessary to decide upon the most parsimonious assignments. (The number of replacements along each lineage is consistent with that found in the shortest distance Wagner tree.) Each set of homoplastic replacements was examined to determine whether the underlying mutational mechanisms might have been parallel or convergent[2,21]. (An example of parallel replacements would be an Arg to Lys change on both lineages. An example of convergent replacements would be Arg to Lys on one lineage and Thr to Lys on the other.) Lys 14 and Glu 50 may have been the result of convergent replacements, while the others appear to have been in parallel.

gene duplication model were correct, an alternative tree (Table 2, tree F) would have been favoured. The duplication model further predicts that within a given primate both *X* and *Y* lysozymes may be present; however, tissue surveys done with electrophoretic and immunological methods give no evidence of two distantly related types of lysozyme within humans or monkeys[1,2]. All evidence to date suggests that these three primate lysozymes are the products of equivalent (orthologous) genes. Thus, gene duplication is unlikely to explain the link between cow and langur stomach lysozymes.

Convergent or parallel evolution (homoplasy). Sequences can also be similar because of independent acquisition of identical amino acids through parallel or convergent replacements, a phenomenon known as homoplasy[19–21]. If the incidence of homoplasy between two distantly related sequences is unusually high, then a parsimony tree is likely to link them[15,22]. To test for homoplasy in the lysozyme sequences we followed the recommendation of Sneath and Sokal[21]: accordingly, the amino-acid replacements were assigned in the simplest way along the lineages of the biological tree (Fig. 2, tree A). As illustrated in Fig. 3, five homoplastic replacements (producing Lys 14, Lys 21, Glu 50, Asp 75 and Asn 87 in cow and langur lysozymes) were found to occur along the cow and langur lineages. This number is significantly greater (χ^2 test, $P > 0.95$) than the number (0 or 1) of homoplastic replacements found to occur along the other pairs of lineages[2]. Therefore, these five amino-acid replacements are unlikely to have occurred by chance (that is, neutral drift) alone. In addition, two replacements (producing Leu 17 and Ser 101) appear to have occurred in parallel along the lineage leading to cow and along the lineage leading to the Old World monkeys (baboon and langur). Four of the resulting amino acids (Lys 21, Glu 50, Asp 75 and Asn 87) are found only in colobine and ruminant stomach lysozymes (Table 1). Thus, homoplasy is the most plausible explanation for tree B (Fig. 2), since it can account for both the significant number of uniquely shared amino acids and the relatively large sequence difference between langur and cow stomach lysozymes (Table 1).

About half of the replacements which occurred on the langur lysozyme lineage after it diverged from that of the baboon made the langur sequence more similar to cow lysozyme than was the ancestral monkey enzyme (Fig. 3); that is, langur lysozyme converged in sequence upon cow lysozyme. In contrast, baboon and human lysozymes followed normal divergent paths. So, half or more of the replacements along the langur lysozyme lineage may have been fixed by positive darwinian selection. Consistent with this interpretation, langur lysozyme appears to have evolved about twice as fast as the other primate lysozymes (Fig. 3).

The possibility that sequence convergence has accompanied the convergent regulatory and functional evolution of langur and cow lysozymes was at first surprising. Comparative biochemistry has repeatedly shown that a given function can be fulfilled by a vast array of sequences and had reinforced the notion that sequence evolution, whether dominated by drift or positive selection, is mainly divergent. Lysozyme seemed to be typical in these regards: in their amino-acid sequences, the *c*, *g* and phage classes of lysozymes have diverged greatly and yet have the same bacteriolytic function[23]. Furthermore, the well-characterized cases of functional convergence in proteins are not the result of convergent sequence evolution. Haemoglobin and haemocyanin, for example, are functionally convergent but these two types of protein exhibit no sequence similarity. Likewise, subtilisin and the trypsin-like serine proteases independently evolved similar active sites but did so with different primary and tertiary structures[24].

Despite the predominantly divergent nature of most sequence evolution, parallel and convergent events often occur; these usually appear to be scattered randomly across species[22] and only rarely have functional explanations for specific replacements been proposed[25]. Moreover, when selection for a new protein function is imposed on bacterial populations in the laboratory, the same way of solving the problem is sometimes encountered[26]. So, there may be a limited number of ways of converting a conventional mammalian lysozyme into one that would function well in the stomach fluid of a foregut fermenter. To understand why langur lysozyme has gained sequence similarity to cow stomach lysozyme, studies must be made of the functional consequences of the homoplastic replacements identified by our phylogenetic analysis. For evaluation of some of the possible consequences, see ref. 27.

We thank P. Dolhinow for supplying langur tissues; S. Kirsher for technical assistance; J. Jollès, P. Jollès and H. McKenzie for unpublished sequences; J. Beintema, B. Bowman, R. Cann, D. Dobson, J. Felsenstein, W. Fitch, P. Hornbeck, J. Kirsch, T. Kocher, K. Milton, H. Ochman, V. Sarich, D. Shaw, M. Stoneking, and especially E. Prager for discussions. This work received support from NIH.

Received 20 July; accepted 1 October 1987.

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ERRATUM

Location of the ATPase site of myosin determined by three-dimensional electron microscopy

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Nature **329,** 635-638 (1987).

IN this letter Fig. 1*c* was incorrectly printed alongside Fig. 4*a* and *b*.