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Coordinated Amino Acid Changes in the Evolution of Mammalian Defensins

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Abstract. The mammalian defensin molecule is a short, highly cationic peptide cytotoxic to both microbial and mammalian cells which is cleaved from a precursor including a signal peptide and a highly anionic propiece. A phylogenetic analysis of 28 complete sequences from five mammalian species (mouse, rat, guinea pig, rabbit, and human) showed species-specific clusters of sequences, indicating that the genes duplicated after divergence of these species. Comparison of rates of synonymous and nonsynonymous nucleotide substitution suggested that gene duplication has often been followed by a period in which diversification of the mature defensins at the amino acid level has been selectively favored. In some comparisons, it appeared that amino acid differences in this region have appeared in a nonrandom fashion so as to change the pattern of residue charges. Because it has been hypothesized that the negative charge in the propiece serves to balance the positive charge in the mature defensin and thus to prevent cytotoxicity prior to cleavage, we used a maximum likelihood method of reconstructing ancestral states in order to test whether this balance has been maintained over evolutionary time in spite of rapid diversification of the mature defensin at the amino acid level. Reconstructed ancestral sequences always maintained a charge balance between mature defensin and propiece, and changes in the net positive charge of the mature defensin were balanced by corresponding changes in the propiece. The results support the hypothesis that, in the evolution of these proteins, amino acid changes have occurred in a coordinated fashion so as to preserve an adaptive phenotype.

Key words: Mammalian defensin — Amino acid — Propiece

Introduction

The concept of epistatic fitness interactions, or fitness effects arising from interactions among alleles at different genetic loci, has a long history in evolutionary biology (Haldane 1931; Wright 1931). At the level of amino acid sequences, this concept has been extended to cover interactions among residues at different sites within a single polypeptide chain. For example, it has been hypothesized that the presence of a particular residue at one position might permit or even favor the presence of certain other residues at other positions and thus that amino acid positions may evolve in a coordinated fashion (Fitch and Markowitz 1970). The potential importance of such interactions was supported early in the history of molecular biology by experimental studies with *E. coli* in which it was shown that the effect on enzyme activity of a mutation at one site can depend on the amino acid present at another site (Yanofsky et al. 1964). However, so far, few convincing cases have been described of coordinated amino acid changes over evolutionary time.

The defensins of vertebrates are antimicrobial peptides that are stored in cytoplasmic granules of Paneth cells of the intestine, neutrophils, and macrophages (Ganz et al. 1989). The mature defensin is a highly cat-

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Fig. 1. Alignment of mammalian defensin primary translation products, showing major functional regions.

ionic peptide of 29–34 amino acids; it is cleaved from a primary translation product consisting of a signal peptide (19 amino acids), a propiece (37–51 amino acids), and the mature peptide (Michaelson et al. 1992). Defensins are cytotoxic to mammalian and bacterial cells, presumably as a result of their ability to form pores in lipid bilayers (Kagan et al. 1990). Because cationic proteins are often cytotoxic (Antohi and Brumfield 1984), the cationic character of defensins is thought to play an important role in their cytotoxicity, although it is evidently not the only factor at work (Lichtenstein et al. 1986; Michaelson et al. 1992). Observing that the propiece has an anionic character, Michaelson et al. (1992) proposed that the propiece plays a role in neutralizing the cytotoxicity of the defensin until it is ready for use in an antimicrobial attack. In support of this hypothesis, they showed that for seven mammalian defensins, the net negative charge of the propiece showed a linear relationship with the net positive charge of the mature defensin (Michaelson et al. 1992).

Because mammalian defensins differ with respect to the net positive charge of the mature defensin, these molecules provide a potential test case for the hypothesis of coordinated evolution of different amino acid sites within a protein. If it is necessary that the propiece be sufficiently anionic to neutralize the mature defensin, it is expected that addition of new positively charged residues to the mature defensin will be compensated by corresponding increases in the net negative charge of the propiece. We addressed this question by phylogenetic analysis, by examining the pattern of nucleotide substitution in defensin genes, and by using a maximum-likelihood method for reconstructing ancestral amino acid sequences and thus the pattern of amino acid changes over evolutionary time (Yang et al. 1995).

Methods

Twenty-eight complete defensin gene sequences from five species of mammals were used in analyses. The sequences (with Genbank accession numbers in parentheses were as follows: (1) mouse *Mus musculus:* M-cor (X15617), M-def1 (U02994, U02995), M-def2 (U02996, U02997), M-def3 (U02998, U02999), M-def5 (U03000, U03001), M-def6 (U03002, U03003); M-4C-2 (U12564), M-4C-4 (U12565), M-4C-5 (U12566), M-CRS4C (S77610), M-CRS4C-2 (U12564), M-CRS4C-5 (S77621); (2) rat *Rattus norvegicus:* R-NP1 (U16686), R-NP2 (U16685), R-NP3 (U16683), R-NP4 (U16684); (3) guinea pig *Cavia pocellus:* Cp-1A (D14119), Cp-1B (D14118); *Cavia ''cutleri''* Cc-1A (X57705); (4) rabbit *Oryctolagus cuniculus:* Rab-NP3 (M64599); Rab-NP4 (M64601); Rab-NP5 (M64602); Rab-MCP1 (M28883); Rab-MCP2 (M28072); and (5) human *Homo sapiens:* H-NP3 (X13621), H-P4 (U18745), H-D5 (M97925), H-D6 (U33317).

Sequences were aligned at the amino acid level using the CLUSTAL V program (Higgins et al. 1992), and the alignment was corrected by eye in some respects (Fig. 1). In computing pairwise distances among members of a set of amino acid or DNA sequences, any site at which the alignment postulated a gap in any one sequence in the set was excluded from all pairwise distance computations. Phylogenetic trees of the 28 amino acid sequences were constructed by (1) the neighbor-joining (NJ) method Saitou and Nei (1987), based on the proportion of amino acid differences, and (2) the maximum parsimony (MP) method (Swofford 1990). The significance of internal branches in the NJ tree was tested by Rzhetsky and Nei's (1992) standard error test. The reliability of clustering patterns in the MP tree was tested by bootstrapping, which involves repeated sampling (with replacement) from the data set (Felsenstein 1985). Previously published phylogenies of defensins (Michaelson et al. 1992; Yount et al. 1995) have used smaller numbers of sequences and have been constructed by the UPGMA method, which is inappropriate for most most molecular data sets because it assumes a constant rate of evolution in all branches.

In pairwise comparisons among selected sequences, the number of synonymous nucleotide substitutions per synonymous site (d_S) and the number of nonsynonymous nucleotide substitutions per nonsynonymous site (d_N) were estimated by Nei and Gojobori's (1986) method. In order to test the hypothesis that amino acid differences occurred at random with respect to residue charge, the method of Hughes et al. (1990) was used. Briefly, this method divides nonsynonymous nucleo-

tide differences between sequences into those that are conservative with respect to some qualitative amino acid property and those that are radical (nonconservative) with respect to the same property. The proportion of conservative nonsynonymous differences per conservative nonsynonymous site (p_{NC}) and the proportion of radical nonsynonymous differences per radical nonsynonymous site (p_{NR}) are then computed. If $p_{NR} > p_{NC}$, this indicates that nonsynonymous differences occur in such a way as to change the property of interest to a greater extent than expected under random substitution. In computing p_{NC} and p_{NR} with respect to charge, we categorized amino acid residues as positive (H, K, R), negative (D, E), or neutral (all others); and any nonsynonymous difference causing a change of category was counted as a radical difference. Standard errors of mean d_S, d_N, p_{NC}, and p_{NR} were estimated by Nei and Jin's (1989) method.

Using a subset of the sequences for which both NJ and MP methods produced a stable phylogeny (see Results), we used the method of Yang et al. (1995) to reconstruct ancestral amino acid sequences at nodes of this phylogeny. This method uses a maximum-likelihood approach based on an empirically derived matrix of amino acid substitution probabilities. Because this method does not reconstruct sequences at sites at which the alignment postulated an insertion/deletion events, we reconstructed the occurrence of these events using maximum parsimony.

Results

Phylogenetic Analysis

Figure 2 shows the NJ tree of mammalian defensins. Sequences for the mouse, rat, guinea pig, and rabbit formed species-specific clusters, each of which was supported by a statistically significant internal branch (Fig. 2). The human sequences clustered together, apart from the other species, but in this case the cluster was not separated from others by a significant branch (Fig. 2). Because of the lack of an outgroup, this phylogenetic tree cannot be definitively rooted; it is rooted in Fig. 2 to correspond to the known phylogeny of placental mammals, with Rodentia as an outgroup to Primates and Lagomorpha (Li et al. 1990). The simplest interpretation of the pattern of species-specific clustering is that defensin genes have duplicated independently in the species examined after the species diverged from common ancestors. Even between rat and mouse, which have been estimated to have diverged 17–25 Mya (Janke et al. 1994), none of the loci are orthologous. Within the mouse genes, there were two major clusters (designated 1 and 2 in Fig. 2). In contrast to the mouse, rat, and rabbit genes, the deep branches within the human gene cluster were very short; thus interrelationships among the human genes were poorly resolved.

MP analysis of these data produced eight equally parsimonious trees. The strict consensus tree of these was very similar to the NJ tree (data not shown). The species-specific clusters of genes received high bootstrap support in the MP tree: The mouse and guinea pig clusters each received 100% support, the rat cluster 99%, and the rabbit cluster 81%. As with the NJ tree, the human genes did not form a strongly supported cluster (data not shown).

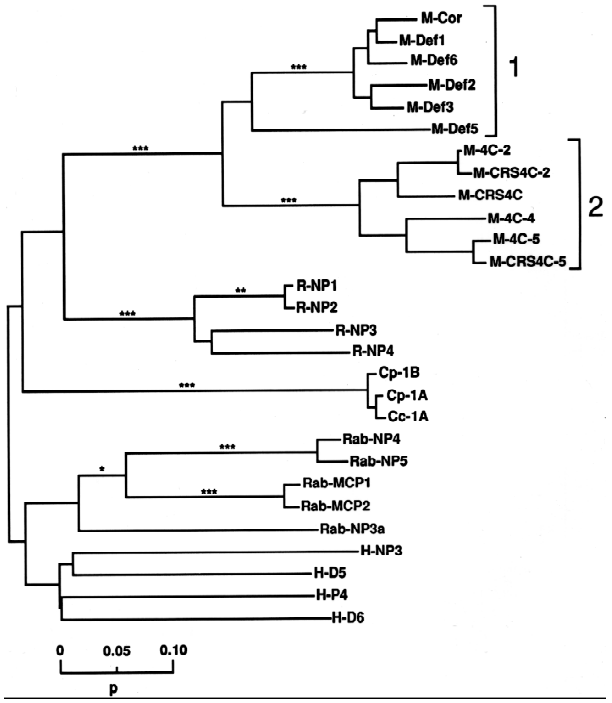

Fig. 2. NJ tree based on proportion difference (*p*) at 76 aligned amino acid sites of mammalian defensins. Tests of significance of internal branches: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Prefixes of sequence symbols indicate the species as follows: *Cc—Cavia cutleri; Cp—Cavia porcellus; H*—human; *M*—mouse; *R*—rat; *Rab*—rabbit.

Pattern of Nucleotide Substitution

In order to examine the extent of constraint at the amino acid level in different regions of the molecule, we computed d_S and d_N for all pairwise comparisons within five gene families (mouse 1 and 2, rat, guinea pig, and rabbit; Table 1). The human genes were not included in this analysis because d_S was undefined in some comparisons among them (data not shown). In the case of all five families, d_N showed a consistent pattern of being highest in the mature defensin, intermediate in the propiece and lowest in the signal peptide, a trend that was highly significant by a Friedman Rank Sum analysis of variance (Table 1). By contrast, d_S showed no consistent pattern of difference among the three regions (Table 1). These results indicate that the mature defensin is the least conserved region at the amino acid level, while the signal peptide is the most conserved.

In some individual comparisons d_N in the mature defensin was substantially higher than d_S for the same comparison. This is a highly unusual pattern of nucleotide substitution, since in most genes d_S exceeds d_N (Nei 1987, pp 79–83). Because $d_S > d_N$ is predicted by the neutral theory of molecular evolution (Kimura 1977), $d_N > d_S$ is evidence of positive Darwinian selection acting to promote diversity at the amino acid level (Hughes and Nei 1988). For example, mean d_N exceeds mean d_S for comparisons between *M-def5* and other mouse family 1 genes (Table 1).

Table 1. Mean numbers of synonymous (d_S) and nonsynonymous (d_N) nucleotide substitutions per 100 sites (± S.E.) in within-species comparisons of mammalian defensins[a]

	Signal peptide		Propiece		Mature defensin	
	d_S	d_N	d_S	d_N	d_S	d_N
Mouse 1: *M-def5* vs others	15.9 ± 8.3	5.6 ± 2.4	18.9 ± 6.3	16.7 ± 4.2	13.0 ± 5.4	31.4 ± 7.1*
All	21.0 ± 8.3	6.0 ± 2.5	15.9 ± 5.3	7.1 ± 1.7	10.5 ± 5.0	13.3 ± 3.0
Mouse 2	33.7 ± 14.2	2.9 ± 2.0*	15.5 ± 5.0	7.8 ± 2.1	32.2 ± 10.3	14.3 ± 3.5
Rat	9.4 ± 6.1	3.0 ± 2.2	9.1 ± 4.6	9.7 ± 2.6	19.8 ± 7.4	21.4 ± 4.6
Guinea pig	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.7 ± 0.7	2.8 ± 2.8	2.0 ± 1.4
Rabbit	5.2 ± 3.8	4.7 ± 2.4	47.6 ± 12.5	25.4 ± 4.3	31.5 ± 9.3	38.8 ± 6.5

[a] Sequence families are as in Fig. 2. Tests of the hypothesis that $d_S = d_N$: * $P < 0.05$. The hypothesis of a difference in median d_S and d_N among regions was tested by the Friedman Rank-Sum Test (a nonparametric analysis of variance for blocked data; Hollander and Wolfe 1973). For d_S, S = 0.7 n.s.; for d_N, S = 10.0 ($P = 0.001$)

When d_N was plotted against d_S for all within-family comparisons for the five families of genes analyzed in Table 1, the pattern in the mature defensin was strikingly different from that seen in the two other regions (Fig. 3). (Note that between-family comparisons were not included because in many of these d_S was undefined; data not shown.) In the signal peptide (Fig. 3A), d_S exceeded d_N in every comparison; and in the propiece d_S exceeded d_N for most comparisons (Fig. 3B). By contrast, in the mature defensin, d_N often exceeded d_S, sometimes greatly so, especially in comparisons where d_S was low (Fig. 3C). Because synonymous substitutions, being selectively neutral or nearly so, should accumulate more or less regularly with time, such a pattern of substitution suggests that, in the defensin gene family, gene duplication has often been followed by a rapid burst of nonsynonymous substitutions and that the rate of nonsynonymous substitution subsequently slows down.

In the case of the rabbit defensin genes, d_S and d_N in both the propiece and the mature defensin were significantly greater ($P < 0.01$) than the corresponding values in the signal peptide; indeed, d_S and d_N in other regions were five to nine times as high as the corresponding values for the signal peptide (Table 1). No such pattern was seen in the genes from other species (Table 1). This observation suggests that in the rabbit the signal peptide region may have been homogenized among loci by past interlocus recombination events.

When conservative and radical nonsynonymous differences were compared among rodent defensin gene families, p_{NR} was found to be significantly higher than p_{NC} in the mature defensin in several comparisons (Table 2). No such pattern was seen in the signal peptide or propiece (data not shown). This suggests that over long periods of evolutionary time, one of the ways that mammalian defensins have diversified is to alter the pattern of residue charges in the mature defensin region.

Reconstruction of Ancestral Sequences

Michaelson et al. (1992) plotted net charge in the propiece against that in the mature defensin in the case of seven mammalian defensins; we repeated this type of analysis for our sample of 28 defensins (Fig. 4A). A significant negative correlation was observed between net charge in the propiece and net charge in the mature defensin (Fig. 4A). Moreover, as shown in Fig. 4A, the negative charge in the propiece usually balanced the positive charge in the mature defensin so that the net charge of the two together was slightly negative. In four cases the negative charge in the propiece exactly balanced the positive charge in the mature defensin so that the net charge was neutral; and in one case the net charge was slightly positive (Fig. 4A).

In order to test whether this balancing of charges in propiece and mature defensin has occurred throughout the evolutionary history of mammalian defensins, we reconstructed ancestral sequences using the method of Yang et al. (1995). This method is based on a phylogenetic tree, and the reliability of the method is likely to improve if the phylogeny used is reliable. For this reason, we used the phylogeny based on 23 sequences illustrated in Fig. 5. Because the relationships among human defensins and between human and other mammalian defensins were unstable in both NJ (Fig. 2) and MP trees, we did not include human sequences. Because this method does not consider insertion/deletion events, we also excluded R-NP3, which has a large deletion in the propiece (Fig. 1).

In Fig. 4B, the net charge of the propiece is plotted against that of the mature defensin for the 22 reconstructed internal nodes of the phylogeny. There was a significant negative linear correlation between net charge values for the two regions, as for the 28 extant sequences (Fig. 4A,B). The net charge of the propiece and the mature defensin together was zero or less in the case of all reconstructed sequences (Fig. 4B).

There were 36 internal branches in the phylogeny of Fig. 5 at which the reconstruction hypothesized at least one amino acid replacement to have taken place. We compared numbers of amino acid changes in these branches pairwise within branches because the branches in the tree varied considerably in length (Fig. 6). There

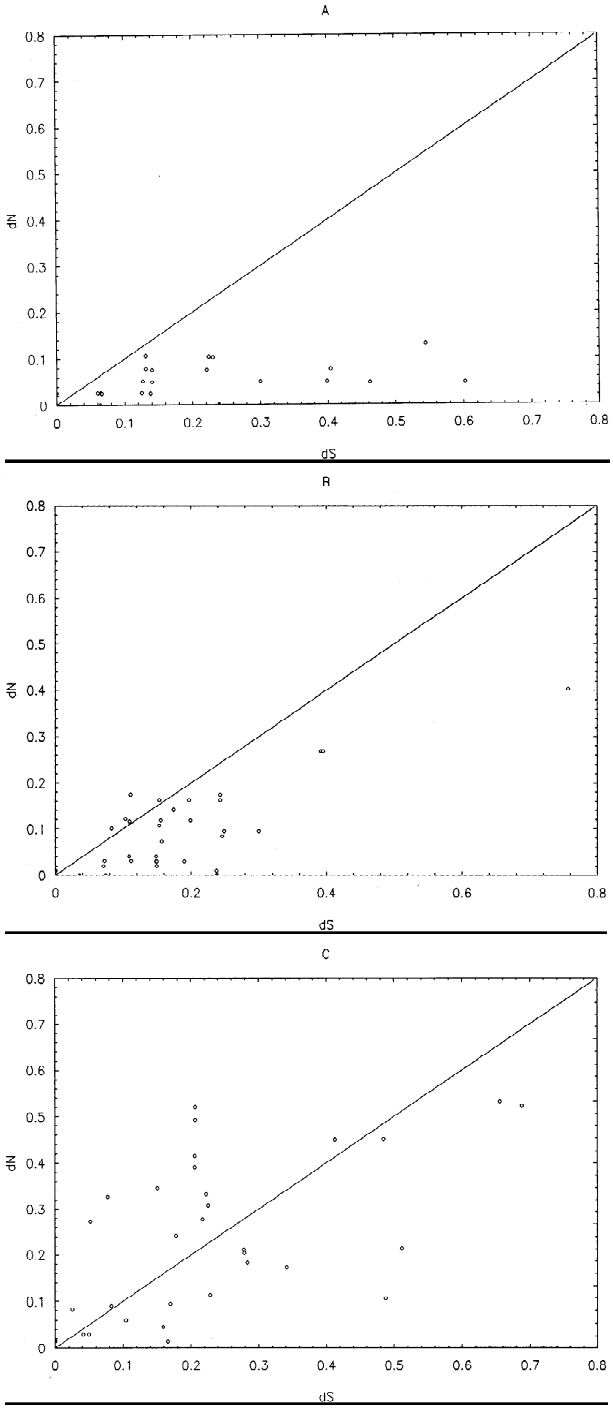

Fig. 3. Plots of numbers of nonsynonymous substitutions per site (d_N) vs number of synonymous substitutions per site (d_S) in within-family comparisons of different regions of mouse, rat guinea pig, and rabbit defensin genes: **A** signal peptide; **B** propiece; **C** mature defensin. In each case, the line is a 45° line ($Y = X$).

were significantly more changes in the propiece than in the mature defensin involving no charge change, involving the subtraction of a negative charge, and involving the addition of a negative charge (Fig. 6). Indeed, no change involving the addition of a negative charge was hypothesized to occur at any branch in the mature defensin (Fig. 6). Changes involving addition of a positive

Table 2. Mean numbers of conservative (p_{NC}) and radical (p_{NR}) nonsynonymous substitutions per 100 sites (± S.E.) in comparisons of mature defensin regions between families of rodent defensin genes[a]

		p_{NC}	p_{NR}
Mouse 1	vs mouse 1	30.9 ± 7.3	17.4 ± 6.7
	vs mouse 2	55.0 ± 8.5	68.8 ± 10.2
	vs rat	49.6 ± 7.7	44.6 ± 9.4
	vs guinea pig	41.6 ± 7.6	66.8 ± 9.7*
Mouse 2	vs mouse 2	7.6 ± 2.8	22.6 ± 6.1*
	vs rat	50.2 ± 8.2	82.3 ± 11.1*
	vs guinea pig	57.7 ± 8.2	69.3 ± 11.8
Rat	vs rat	15.7 ± 4.1	22.1 ± 6.6
	vs guinea pig	39.9 ± 7.1	55.3 ± 10.3
Guinea pig	vs guinea pig	1.4 ± 1.4	3.0 ± 3.0

[a] Sequence families are as in Fig. 2. Tests of the hypothesis that $p_{NC} = p_{NR}$: * $P < 0.05$

charge occurred at about equal rates in the propiece and in the mature defensin, while subtraction of a positive charge occurred much more frequently in the mature defensin than in the propiece (Fig. 6). Thus, the regulation of the net charge of both the mature defensin and the propiece over evolutionary time seems to have occurred mainly by addition and subtraction of negatively charged residues in the propiece, by addition of positively charged residues in the propiece, and by addition and subtraction of positively charged residues in the mature defensin. For all 36 branches, when the net charge change in the propiece was plotted against the net charge change in the mature defensin, a significant negative linear correlation was found (Fig. 4C). Thus, when the mature defensin became more positive in charge, the propiece either showed no change in charge or actually became more negative, whereas when the mature defensin became less positive in charge, the propiece tended to become less negative.

It is of interest to examine the pattern of residue change reconstructed for particular branches, particularly those at which patterns of nucleotide difference suggested that nonsynonymous differences occurred in a nonrandom way so as to favor charge changes (Table 2). One such branch was the branch leading to the mouse 2 family (labeled 1 in Fig. 5). According to our reconstruction, on this branch in the mature defensin there were 10 amino acid changes involving charge change in the mature defensin: six changes involving subtraction of a positive charge, three involving addition of a positive charge, and one involving subtraction of a negative charge. The net charge change in the mature defensin thus changed from +8 to +6. In the same branch, there was one change in the propiece involving subtraction of a negative charge, balanced by one subtraction of a positive charge; the net charge in the propiece remained at −11. Thus, in spite of the extraordinary number of charge changes in the mature defensin, the net charge of mature defensin plus propiece remained negative. Addition of

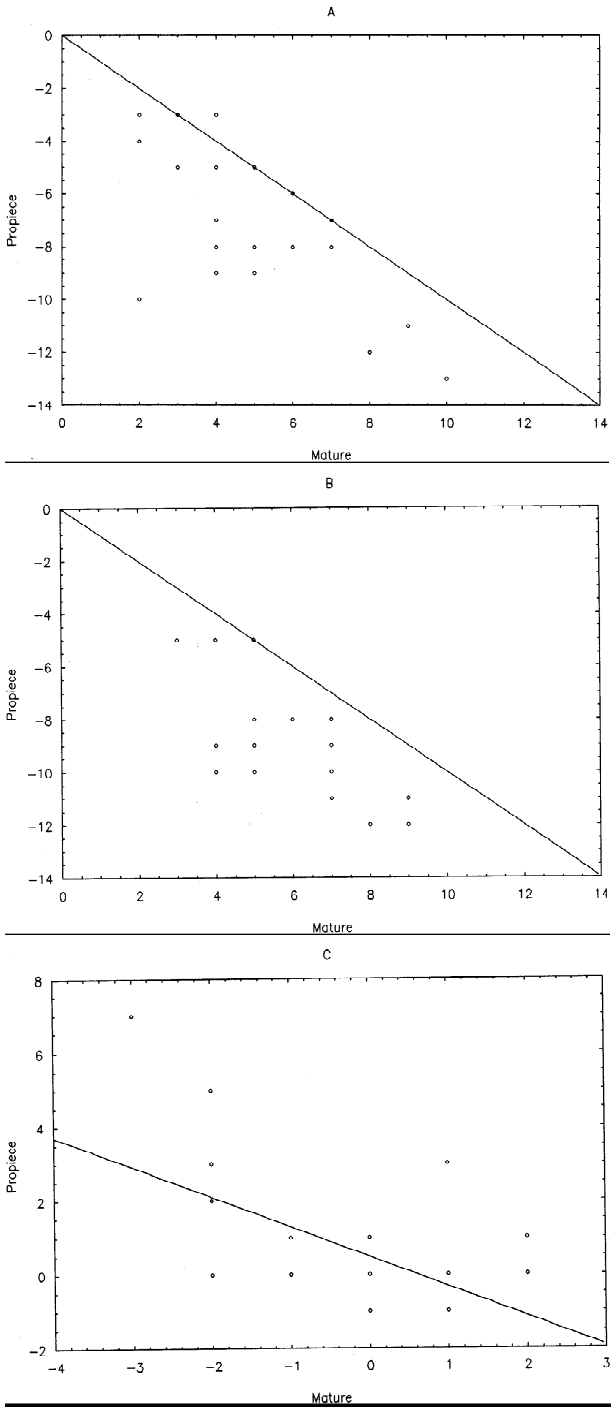

Fig. 4. **A** Net charge in the propiece vs that in the mature defensin for 28 mammalian defensins ($r = -0.742$; $P < 0.001$). The line is a 45° line ($Y = X$). **B** Net charge in the propiece vs that in the mature defensin for 22 reconstructed ancestral sequences corresponding to the nodes of Fig. 5 ($r = -0.755$; $P < 0.001$). The line is a 45° line ($Y = X$). **C** Charge change in the propiece vs that in the mature defensin, as reconstructed to have occurred in 36 branches of the phylogeny of Fig. 5 ($r = -0.548$; $P < 0.001$). The line is the linear regression line $Y = 0.486 - 0.808X$).

three positively charged residues in the mature defensin and subtraction of one negatively charged residue were more than compensated by subtraction of positive charges from the same region.

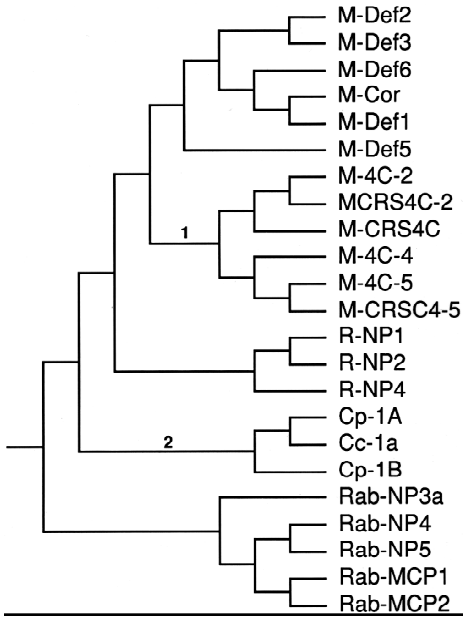

Fig. 5. Phylogenetic tree used for reconstruction of ancestral sequences. *Prefixes* indicating species are as in Fig. 2.

In the branch leading to the guinea pig genes (labeled 2 in Fig. 5), there were nine charge changes in the mature defensin: In six cases a positive charge was subtracted, while in two cases a positive charge was added and in one case a negative charge was subtracted. The charge of the mature defensin changed from +6 to +3. In the same branch, there were 13 charge changes in the propiece: In six cases a positive charge was added, in four cases a negative charge was subtracted, in one case a positive charge was subtracted, and in two cases a negative charge was added. The net charge of the propiece changed from −11 to −4. Thus, in this branch, the mature defensin became less cationic, while the propiece became correspondingly less anionic.

Discussion

When certain closely related defensin genes were compared, the rate of nonsynonymous nucleotide substitution substantially exceeded the synonymous rate in the mature defensin region (Fig. 3C). In more distant comparisons, however, the synonymous rate exceeded the nonsynonymous rate (Fig. 3C). In other gene regions, the synonymous rate was higher in most comparisons (Fig. 3A,B). The simplest explanation for these observations is that, after gene duplication, certain pairs of newly duplicated mammalian defensin genes have been subject to positive Darwinian selection favoring diversification of the mature defensin at the amino acid level. A similar pattern of nucleotide substitution has been observed in certain other multigene families, including serine protease inhibitors (Hill and Hastie 1987) and immunoglobulin V region genes (Tanaka and Nei 1989).

It is not known what factors might favor changes in the amino acid sequence of mammalian defensins. One

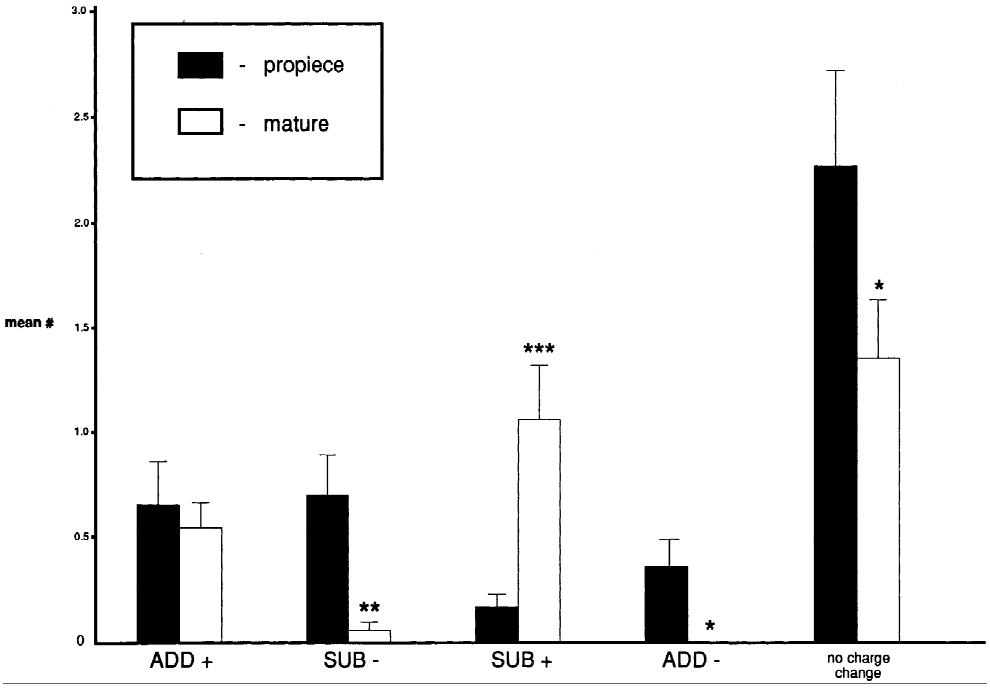

Fig. 6. Mean numbers (with S.E.) of amino changes in 36 branches of the tree in Fig. 5 reconstructed by the method of Yang et al. (1995); only branches in which at least one amino acid change was hypothesized to have occurred were included. Changes were categorized as those involving addition (ADD+) or subtraction (SUB+) of a positively charged residue; those involving addition (ADD−) or subtraction (SUB−) of a negatively charged residue; and those involving no charge change. For each category, the hypothesis that the number of changes occurring in the propiece equaled that in the mature defensin was tested by paired sample *t*-test: ** $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

possibility is that defensins have diversified in response to changes in the microbial species to which a given host is exposed. The fact that mammalian defensins have diversified in a species-specific manner is consistent with this hypothesis, since in adapting to a new niche each species is likely to encounter a new microbial community.

Gene duplication is believed to be necessary for the evolution of new gene function (Li 1982), but the mechanism whereby new gene functions arise remains unknown. The most widely cited hypothesis is that, after gene duplication, one gene copy is redundant and thus free to accumulate mutations at random; by chance such mutations may endow this gene with a new function (Onho 1973). In fact, however, several lines of evidence contradict this hypothesis (Hughes 1994). One of these is the evidence that in several cases gene duplication is known to have been followed not by random substitution in one gene but by a burst of positively selected amino acid changes leading to functional differentiation of the two daughter genes (Hughes 1994). Because the mammalian defensins seem to have evolved according to the latter model, the present case adds to the evidence against the hypothesis that functionally novel genes arise from nonfunctional, redundant gene copies.

In the case of the mammalian defensins, it has been hypothesized that the net positive charge of the mature defensin is balanced by a net negative charge in the propiece, and that this balance of charges is an adaptation to prevent cytotoxic effects prior to cleavage of the propiece (Michaelson et al. 1992). Our reconstruction of ancestral defensin sequences allowed us to examine the pattern of change in the mature defensin and in the propiece over evolutionary time. The results suggest that, in spite of some dramatic changes in the pattern of residue charges in the mature defensin over evolutionary time, coordinate changes have occurred in the propiece and/or in the mature defensin itself so as to keep the net positive charge of the mature defensin low enough so that it is more or less balanced by the negative charge of the propiece. Furthermore, because when the mature defensin becomes less positive, the propiece tends to become less negative (Fig. 4C), these results suggest that it may be deleterious if the net charge of the propiece plus the mature defensin is strongly negative as well as if it is strongly positive.

Like any method of reconstruction of ancestral states, the method used here is subject to some error. However, because the method is based on an empirically derived matrix of amino acid changes among closely related, conserved proteins, it is unlikely to overestimate the frequency of radical amino acid changes, such as those involving a change of residue charge. Thus the reconstructed pattern of a continued balance between the cationic character of the mature defensin and the anionic character of the propiece is likely to refect the actual course of evolution.

Wright (1932) posed as a central problem of evolution the question of how species are able move among multiple adaptive ‘‘peaks’’ in a hyperspace in which fitness is a function of gene combination. In the case of mammalian defensins, this problem might be expressed: How is the transition made from one pattern of residue charges in the mature defensin to another—a process that may be driven by selective pressure arising from the composition of the microbial community to which the species is exposed—while maintaining intact the balance between charges in the propiece and in the mature defensin. Our results suggest that, at least as regards charge balance, mammals have been able to move from one defensin ‘‘peak’’ to another while avoiding adaptive ‘‘valleys’’ in which the charges in these two regions would be strongly unbalanced.

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