transcription of a digestion-specific gene battery in the foregut. As additional genes were co-opted as target genes of FoxA, novel patterns of gene expression would have been superimposed on the developing structure, enabling new cell types or morphologies to emerge.

The concept of gene co-option has been suggested as a major mechanism of evolutionary change at the gene network level[12]. This mechanism also offers a solution to the paradox of finding similar use of homologous genes in functionally analogous, but morphologically dissimilar, body parts. In other words, continuing with the case of the evolution of the foregut as an instructive example, the mechanism of co-option explains how homologous FoxA genes might control the ontogeny of the foreguts in phylogenetically distant organisms with different, non-homologous, digestive tracts. It is therefore reasonable to predict that more controversial evolutionary cases will be clarified as additional developmental gene regulatory networks are dissected. □

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Evaluating adaptive evolution

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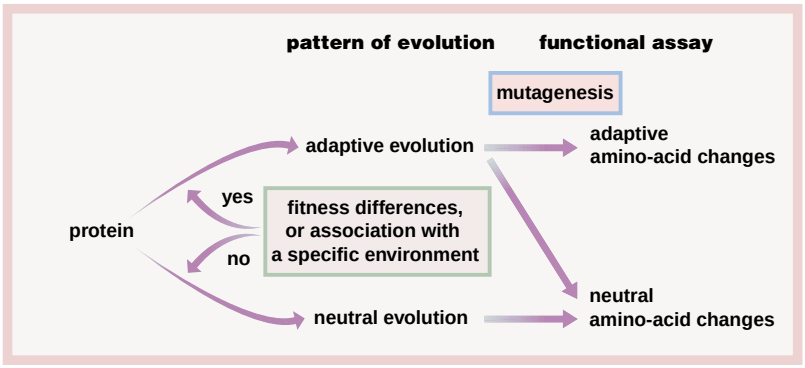
Using comparative sequence analyses, we can identify proteins that may have been subject to positive darwinian selection. To test these statistical results, it is important to develop functional assays and identify amino-acid changes that are responsible for the adaptation of organisms to specific environments. One of the two duplicated pancreatic ribonuclease genes of a leaf-eating colobine monkey, douc langur, is now shown to have adapted to digest bacterial RNAs in the monkey's foregut.

Organisms encounter a diverse array of habitats, from the Himalayas to the deep sea, and adapt to these environments with an equally diverse array of structures and functions. One of the major goals of evolutionary biology is to elucidate mechanisms that drive these adaptive changes at the DNA, protein and functional levels.

In vertebrates, adaptive evolution has been extremely difficult to study, owing to the scarcity of genetic systems in which the functional effects of mutations can be evaluated experimentally. In principle, we can pick proteins with polymorphic amino acids and evaluate selective differences among them. In practice, however, the magnitudes of selective differences are generally so small that it is almost impossible to detect any significant differences in fitness[1]. Alternatively, adaptive evolution is strongly suggested by one type of protein having a strong association with a specific environment. Once a protein is suspected to be under positive darwinian selection, we can identify potentially important amino-acid replacements that may be responsible for adaptation. These hypotheses can then be tested experimentally (see figure). On page 411 of this issue, Jianzhi Zhang and colleagues[2] report such a functional assay and present strong evidence of the adaptive evolution of a duplicate pancreatic ribonuclease gene in the leaf-eating colobine monkey, the douc langur.



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Putting adaptive evolution to the test. Proteins may be classified either as adaptive or as neutral (or nearly neutral), excluding those containing deleterious mutations. Adaptive evolution may be established either by demonstrating fitness differences among proteins with polymorphic amino acids, or by establishing a strong association between one type of protein and a specific environment. Once a protein is suspected to have experienced positive darwinian selection, we need first to identify potential adaptive amino-acid replacements and then to verify their importance by mutagenesis. Using such engineered proteins, amino-acid replacements of a protein under positive darwinian selection can be classified into two groups: amino-acid replacements that cause adaptation and those that do not. Amino-acid replacements in a protein under neutral evolution are not involved in adaptation.

Langur ribonucleases

Douc langurs consume leaves as their primary food source. The pancreatic ribonuclease is necessary for digesting large amounts of RNA derived from the symbiotic microflora of the foregut of these monkeys. The douc langur has two pancreatic ribonucleases, RNASE1 and RNASE1B, the result of a recent duplication[2]. During the divergence of these enzymes, all nine amino-acid replacements detected can be traced exclusively to RNASE1B. RNASE1 and RNASE1B digest yeast tRNAs at the optimal catalytic pH of 7.4 and 6.3, respectively[2]. At pH 6.3, the 'digestive environment' of the small intestine of colobine monkeys, RNASE1B is about six times as active as RNASE1 (ref. 2).

Zhang *et al.*[2] have also evaluated the activity of the enzymes in degrading double-stranded RNA, which presumably occurs outside the digestive system. They find that the enzymatic activity of RNASE1B is over 300-fold lower than that of RNASE1. When the nine amino-acid changes are introduced into RNASE1 individually, the greatest reduction in enzymatic activity is effected the most by Ala32Leu and Asp83Gly. As RNASE1 is expressed in many other tissues in addition to the pancreas, and has an optimal catalytic pH higher than that in the foregut, it is probable that RNASE1 functions outside the digestive system. These observations strongly suggest that RNASE1B is adapted for enhanced RNASE activity in the relatively low pH environment of the colobine foregut[2]. Two amino-acid changes in RNASE1 reduced its activity against double-stranded RNAs. It is not known which of the nine amino acid changes are responsible for the functional specialization of RNASE1B in the 'digestive environment'. This critical question remains to be answered, using the ribonuclease assay against yeast tRNAs.

Two modes

By showing that the number of nucleotide substitutions that change amino acids exceeds that of silent nucleotide substitutions, many proteins are suggested to have undergone adaptive evolution[3]. In particular, major histocompatibility loci in vertebrates and self-incompatibility loci in plants show high levels of allelic diversity and long persistence of allelic lineages[3]. As it is advantageous to have a larger number of alleles at these loci, it is reasonable to invoke some type of 'balancing selection' for maintaining the amino-acid polymorphisms. Adaptation of organisms to a specific environment, however, is based more on 'directional selection' with amino-acid replacements than on 'balancing selection'.

To identify amino-acid replacements that are responsible for adaptive evolution, appropriate mutant proteins need to be constructed and their possible functional changes must be tested. Using such engineered proteins, the molecular bases of adaptive evolution have been demonstrated for two groups of proteins in vertebrates. The bar-headed goose (*Anser indicus*) lives above 4,000 meters in the Himalayas and migrates over Mount Everest, whereas the Andean goose (*Chloephaga melanoptera*) lives at 6,000 meters in the Andes. For these birds in the rarefied atmosphere, the high oxygen affinity of their hemoglobins should have a clear selective advantage. Based on the interface structure of α- and β-hemoglobins, it was suspected that amino-acid replacements Pro119Ala in the α-hemoglobin of the bar-headed goose[4] and Leu55Ser in the β-hemoglobin of the Andean goose[5] might have been responsible for their high affinities for oxygen. Indeed, when the Pro119Ala and Met55Ser substitutions are introduced into the human α- and β-hemoglobins, respectively, both mutants have higher affinity for oxygen[5].

The second example comes from another extreme environment. The coelacanth (*Latimeria chalumnae*) lives near the coast of the Comoros Islands in the Indian Ocean. Living at a depth of approximately 200 meters, this fish receives only a narrow range of light, at approximately 480 nanometers, and uses two photo-sensitive rhodopsin molecules, RH1 and RH2, with optimum light sensitivities of 485 and 478 nanometers, respectively[6]. Compared with their ancestral pigments, these two pigments have shifted their light sensitivities by approximately 15–20 nanometers toward blue, and together detect the entire range of color available to the coelacanth. Mutagenesis experiments show that the co-adaptation of RH1 and RH2 pigments has been achieved by a total of four amino-acid replacements[6].

The future of adaptive evolution

The avian hemoglobin, coelacanth vision and langur ribonucleases show that adaptation of proteins can occur by amino-acid replacements at a small number of critical sites. Golding and Dean[7] have also reviewed several examples of major functional shifts that are caused by just a few amino-acid replacements. Similarly, the evolution of avian ultraviolet vision[8], and that of red-green color vision[9], is due to fewer than five amino-acid replacements. It is interesting to see that, even when proteins are subject to adaptive evolution, a majority of mutants can evolve under neutral evolution (see figure)[10]. All of these analyses are based on information derived from protein phylogeny, structure and engineering, which together provide much deeper insight into the structure–function relationship of evolving proteins than was previously possible. To understand better the molecular basis of adaptive evolution in general, it is essential to establish functional assays for as many genetic systems as possible. □

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