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Adaptive protein evolution at the *Adh* locus in *Drosophila*

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PROTEINS often differ in amino-acid sequence across species. This difference has evolved by the accumulation of neutral mutations by random drift, the fixation of adaptive mutations by selection, or a mixture of the two. Here we propose a simple statistical test of the neutral protein evolution hypothesis based on a comparison of the number of amino-acid replacement substitutions to synonymous substitutions in the coding region of a locus. If the observed substitutions are neutral, the ratio of replacement to synonymous fixed differences between species should be the same as the ratio of replacement to synonymous polymorphisms within species. DNA sequence data on the *Adh* locus (encoding alcohol dehydrogenase, EC 1.1.1.1) in three species in the *Drosophila melanogaster* species subgroup do not fit this expectation; instead, there are more fixed replacement differences between species than expected. We suggest that these excess replacement substitutions result from adaptive fixation of selectively advantageous mutations.

Consider a set of alleles from more than one species and assume that there is no recombination. The alleles are then connected by a single phylogenetic tree, which can be divided into two parts: between-species branches and within-species branches. Within-species branches connect all the alleles within each species to their most recent common ancestor. Between-species branches connect these common ancestors to the common ancestor of the whole phylogeny. A mutation on a between-species branch will appear in all the descendant alleles and thus will be a fixed difference between species, whereas a mutation on a within-species branch will be a polymorphism within a species.

Nucleotide substitutions in a coding region can also be divided into replacement substitutions and synonymous substitutions. Of M possible mutations in a coding region, let M_r be the number of possible neutral replacement mutations and M_s be the number of possible neutral synonymous mutations. All remaining mutations, $M - (M_r + M_s)$, are deleterious. Let μ be the nucleotide mutation rate per nucleotide site, so that the mutation rate for any one of the three possible mutations at a site is $\mu/3$. Under the neutral theory, the expected number of fixed replacement substitutions in a set of alleles is $T_b(\mu/3)M_r$, where T_b is the total time on the between-species branches. The expected number of fixed synonymous substitutions is

$T_b(\mu/3)M_s$. For a particular phylogeny and mutation rate, the number of replacement substitutions is independent of the number of synonymous substitutions. Therefore, the expected ratio of replacement to synonymous fixed substitutions is $T_b(\mu/3)M_r : T_b(\mu/3)M_s$, which reduces to $M_r : M_s$. If T_w is the total time on the within-species branches of the phylogeny, the expected ratio of replacement to synonymous polymorphisms is $T_w(\mu/3)M_r : T_w(\mu/3)M_s$, which also reduces to $M_r : M_s$. Thus, if protein evolution occurs by neutral processes, the ratio of replacement to synonymous fixed substitutions should be the same as the ratio of replacement to synonymous polymorphisms. A G -test of independence¹ can be used to test this null hypothesis.

This test does not require many assumptions. Unlike most tests of the neutral theory, it does not require that populations have reached equilibrium. Recombination could cause different segments of a locus to have different phylogenies, but this would only cause a serious bias in the above test if the time to a common ancestor of a genomic segment was correlated with the ratio of neutral replacement to synonymous sites in that segment. There is no reason to expect such a correlation. Similarly, variation in nucleotide mutation rate among regions of a locus would only be important if nucleotide mutation rate was correlated with the ratio of neutral replacement to synonymous sites, which does not seem likely. We have assumed, for simplicity, that all nucleotide mutations are equally likely. A difference in mutation rate between transitions and transversions, coupled with the greater proportion of transitions that are synonymous, would change the ratio of replacement to synonymous substitutions. But the ratio for fixed substitutions would still equal the ratio for polymorphisms. If there are more synonymous sites than replacement sites with two or three possible neutral mutations, multiple substitutions at a single nucleotide site will make the ratio of replacement to synonymous fixed differences a slight overestimate of $M_r : M_s$. For closely related species the effect is trivially small and even in the most extreme case of complete saturation of neutral mutable sites with substitutions, the overestimation from multiple substitutions is not large enough to account for the results presented below.

We have compared DNA sequences of the coding region of the *Adh* locus in three species in the *Drosophila melanogaster* species subgroup (Table 1). There is substantial evidence that the threonine/lysine polymorphism in the alcohol dehydrogenase of *D. melanogaster* is subject to natural selection^{2,3}, but there has been no evidence to indicate whether any of the replacement differences between species were adaptive. We limited the comparison to closely related species to minimize the number of multiple mutations at single nucleotide sites. The ratio of replacement substitutions to synonymous substitutions that are fixed between species is significantly greater than the ratio of replacement to synonymous polymorphisms (Table 2). About 29% of the fixed differences between species are replacement substitutions, but only 5% of the polymorphisms are replacement substitutions. This is not what would be expected if the observed replacement and synonymous substitutions were selectively neutral. It would be expected, however, if most of the observed replacement substitutions were due to adaptive fixation of selectively advantageous mutations. A substitution which becomes fixed by selection will be a polymorphism for less time than a substitution that becomes fixed by random drift⁴ and so an adaptive substitution will be less likely to appear as a polymorphism than will a neutral substitution.

An alternative explanation for the excess number of fixed replacement differences could be a combination of many slightly deleterious replacement mutations and population sizes which have recently undergone dramatic expansion in all three species. In the ancestral small populations, mildly deleterious replacement mutations are effectively neutral and would therefore accumulate as fixed differences. In recently expanded populations, slightly deleterious replacements are selected against and

TABLE 2 Number of replacement and synonymous substitutions for fixed differences between species and polymorphisms within species

	Fixed	Polymorphic
Replacement	7	2
Synonymous	17	42

A G-test of independence (with the Williams correction for continuity)¹ was used to test the null hypothesis, that the proportion of replacement substitutions is independent of whether the substitutions are fixed or polymorphic. $G=7.43$, $P=0.006$.

More realistic models of selection suggest that adaptation could occur much more quickly^{8,9}, but if we accept the conservative figure and assume there are 10 generations per year in *Drosophila*, it would mean that 50,000 loci could be undergoing adaptive evolution as quickly as *Adh*. Thus, the rate of adaptive protein evolution seen at the *Adh* locus may not be unusually

high, and selective fixation of adaptive mutations may be a viable alternative to the clocklike accumulation of neutral mutations as an explanation for most protein evolution. □

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Development of the light response in neonatal mammalian rods

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THE sensitivity to light is low in many neonatal mammals when compared with that in the adult. In human infants at one month of age, for example, the dark-adapted sensitivity for detection of large stimuli is 50 times lower than in the adult^{1–4}, and in rats the overall sensitivity of the neonatal retina is also low compared with the adult^{5,6}. This low sensitivity in the neonate has been attributed to a number of factors^{5–11}, but the possibility that the photoreceptors themselves might be an important limitation on the overall visual sensitivity has not so far been clearly established. Here we record the light response of single neonatal rat rods and find that the sensitivity is considerably lower than in the adult. The response to a single photoisomerization is normal in the neonate, and the sensitivity deficit can therefore be attributed to a low level of functional rhodopsin. Opsin, the protein component of rhodopsin, must be present in normal amounts, as the sensitivity can be restored to adult levels by treating the retina with 9-*cis* retinal, an active homologue of the native chromophore 11-*cis* retinal. The low sensitivity of photoreceptors in the neonate can therefore be attributed mainly to a low concentration of 11-*cis* retinal in the developing retina.

Rod outer segments begin to appear around the optic disk at about postnatal day 8 (P8) in the rat, and they grow rapidly towards the adult size over the next three weeks¹². We have recorded the membrane current of single rod outer segments, using the suction pipette method¹³, from animals ranging in age from P12 (the earliest time at which any response to light has been detected) to adult (age greater than two months). Figure 1 compares typical recordings obtained from rods from a one-month-old animal and a 13-day-old animal. There are two principal respects in which the responses from neonatal rods differ from those in more mature animals: the light-sensitive current is smaller, and the sensitivity to light is less by more than an

order of magnitude. As a qualitative illustration of the second point, the flash strength giving the smallest response in the P13 rod shown in Fig. 1*b* is the same as that giving the second-largest response in the P35 rod in Fig. 1*a*.

The intensity–response relations for rods from albino rats of various ages are shown in Fig. 2. In each case the points have been fitted by an exponential function¹⁴ (see legend to Fig. 2), which provides a good fit to the intensity–response relation of other mammalian rods¹⁵. The sensitivity is inversely proportional to the light intensity required to produce a half-saturating response. Only a small fraction of rods at P13 responded to light and, of those that did, the sensitivity was on average 49 times less than in the adult. Rods from 17-day-old animals are seven times less sensitive than those from adults, and at P35, when the animals have been weaned and the outer segments are almost of adult size, the sensitivity is still three times less than in the adult. Similar results were obtained from pigmented rats (see Fig. 2 legend).

There are several mechanisms which might account for the low sensitivity of the neonate. Some components of the light-sensitive pathway may not be fully expressed, resulting in a reduced response to a single photoisomerization. A second possibility is that fewer photons of light may be absorbed in the neonatal outer segments, either because of a delay in expression of the gene coding for opsin, or because an insufficient supply of the light-sensitive chromophore 11-*cis* retinal is available. The outer segment length at P13 is one half of adult (see Fig. 2 legend), but this difference does not account for the low sensitivity (see Fig. 3 legend). The possibility that the action spectrum of neonatal rhodopsin might be different can also be discarded¹⁶.

The response to a single photoisomerization has been determined in the experiments shown in Fig. 3 by observing the responses to a series of dim flashes^{15,17}. The single-photon response in this adult (Fig. 3, top) was 0.2 pA, or 4.2% of the maximum response, whereas in the P13 rod (Fig. 3, bottom), which was 56 times less sensitive, the single photon response was 0.42 pA, or 21% of the saturating response. In four experiments on animals aged between P13 and P17 the mean single-photon response was 0.58 pA, and in seven experiments on animals P30 or older, the mean response was 0.50 pA (for further details see legend to Fig. 3). The gain of the light-sensitive pathway is therefore not significantly different in the neonate, and the low sensitivity must be due to a reduced absorption of light in the immature outer segments.

If there is a delay in the expression of the gene coding for opsin, then the absorption of light should be localized to the

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