**In Vivo Introduction of Unpreferred Synonymous Codons Into the Drosophila Adh Gene Results in Reduced Levels of ADH Protein**

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ABSTRACT

The evolution of codon bias, the unequal usage of synonymous codons, is thought to be due to natural selection for the use of preferred codons that match the most abundant species of isoaccepting tRNA, resulting in increased translational efficiency and accuracy. The codon bias model predicts that unpreferred codons would increase proofreading costs and would also be predicted to result in a net decrease in the protein levels.

Under natural selection, the fate of any given mutation depends on the product of the effective population size and selection coefficient, $N_s$ (Kimura 1983). Codon bias results from the dual action of directional selection for preferred codons ($N_s > 0$) and purifying selection against unpreferred codons ($N_s < 0$). Since selection for codon bias is thought to be relatively weak (e.g., in comparison with adaptive substitutions at the amino acid level), the selection-mutation-drift (SMD) model of codon bias predicts that unpreferred codons will persist as a consequence of mutation pressure and genetic drift (Li 1987; Bulmer 1991). Current population genetics theory predicts that $N_s$ for any codon change in Drosophila melanogaster is not significantly different from 0 (Akashi 1995, 1996; McVean and Vieira 2001). In contrast, for D. simulans the estimate is $1.3 < |N_s| < 3.6$ (Akashi 1995). Although D. melanogaster shows less nucleotide diversity than D. simulans, the threefold difference in $N_e$ (Powell 1997) is not large enough to account for the difference in $N_s$ between the two species. This suggests a decrease of $s$ in the recent past (Akashi 1995, 1996; McVean and Vieira 2001).

Thus, the analysis of patterns of molecular evolution using population genetics theory suggests that the fit-
ness effect of an individual synonymous mutation from a preferred codon to an unpreferred codon is likely to be very small, perhaps immeasurable in the lab. However, although \( N_s \) for any codon change in *D. melanogaster* is not statistically different from 0 (Akashi 1995, 1996; McVean and Vieira 2001), each codon family is likely to have a unique selection coefficient. Furthermore, each class of synonymous mutation within a codon family is probably unique. Since we wanted to determine if it was possible to measure the effects of manipulating codon bias, the leucine codons of the alcohol dehydrogenase gene (*Adh*) appeared to be the most promising targets for experimentation for two reasons. First, different codon families exhibit different degrees of codon bias in *D. melanogaster* (McVean and Vieira 2001). By several measures, the leucine codon family is one of the most highly biased in the *D. melanogaster* genome (Li 1987; Moriyama and Powell 1997; McVean and Vieira 2001).

Second, *Adh* is a highly expressed gene with a high level of codon bias. *Adh* is among the top 2% most highly biased genes in the *D. melanogaster* genome (see Duret and Mouchiroud 1999, online material). Accordingly, we expected unpreferred changes in the leucine codon family would be the most likely to result in measurable differences in ADH expression following the experimental introduction of unpreferred codons.

**MATERIALS AND METHODS**

**Experimental procedures:** *Adh* constructs were derived from an 8.6-kb Sad-Clad fragment of the *Wa-F* allele (Kefelman 1983). Mutagenesis was performed on a pUC18 plasmid containing the 8.6-kb fragment using the Quick-change mutagenesis kit (Stratagene, La Jolla, CA). A single nucleotide substitution was made at codon 16 (CTG to CTA) to create the 1 Leu mutant construct. For the 6 Leu mutant construct, nucleotide substitutions were made at codons 5 (TTG to CTA), 16 (CTG to CTA), 21 (CTG to CTA), 27 (CTG to CTA), 28 (CTG to CTA), and 32 (CTG to CTA). With the exception of codon 5, the 10 Leu mutant construct contained the same substitutions as the 6 Leu construct, with an additional five substitutions at codons 35 (CTG to CTA), 36 (CTG to CTA), 50 (CTG to CTA), 76 (CTG to CTA), and 77 (CTG to CTA). Mutant clones were sequenced to ensure that the desired mutation(s) were present before proceeding. The 8.6-kb Sad-Clad fragment was subcloned into a Clad site added to the YES transformation vector ( Parsch et al. 1997). The YES vector is a pElement vector containing the *D. melanogaster* yellow gene as a selectable marker (Patton et al. 1992).

Germline transformation was performed by microinjection of *y w, AdhH5; Δ2-3, Sh/TM6* embryos. A splicing defect in the *AdhH5* allele results in no detectable ADH protein (Benyajati et al. 1982). The source of transposase used was from the Δ2-3 P insertion on the third chromosome (Robertson et al. 1988). Injected survivors were crossed to a *y w, AdhH5* stock and transformants were identified by body color. Mobilization crosses were performed to generate additional lines with insertions at unique chromosomal locations. Transformant lines containing insertions on the X chromosome were crossed to the *y w, AdhH5; Δ2-3, Sh/TM6* stock. *y*; *Sh* offspring (containing both the YES insertion and the source of transposase) were then crossed to the *y w, AdhH5* stock. Flies containing mobilized insertions were identified as *y* offspring where the *y* marker was not segregating with the same chromosome as the parental insert.

Lines containing single insertions were identified through Southern blotting using an *Adh*-specific probe spanning ~1.5 kb of the *Adh* 5′ flanking sequence (Parsch et al. 1997). Insert DNA from two to three independent lines within each genotype was PCR amplified and sequenced to verify the correct haplotype with respect to the respective mutations.

Transformed males were crossed to the *y w, AdhH5* stock to produce *y* offspring heterozygous for the *Adh* insertion. Two crosses were performed for each line. For each cross, five males and five females were mated, and five male progeny were collected at age 6–8 days and used for preparation of crude protein extracts, which were used in the ADH assays.

A standard protocol was used for performing ADH assays (Maroni 1978) using isopropanol as the substrate. Total protein content of the crude extracts was determined through the Lowry method (Lowry et al. 1951). ADH activity was measured as micromole of NADH reduced per minute per milligram of total protein. The entire procedure (ADH activity and protein content) was repeated at two different time blocks, representing a total of four measurements per line (= two crosses per line × two measurements per cross). A nested ANOVA was used to test the null hypothesis of no differences in ADH activity between genotypes. Post hoc tests were performed to test for significant differences in pairwise comparisons.

**Data analyses:** We used two population genetic methods to obtain rough estimates of the fitness effects of our mutations. First, we applied the saturation theory of molecular evolution (Hartl et al. 1985) to our empirical data on the relationship between the number of unpreferred mutations and corresponding reduction in ADH activity. The saturation theory of molecular evolution explores the relationship between enzymatic activity and fitness. Using saturation theory, Hartl et al. 1985) derived the relation between ADH activity and fitness from the frequency of null *Adh* alleles in natural populations (Langley et al. 1981). From this they estimated the standardized amount or activity (*a* = 538.50) of the *Adh* gene product in natural populations. We used this estimate of *a* to obtain the value of *s* from our data. First, we performed a linear regression on percentage of activity (relative to the control mean) vs. number of unpreferred mutations. The relative activity was calculated as a percentage of the average activity (micromole NADH reduced per minute per milligram protein × 100) among control (*Wa-F* transformant) lines. The slope of the linear regression (\( y = -2.13x + 95.87, R^2 = 0.23 \)) indicated a significant reduction in ADH activity with number of unpreferred mutations (*P* < 0.001). Higher-order regressions did not improve the fit to the data. We observed a 2.13% decrease in activity per unpreferred mutation, a value that we then used to calculate *a*, which is simply equal to *a* = (2.13% × *a*) (Hartl et al. 1985). Next, we obtained *f(a)*, the fitness of an individual with a single unpreferred mutation using the relation *f(a) = a/(1 + a)*. Finally, the selection coefficient is given by *s = 1 – f(a)/f(a)*, which yielded an estimate of \(|s| = 4.0 \times 10^{-5}\). The value of the standardized ADH activity, *a* = 538.5 (Hartl et al. 1985), is dependent on the frequencies of the *Adh*-Fast and *Adh*-Slow alleles in the populations surveyed (Langley et al. 1981). If their estimate of *a* relates to some average of the two variants in the population, then the value of *a* based on *Adh*-Fast alone would be >538.5 (by a factor of two at the most), resulting in a smaller selection coefficient. Nevertheless, doubling the value of *a* would halve the value of \(|s|\), but \(|s|\) would still be over an order of magnitude >10^{-6}. 
TABLE 1

ADH activities of control and mutant transgenic fly lines

<table>
<thead>
<tr>
<th>Genotype</th>
<th>No. of independent insertion lines assayed</th>
<th>Average ADH activity (± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wa-F (control)</td>
<td>n = 10 lines</td>
<td>98.75 ± 12.79</td>
</tr>
<tr>
<td>1 Leu</td>
<td>n = 9 lines</td>
<td>89.92 ± 6.24</td>
</tr>
<tr>
<td>6 Leu</td>
<td>n = 16 lines</td>
<td>80.33 ± 10.82</td>
</tr>
<tr>
<td>10 Leu</td>
<td>n = 15 lines</td>
<td>75.01 ± 22.35</td>
</tr>
</tbody>
</table>

ADH activity is expressed in standard units (micromole NADH reduced per minute per milligram of total protein × 100).

Second, following Bulmer (1991), we used the SMD model to obtain a crude estimate of the fitness effects of our introduced CTA mutations. The diffusion approximation of the SMD model with genic selection can be extended from a two- or fourfold degenerate codon family to a family with six codons under the assumption that the mutation rate between all codons is equal. According to equation 4 of Li (1987), the expected frequency of codon $i$ within a family is then approximately proportional to $\exp(4N_s)$, where $N_s < 0$ is the selection intensity against codon $i$. In Adh of D. melanogaster (and in all species of the melanogaster subgroup), the observed frequency of the CTA codon is 0 (Nakamura et al. 2000). This may suggest that $N_s < -1$ (or even $N_s < 0$). For unequal mutation rates (more appropriate for the Leu codon family), a solution of the diffusion equation of the SMD model is not available (Ewens 1979). However, assuming that the unpreferred codon CTA is much stronger selected against than the suboptimal codons TTG and CTC of the Leu family, a timescale argument suggests a similar result as in the case of equal mutation rates.

Folding free energies of the 1 Leu, 6 Leu, and 10 Leu mature mRNA sequences were calculated on the mFOLD server (Mathews et al. 1999). Phylogenetically conserved pairing regions were identified using the PIRANAH software program (Parzsch et al. 2000).

RESULTS AND DISCUSSION

Three classes of mutant genotypes were constructed using P-element-mediated germline transformation. We introduced 1 (1 Leu), 6 (6 Leu), or 10 (10 Leu) mutations from preferred leucine codons (CTG or CTC; Akashi 1995) to unpreferred leucine codons (CTA) in the Adh transgene and compared the level of ADH activity in these lines to transformant lines containing the unaltered native transgene (control, Wa-F allele). Since the amino acid sequences of all four genotypes were identical and the only differences among the genotypes were in synonymous mutations in coding regions, any differences in ADH activity could be attributed to differences in the expression of the transgene (in an otherwise Adh-null background of Adh<sup>4</sup>ut, splicing defect).

The introduction of unpreferred codons resulted in a measurable decrease in ADH activity. The average ADH activities of the Wa-F controls and 1 Leu, 6 Leu, and 10 Leu lines were 98.8, 88.9, 80.3, and 75.0, respectively (Table 1). Differences in ADH activity among the four genotypes were highly significant ($P < 0.01$, Table 2). The mean ADH activity of the Wa-F control lines was significantly greater than that of both the 6 Leu ($P < 0.05$) and 10 Leu ($P < 0.01$) lines (Table 3).

The prediction of population genetics theory that $N_s$ for any codon change in D. melanogaster is not significantly different from 0 (Akashi 1996; McVean and Vieira 2001) is difficult to reconcile with our data, which demonstrate that the effects of unpreferred synonymous substitutions are experimentally measurable. Indeed, a population genetics model that relates enzyme flux to fitness in a simple linear fashion (Hartl et al. 1985) indicates that a value of $|\sigma|$ on the order of $10^{-3}$ would be consistent with our observations (see MATERIALS AND METHODS). Assuming the standard estimate of $N_e = 10^6$ for D. melanogaster inferred from levels of neutral variation (Powell 1997), this value of $s$ would then result in a very large estimate of $|N_e|s$. Finally, estimation based on an extension of the SMD model from twofold degenerate to sixfold degenerate codons may also suggest a value of $|N_e|s > 1$ for our changes to the unpreferred Leu codon CTA. This is based on the observation that the frequency of CTA codons in all D. melanogaster Adh alleles sequenced to date is 0 (Nakamura et al. 2000). In fact, the frequency of CTA codons in Adh in all species of the melanogaster subgroup is 0 (Nakamura et al. 2000).

The discrepancy between these estimates may arise from several sources. On the one hand, the underlying population genetic models rest on various assumptions.

TABLE 2

Results from statistical analysis of ADH activity in control and mutant transgenic fly lines

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Degrees of freedom</th>
<th>Sum of squares</th>
<th>Mean square</th>
<th>$F$</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Among genotypes</td>
<td>3</td>
<td>15294.24</td>
<td>5098.08</td>
<td>5.17</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Among lines</td>
<td>46</td>
<td>45379.93</td>
<td>986.52</td>
<td>7.93</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Within cross</td>
<td>50</td>
<td>6217.21</td>
<td>124.34</td>
<td>1.23</td>
<td>&gt;0.1 NS</td>
</tr>
<tr>
<td>Trial</td>
<td>100</td>
<td>10073.57</td>
<td>100.74</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>199</td>
<td>76064.95</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NS, not significant.
scripts were more difficult to translate due to interference. We are grateful to J. Baines, Y. Chen, and J. Parsch for assistance.

On the other hand, Adh-specific effects may also play a role. The 6 Leu and 10 Leu line constructs contained one and two sets, respectively, of consecutive unpreferred codons. In highly expressed genes of bacteria, the tandem arrangement of rare codons has been shown to sequester cognate tRNAs in the P site, causing the translation of these codons to be rate limiting (Varenne et al. 1989; Ivanov et al. 1997). Furthermore, unpreferred codons were all introduced in the 5’ region of the gene, where their effects on translation may be more pronounced if translation initiation is rate limiting. However, in prokaryotes codon bias is less extreme at the 5’ end of genes, possibly facilitating ribosome binding (Eyre-Walker and Bulmer 1993).

It is also possible that the reduction in ADH protein production may not be due to codon bias alone. Perhaps the introduced substitutions altered the secondary structure of the Adh mRNA transcript, and the mutant transcripts were more difficult to translate due to interference from secondary structures. To address this possibility, we compared the folding free energies of the Wa-F, 1 Leu, 6 Leu, and 10 Leu transcripts using mFOLD (Mathews et al. 1999). We observed no appreciable differences in free energies, indicating that global secondary structure was not significantly altered by the introduced mutations. We also tested for the alteration of individual structural elements (i.e., hairpins) using a maximum-likelihood-based phylogenetic comparative approach to predicting mRNA secondary structures (Parsch et al. 2000). None of the sites targeted for mutation were predicted to be involved in strongly conserved structures. Previous analyses also indicated that the coding sequences of the Adh gene are unlikely to contain strongly conserved individual structural elements (Carlini et al. 2001). Therefore, the predicted changes in secondary structure are minor and unlikely to be the major factor accounting for the relatively large changes in protein activity we observed. We conclude that the observed differences in protein activity are likely due to effects at the level of translation. The introduction of unpreferred codons decreased the rate and accuracy of translation and/or increased proofreading costs (Bulmer 1991).

In summary, our results are important for at least two reasons. First, if the population genetic estimates of Ns are indeed as small as currently thought, our observations show that the consequences of very small selective differences can be observed. This will encourage more experimental work on fitness-related traits in eukaryotes, which thus far has not been undertaken because the effects of small fitness differences were thought to be immeasurable. Even granting that the actual fitness differences are immeasurable in the lab, our findings indicate that the effects on the phenotype may be substantial (e.g., each unpreferred codon resulted in an ~2.13% drop in activity) and may be worthy of further investigation. However, we point out that we deliberately selected the most biased codon family and introduced a strongly unpreferred codon (CTA) in place of preferred codons, so that average selection coefficients are likely to be much smaller. Second, should the selection intensity on synonymous positions be larger than currently believed, our observations are expected to stimulate more work on codon bias evolution and the theory of weak selection in general. Several avenues of future research include replacing unpreferred codons with preferred codons, examining other codon families, or measuring the level of expression of other highly expressed genes in different genetic backgrounds (e.g., wild-type Adh vs. 10 Leu Adh) to examine the effects of ribosome competition. These studies would complement work previously conducted in prokaryotes (Sorensen et al. 1989; Andersson and Kurland 1990) and would address the generality of the results of this study to eukaryotic systems.

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**LITERATURE CITED**


acid substitution, and larger proteins in D. melanogaster. Genetics 144: 1297–1307.


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