

Deletion of a Conserved Regulatory Element in the *Drosophila Adh* Gene Leads to Increased Alcohol Dehydrogenase Activity but Also Delays Development

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ABSTRACT

In vivo levels of enzymatic activity may be increased through either structural or regulatory changes. Here we use *Drosophila melanogaster* alcohol dehydrogenase (ADH) in an experimental test for selective differences between these two mechanisms. The well-known ADH-Slow (S)/Fast (F) amino acid replacement leads to a twofold increase in activity by increasing the catalytic efficiency of the enzyme. Disruption of a highly conserved, negative regulatory element in the *Adh* 3' UTR also leads to a twofold increase in activity, although this is achieved by increasing *in vivo Adh* mRNA and protein concentrations. These two changes appear to be under different types of selection, with positive selection favoring the amino acid replacement and purifying selection maintaining the 3' UTR sequence. Using transgenic experiments we show that deletion of the conserved 3' UTR element increases adult and larval *Adh* expression in both the ADH-F and ADH-S genetic backgrounds. However, the 3' UTR deletion also leads to a significant increase in developmental time in both backgrounds. ADH allozyme type has no detectable effect on development. These results demonstrate a negative fitness effect associated with *Adh* overexpression. This provides a mechanism whereby natural selection can discriminate between alternative pathways of increasing enzymatic activity.

THE amount of activity of a given enzyme in an organism may be increased by either of two evolutionary mechanisms: a structural change that alters the catalytic efficiency of the enzyme or a regulatory change that alters the amount of enzyme produced. These two mechanisms are often considered equivalent in models of enzyme evolution because they result in the same phenotype when measured as *in vivo* enzymatic activity. The phenotypes induced by these alternative mechanisms may differ greatly, however, when measured as *in vivo* mRNA or protein concentration. Thus, it is possible that natural selection may be able to discriminate between these two routes of increase in enzymatic activity. The *Drosophila* alcohol dehydrogenase (ADH; EC 1.1.1.1) enzyme provides an excellent opportunity to investigate this possibility. ADH is one of the most abundant proteins in *Drosophila melanogaster* (BENYAJATI *et al.* 1980) and is encoded by a single gene, *Adh*. ADH functions primarily in the detoxification of ingested alcohols, and it is thought that the evolution of this enzyme, accompanied by adaptation to survival in alcohol-rich environments such as rotting fruits, has played an important role in the evolution of the family Drosophilinae,

particularly the subfamily Drosophilinae (ASH-BURNER 1998).

ADH protein and DNA sequence variation have been studied extensively in *D. melanogaster*. Electrophoretic studies of allozyme polymorphism have identified two forms of the ADH enzyme that are present at high frequency in populations worldwide (OAKESHOTT *et al.* 1982). The two forms, designated ADH-Slow (S) and ADH-Fast (F), differ by a single lysine → threonine amino acid replacement (FLETCHER *et al.* 1978). The amino acid replacement leads to an approximately twofold increase in catalytic activity in the ADH-F form (CHOUDHARY and LAURIE 1991). The amino acid replacement, however, does not significantly affect the total amount of enzyme or *Adh* mRNA in the fly (CHOUDHARY and LAURIE 1991). Several lines of evidence suggest that natural selection favors increased ADH activity and/or the S → F replacement in at least some environments: (1) ADH activity is positively correlated with ethanol tolerance in laboratory experiments, and is also positively correlated with ethanol concentration in natural environments (CHAKIR *et al.* 1993; MERCOT *et al.* 1994); (2) ADH-S and ADH-F alleles show similar latitudinal clinal distributions on different continents (OAKESHOTT *et al.* 1982; BERRY and KREITMAN 1993), suggesting a common selective gradient; (3) intra- and interspecific DNA sequence comparisons indicate that ADH-S is the ancestral form of the enzyme and that ADH-F has risen to high frequency worldwide in the recent past (KREITMAN 1983; AQUADRO *et al.* 1985; SUL-

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LIVAN *et al.* 1990); and (4) statistical tests based on levels of nucleotide polymorphism and divergence reject a pattern of neutral molecular evolution at the *Adh* locus in *D. melanogaster* (HUDSON *et al.* 1987; KREITMAN and HUDSON 1991; BEGUN *et al.* 1999).

A complex polymorphism, designated as $\nabla 1$, in the first (adult) *Adh* intron that is tightly linked to the S/F replacement site has also been shown to affect ADH activity levels (LAURIE and STAM 1994). Experimental replacement of the S-linked $\nabla 1$ sequence with that of the F-linked results in a 15–20% increase in ADH activity (LAURIE and STAM 1994). The $\nabla 1$ polymorphism, like the ADH allozymes, shows a geographical cline in frequency and may be a target of positive selection (BERRY and KREITMAN 1993). Interestingly, the increase in ADH activity associated with the F-linked $\nabla 1$ sequence is not accompanied by an increase in *Adh* mRNA concentration (LAURIE and STAM 1994).

We have identified previously a negative regulatory element in the *Adh* 3' untranslated region (UTR; PARSCH *et al.* 1999). Deletion of this highly conserved 8-bp sequence in the ADH-F background leads to a twofold increase in *in vivo* ADH activity. The relative change in activity is thus nearly identical to the one caused by the S \rightarrow F amino acid replacement. The 3' UTR deletion, however, does not affect the amino acid sequence of the ADH enzyme; activity is increased through twofold greater *in vivo* concentration of *Adh* mRNA and protein than in nondeletion flies (PARSCH *et al.* 1999). The conservation of this 3' UTR sequence implies strong purifying selection against mutations that disrupt the 8-bp motif. As discussed above, there is no evidence for purifying selection against ADH-F alleles. On the contrary, most evidence indicates positive selection on the amino acid replacement. Why, then, is there selection against disruption of the 3' UTR motif? We have hypothesized that since mutation of the 3' UTR sequence increases ADH activity by increasing *in vivo* *Adh* mRNA and protein concentrations, while the S \rightarrow F amino acid replacement increases activity solely by altering the catalytic efficiency of the enzyme, there may be deleterious effects associated with increased *Adh* mRNA and protein levels (PARSCH *et al.* 1999).

To test this hypothesis, we first demonstrate that deletion of the conserved 3' UTR motif results in twofold greater ADH activity in the ADH-S background. This is necessary because previous experiments used only the ADH-F background (PARSCH *et al.* 1999). We then compare the developmental times (from egg to adult) of transgenic flies carrying wild-type and deletion 3' UTR sequences in both the ADH-F and ADH-S backgrounds. We find that in both backgrounds, flies with the 3' UTR deletion show a significant increase in developmental time relative to wild type. Importantly, wild-type ADH-F flies and 3' UTR-deletion ADH-S flies, which are nearly identical in ADH activity, also show a significant difference in developmental time. Thus the effect cannot be

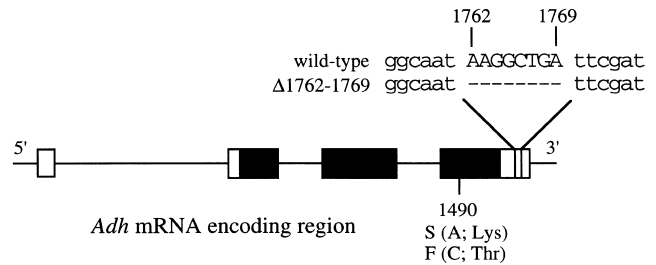


FIGURE 1.—Schematic representation of the *Adh* mRNA encoding region. Exons are represented as boxes; introns as lines. The protein encoding regions are shown as solid boxes. The F/S amino acid replacement site is indicated, as well as the highly conserved 8-bp 3' UTR sequence that is deleted by the $\Delta 1762$ –1769 mutation. Numbering is from KREITMAN (1983).

explained by differences in ADH activity, but must be a result of the underlying difference in *Adh* mRNA and/or protein concentration. These results are consistent with our hypothesis and suggest that the different mechanisms of increasing ADH enzymatic activity are not selectively equivalent.

MATERIALS AND METHODS

Site-directed mutagenesis and plasmid construction: Wild-type and mutant ADH-F constructs were derived from an 8.6-kb *SacI-ClaI* fragment of the *Adh* *Wa-f* allele (KREITMAN 1983) and have been described previously (PARSCH *et al.* 1999). For the ADH-S constructs, the 8.6-kb *SacI-ClaI* fragment of the *Wa-s* allele (KREITMAN 1983) was used. A 3.2-kb *Sall-ClaI* fragment containing the entire *Adh* mRNA encoding region was subcloned into a pUC18 plasmid and used as a template for site-directed mutagenesis following the QuikChange (Stratagene, La Jolla, CA) procedure. The mutagenesis primers were 5' GATATTACGCAAGGCAATTCGATGCACACTCACATTC 3' and its complement. These primers correspond to positions 1743–1790 in KREITMAN's (1983) consensus sequence, with bases 1762–1769 eliminated. Note, however, that the original consensus sequence contains a sequencing error (an extra T at position 1761; LAURIE *et al.* 1991) that has been corrected in our primer design. Thus, there is not perfect correspondence between our sequence and that of KREITMAN (1983). For consistency, we use KREITMAN's (1983) coordinates to designate the highly conserved 8-bp motif as bases 1762–1769. The mutagenesis procedure resulted in the specific deletion of the conserved 8-bp 3' UTR motif, which was confirmed by direct DNA sequencing. Following mutagenesis, a *BamHI-ClaI* restriction fragment containing the mutant 3' UTR was used to replace the corresponding fragment in the original 8.6-kb *SacI-ClaI* clone. The entire *BamHI-ClaI* region was then sequenced to ensure that the 8-bp motif was deleted and that no other sequence changes had occurred. The constructs were designated as wt-S, $\Delta 1762$ –1769-S, wt-F, and $\Delta 1762$ –1769-F, where wt and $\Delta 1762$ –1769 indicate the wild-type and mutant 3' UTR, and S and F indicate the *Wa-s* and *Wa-f* backgrounds (Figure 1).

Germline transformation: Wild-type and mutant *Adh* fragments were inserted into the polycloning region of the YES transformation vector (PATTON *et al.* 1992). This vector contains the *D. melanogaster* *yellow* (*y*) gene as a phenotypic marker and also contains binding sites for the *suppressor of Hairy-wing*

protein, which flank the target DNA and serve as an insulator of chromosomal position effects (PATTON *et al.* 1992). All constructs were introduced into the *y w; Adh^{h6}; Δ2-3, Sb/TM6* genetic background (ADH-null) through germline transformation (RUBIN and SPRADLING 1982; SPRADLING and RUBIN 1982). Some insertions on the *X* and third chromosomes were mobilized to new locations through genetic crosses using the $\Delta 2-3$ *P* element as a source of transposase (ROBERTSON *et al.* 1988; PARSCH *et al.* 1997). Following transformation or mobilization, all lines were crossed to a *y w; Adh^{h6}* stock to remove the source of transposase. To avoid possible effects of dosage compensation on *Adh* expression (LAURIE-AHLBERG and STAM 1987; PARSCH *et al.* 1997), only autosomal-insertion lines were used for further analysis. In addition, we limited our analysis to transformed lines containing only a single *Adh* insertion, as determined by Southern blotting (PARSCH *et al.* 1997).

Measurement of ADH activity: For measurements of adult activity, we used a total of 32 transformed lines: 8 wt-S, 7 $\Delta 1762-1769$ -S, 10 wt-F, and 7 $\Delta 1762-1769$ -F. Transformed males were crossed to *y w; Adh^{h6}* females to produce offspring heterozygous for the *Adh* insertion. For each cross, five males and five females were placed in each of two vials and the pooled progeny were used for ADH assays. Total soluble protein was extracted from five 6- to 8-day-old heterozygous males and ADH activity was determined spectrophotometrically (MARONI 1978) using isopropanol as the substrate. The total protein concentration of each preparation was estimated using the method of LOWRY *et al.* (1951). Units of ADH activity were measured as micromoles of NAD reduced per minute per milligram of total protein. Two separate ADH assays were performed on each protein preparation, and two separate protein preparations were made from each line. Thus, a total of four ADH assays were performed for each transformed line. Activity differences between the different transformant classes were tested by analysis of variance (ANOVA), using a model that accounts for intraclass position-effect variation (LAURIE-AHLBERG and STAM 1987). Statistical analyses were performed with the MacAnova software package (OEHLERT and BINGHAM 1998).

For measurements of larval activity, we used homozygous lines established from transformants showing adult ADH activities typical of their respective transformant classes. Larvae were collected at each instar stage from two independent homozygous lines within each transformant class. Soluble proteins were extracted from either 20 (first instar), 10 (second instar), or 5 (third instar) larvae and ADH activity was assayed as described above. ADH histochemical staining was performed on hand-dissected third instar larvae using standard techniques (ASHBURNER 1989).

Developmental time-course assays: Three separate experiments (hereafter referred to as E1, E2, and E3) were performed to assess the rate of development of wild-type and 3' UTR-deletion transformants. Experiments were performed at two different locations and by three different investigators: E1 by J. A. Russell (in Rochester, NY); E2 and E3 by J. Parsch and I. Beerman, respectively (in Cambridge, MA). E1 compared wt-F and $\Delta 1762-1769$ -F transformants, with all flies maintained at 22°. E2 and E3 compared wt-S, $\Delta 1762-1769$ -S, wt-F, and $\Delta 1762-1769$ -F transformants, with all flies maintained at 25°. Otherwise, the experimental protocol was identical for each of the three experiments except where noted. Flies were reared in 25 × 95-mm vials containing 7–8 ml of standard cornmeal-molasses medium. Males and virgin females were collected from homozygous lines and aged 2–5 days. Within each transformant class, a single male from one homozygous line was placed in a vial with a female from a

different homozygous line and mating was allowed to occur over a 12-hr period. This mating scheme results in progeny carrying two nonallelic copies of the same *Adh* transgene and was employed to eliminate the possibility that homozygous deleterious mutations caused by the random insertion of our transposable element vectors into the genome might affect the rate of development. For each experiment, three to five homozygous lines from within each transformant class were used for developmental assays. In addition, three to six replicates of each cross (and of the reciprocal cross) were performed. After mating, the male was discarded and the female was placed in a fresh vial, which became the day 1 vial. Females were allowed to lay eggs in the vial for a period of 24 hr, and then were transferred to another fresh vial. This process was repeated for the following 4 days, when the female was discarded. Each female thus produced five vials of eggs that were allowed to develop in uncrowded conditions (<50 eggs per vial). Vials in which the female did not lay eggs were discarded and not considered in further analyses. In total, the number of crosses producing fertile eggs in each experiment ranged from 31 to 71. The number of pupae and the number of emerging adults in each vial were recorded every 24 hr. In E1 and E2, the number of eggs and the number of hatched larvae were recorded for a subset of the vials (the day 1 and day 5 vials) over the first 5 days of development.

RESULTS

Effect of the 3' UTR deletion in ADH-F/S backgrounds: We have demonstrated previously that deletion of the conserved 8-bp 3' UTR motif leads to a significant (approximately twofold) increase in ADH activity in the ADH-F background (PARSCH *et al.* 1999). Although this region of the 3' UTR is identical in *Adh-f* and *Adh-s* alleles (KREITMAN 1983; LAURIE *et al.* 1991), it was not clear whether or not deletion of the 8-bp sequence would lead to a similar increase in activity in the ADH-S background. Flies homozygous for ADH-F typically have two to three times the *in vivo* ADH activity of those homozygous for ADH-S (LAURIE *et al.* 1991). A large portion of this difference can be attributed to the S/F amino acid replacement (CHOUDHARY and LAURIE 1991). Other nonreplacement differences (including $\nabla 1$) between *Adh-f* and *Adh-s* alleles, however, have also been shown to affect *Adh* expression (LAURIE and STAM 1994; STAM and LAURIE 1996). It is thus possible that interactions between the S/F site (or other linked nonreplacement sites) and the conserved 3' UTR sequence may affect the overall level of *Adh* expression. To test this, we specifically deleted the 8-bp 3' UTR motif in the *Adh-s* background and compared the ADH activities of wt-S and $\Delta 1762-1769$ -S transformed lines (Figure 2). The activities were also compared to those of transformed lines containing the wt-F and $\Delta 1762-1769$ -F *Adh* constructs (Figure 2; PARSCH *et al.* 1999). Our results indicate that the 8-bp 3' UTR deletion leads to a highly significant increase in ADH activity in both the ADH-S ($P < 0.001$) and ADH-F ($P < 0.001$) backgrounds. Note, however, that there is no difference in activity between $\Delta 1762-1769$ -S and wt-F transformants ($P = 0.414$). This

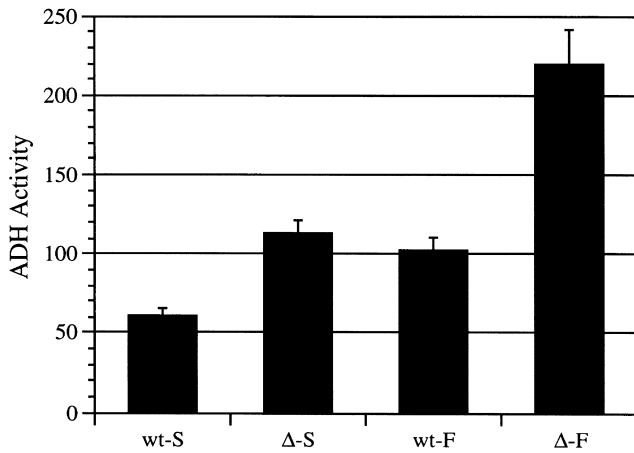


FIGURE 2.—Average adult ADH activity of the four different transformant types. Wild-type and $\Delta 1762$ – 1769 3' UTRs are represented as wt and Δ , respectively. ADH allozyme type is indicated as either F or S. ADH activity is given in units of micromoles of NAD reduced per minute per milligram of total protein (multiplied by 100). Error bars represent ± 1 SE.

indicates that disruption of the conserved 3' UTR motif has the same effect on *in vivo* ADH activity as the S \rightarrow F amino acid replacement.

ADH activity at different larval stages: In wild-type flies, *Adh* gene expression is known to vary over the course of development, beginning with low levels at the first instar larval stage and increasing through the larval stages until the highest levels are reached in the adult (CORBIN and MANIATIS 1989). To determine if the 8-bp 3' UTR deletion had any effect on the developmental pattern of *Adh* expression in either the ADH-S or ADH-F background, we measured ADH enzymatic activity of wt-S, $\Delta 1762$ – 1769 -S, wt-F, and $\Delta 1762$ – 1769 -F

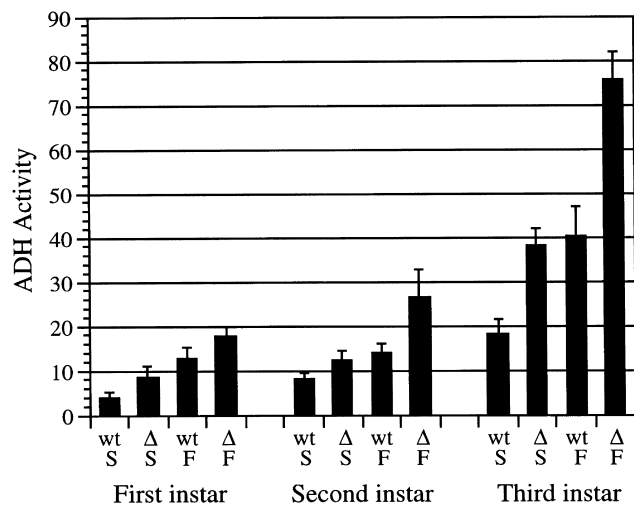


FIGURE 3.—Average ADH activity of wt-S, $\Delta 1762$ – 1769 -S, wt-F, and $\Delta 1762$ – 1769 -F transformants at different larval stages. $\Delta 1762$ – 1769 is indicated by Δ . Units of ADH activity are the same as in Figure 2. Error bars represent the range of observed activities.

flies at each larval instar stage (Figure 3). The pattern of ADH activity at each larval stage is very similar to that seen in adults: the 3' UTR deletion leads to a large increase in activity at each stage in both backgrounds. The activities of $\Delta 1762$ – 1769 -S and wt-F are indistinguishable at each stage (Figure 3).

Tissue-specific expression patterns: SIEGAL and HARTL (1999) observed tissue-specific silencing of some *Adh* transgenes due to generalized position effects. In these cases, *Adh* expression was abolished specifically in larval gastric ceca and the transformed lines showed ADH activity levels lower than average for their specific transformant class (SIEGAL and HARTL 1999). To test for such effects in our transformed lines, we performed histochemical staining of ADH activity in dissected larval tissues. We did not observe differences in the ADH staining pattern among any of our transformant lines, particularly in the gut or gastric ceca (Figure 4). These results indicate that the qualitative pattern of *Adh* expression is not altered by the 3' UTR deletion. Differences in the overall amount of ADH activity must, therefore, be due to quantitative differences in *Adh* expression in the tissues where it is normally expressed.

Developmental time-course results: The results of the three developmental time-course experiments (E1, E2, and E3) are summarized in Table 1. Despite being performed at two different locations, by three different investigators, and in three different time blocks, the results are remarkably consistent. The most notable difference among experiments is that the developmental times are overall slower in E1 than in E2 or E3 (Table 1). This difference can be attributed to the experiments being performed at different temperatures (22° for E1, 25° for E2 and E3), as development is known to be slower at lower temperatures (ASHBURNER 1989). The effects of other environmental differences among the three experiments, however, appear to be small, and the relation of developmental times is in complete agreement across experiments. $\Delta 1762$ – 1769 -S and $\Delta 1762$ – 1769 -F flies show an increase in the time required to reach both the pupal and adult stages relative to their wild-type counterparts (Table 1). Nested ANOVA was performed separately for each experiment to test for significant effects of either allozyme (ADH-S/F) or UTR (wt/ $\Delta 1762$ – 1769) on the time required to reach the larval, pupal, or adult stage (Table 2). In all three experiments, we detect a highly significant effect of UTR on the time to reach both the pupal and adult stages (Table 2). There is, however, no significant effect of allozyme on developmental time at any stage (Table 2). Under the ANOVA model, UTR was nested within allozyme to test for significant effects of UTR in both the ADH-S and ADH-F backgrounds. A nonhierarchical model produced essentially the same results (not shown). Note that the difference in time to reach the adult stage between wild-type and $\Delta 1762$ – 1769 flies in each experiment is nearly identical to the difference in

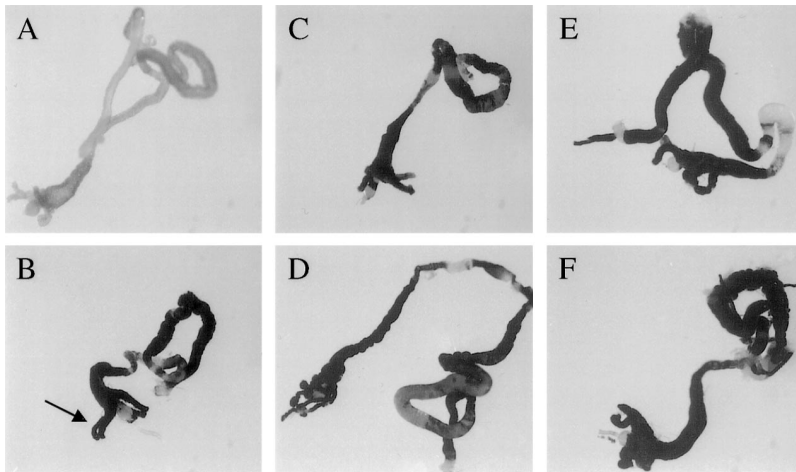


FIGURE 4.—Histochemical staining of ADH activity in the digestive system of third instar larvae. (A) *Adh*ⁿ⁶ (ADH-null), (B) wild-type *Adh* (*Wa-s* allele), (C) wt-S transformant, (D) Δ 1762–1769-S transformant, (E) wt-F transformant, (F) Δ 1762–1769-F transformant. Staining in the gastric ceca is indicated by an arrow in B.

time to reach the pupal stage. This indicates that the slowdown in development in Δ 1762–1769-F and Δ 1762–1769-S flies can be completely explained by an increase in the time to reach the pupal stage. Figure 5 shows the cumulative fraction of flies reaching the pupal or adult stage on each day of the three experiments. Again, the

results are consistent across experiments. Overall, flies with the wild-type 3' UTR reach the pupal and adult stages faster than their Δ 1762–1769 counterparts in both the ADH-S and ADH-F backgrounds. The effect is strongest on the 3–4 days following the first appearance of either pupae or adults (Figure 5).

TABLE 1
Summary of developmental time-course experiments

Stage	Experiment ^a	Type	n ^b	Mean time \pm SE ^c
Larval	E1	wt-F	1647	2.07 \pm 0.010
		Δ 1762–1769-F	523	2.08 \pm 0.020
	E2	wt-S	254	1.99 \pm 0.060
		Δ 1762–1769-S	293	2.01 \pm 0.057
		wt-F	259	1.97 \pm 0.049
		Δ 1762–1769-F	238	1.97 \pm 0.052
Pupal	E1	wt-F	4284	9.22 \pm 0.012
		Δ 1762–1769-F	1225	9.55 \pm 0.025
	E2	wt-S	423	8.40 \pm 0.087
		Δ 1762–1769-S	423	8.77 \pm 0.086
		wt-F	514	8.21 \pm 0.076
		Δ 1762–1769-F	567	8.79 \pm 0.078
	E3	wt-S	324	8.27 \pm 0.063
		Δ 1762–1769-S	774	8.54 \pm 0.050
		wt-F	690	8.12 \pm 0.050
		Δ 1762–1769-F	899	8.65 \pm 0.046
Adult	E1	wt-F	4252	14.17 \pm 0.014
		Δ 1762–1769-F	1188	14.49 \pm 0.029
	E2	wt-S	396	13.10 \pm 0.085
		Δ 1762–1769-S	309	13.43 \pm 0.105
		wt-F	440	12.75 \pm 0.073
		Δ 1762–1769-F	534	13.69 \pm 0.090
	E3	wt-S	321	13.27 \pm 0.071
		Δ 1762–1769-S	729	13.65 \pm 0.061
		wt-F	662	13.14 \pm 0.058
		Δ 1762–1769-F	881	13.73 \pm 0.052

^a E1 compared only wt-F and Δ 1762–1769-F flies. E3 compared only pupal and adult times. E1 flies were raised at 22°; E2 and E3 at 25°.

^b Sample size (the number of larvae, pupae, or adults scored).

^c Average time in days (\pm standard error) to reach the given stage.

TABLE 2
F statistics (and *P* values) for ANOVA of developmental time-course results

Source ^a	Stage	E1	E2	E3
Allozyme	Larval	—	0.31 (0.58)	—
	Pupal	—	2.05 (0.15)	0.74 (0.39)
	Adult	—	0.03 (0.85)	0.86 (0.35)
UTR	Larval	0.05 (0.60)	0.03 (0.97)	—
	Pupal	148.61 (<0.0001)	16.44 (<0.0001)	34.54 (<0.0001)
	Adult	105.03 (<0.0001)	35.71 (<0.0001)	35.40 (<0.0001)

^a Nested ANOVA was performed separately for each experiment with UTR (wt/ Δ 1762–1769) nested within allozyme type (ADH-S/F).

DISCUSSION

A number of 3' UTR sequence motifs that affect gene expression are known, the majority of which act post-transcriptionally by altering mRNA stability, localization, or translation initiation (reviewed by CURTIS *et al.* 1995; ST. JOHNSTON 1995; ROSS 1996). In *Drosophila*, three 3' UTR-specific sequence elements involved in the post-transcriptional regulation of genes in the *Bearded* and *Enhancer of split* complexes have been described (LAI and POSAKONY 1997; LEVITEN *et al.* 1997; LAI *et al.* 1998). These sequences, the Bearded (Brd) box (AGCTTTA), GY box (GTCTTCC), and K box (TGTGAT), are highly conserved within paralogous gene families in *D. melanogaster*, and also in orthologous genes in *D. virilis* and *D. hydei* (LAI and POSAKONY 1997; LAI *et al.* 1998). Experimental deletion of these motifs leads to overexpression of their respective genes and results in defects in neural development (LAI and POSAKONY 1997; LAI *et al.* 1998). Similarly, the *Drosophila Dlmo* gene contains several Brd-like motifs in its 3' UTR (SHORESH *et al.* 1998). *Beadex* mutations, which disrupt the *Dlmo* 3' UTR, cause *Dlmo* overexpression and result in a characteristic scalloping of the wings (SHORESH *et al.* 1998). These examples demonstrate that there may be deleterious fitness effects associated with disruption of conserved 3' UTR motifs and the resulting gene overexpression.

Deletion of the *Adh* 3' UTR motif (AAGGCTGA) results in a twofold increase in *Adh* expression in both the ADH-S and ADH-F backgrounds (Figures 2 and 3). This overexpression, however, does not result in any obvious phenotypic abnormalities, and flies overexpressing *Adh* appear normal with respect to fertility and viability. The strong conservation of the 8-bp 3' UTR motif, however, suggests that there are deleterious fitness effects associated with mutations in this region (PARSCH *et al.* 1999). This is somewhat unexpected given that there is apparently no deleterious effect of increased ADH activity caused by the S \rightarrow F amino acid replacement. On the contrary, most evidence indicates positive selection for increased ADH activity. Why, then, is a negative regulatory element so highly conserved in

Adh? Our developmental time-course results provide an answer to this question. The deleterious effect is not due to increased ADH activity *per se*, but is instead due to the increased level of *Adh* expression caused by disruption of the 8-bp 3' UTR motif. In both the ADH-S and ADH-F backgrounds, Δ 1762–1769 flies show a highly significant increase in developmental time relative to flies with the wild-type 3' UTR (Table 2). It is possible that the excess metabolic energy spent producing ADH is in part responsible for the developmental slowdown in Δ 1762–1769 flies. Also, it is generally assumed that the presence of free ribosomes is the limiting factor in translation. Thus, the increased levels of *Adh* mRNA in the Δ 1762–1769 mutants may occupy a substantial fraction of free ribosomes, leaving them unavailable for the translation of other proteins. The effects may be most dramatic during development, when a large number of proteins need to be synthesized during a relatively short period of time, and may be particularly strong in the case of *Adh*, because it is expressed at very high levels, accounting for 1–2% of the total translational activity in wild-type adult flies (BENYAJATI *et al.* 1980).

Consistent with the above interpretation is the observation that ADH allozyme type does not significantly affect developmental rate (Table 2). Thus, the developmental slowdown cannot be attributed to differences in *in vivo* ADH activity. Furthermore, Δ 1762–1769-S and wt-F transformants, which are nearly identical in their levels of ADH activity (Figures 2 and 3), show a highly significant difference in developmental rate. This indicates that the developmental slowdown can only be caused by differences in *Adh* mRNA or protein concentration, not by differences in ADH activity. Also consistent with our hypothesis is the observation that the developmental slowdown occurs almost entirely between hatching and pupation. *Adh* is not expressed in the developing embryo, and we find no difference in the hatching times among our four different transformant types (Tables 1 and 2). *Adh* expression begins during the first instar larval stage and increases throughout larval development (Figure 3; CORBIN and MANIATIS

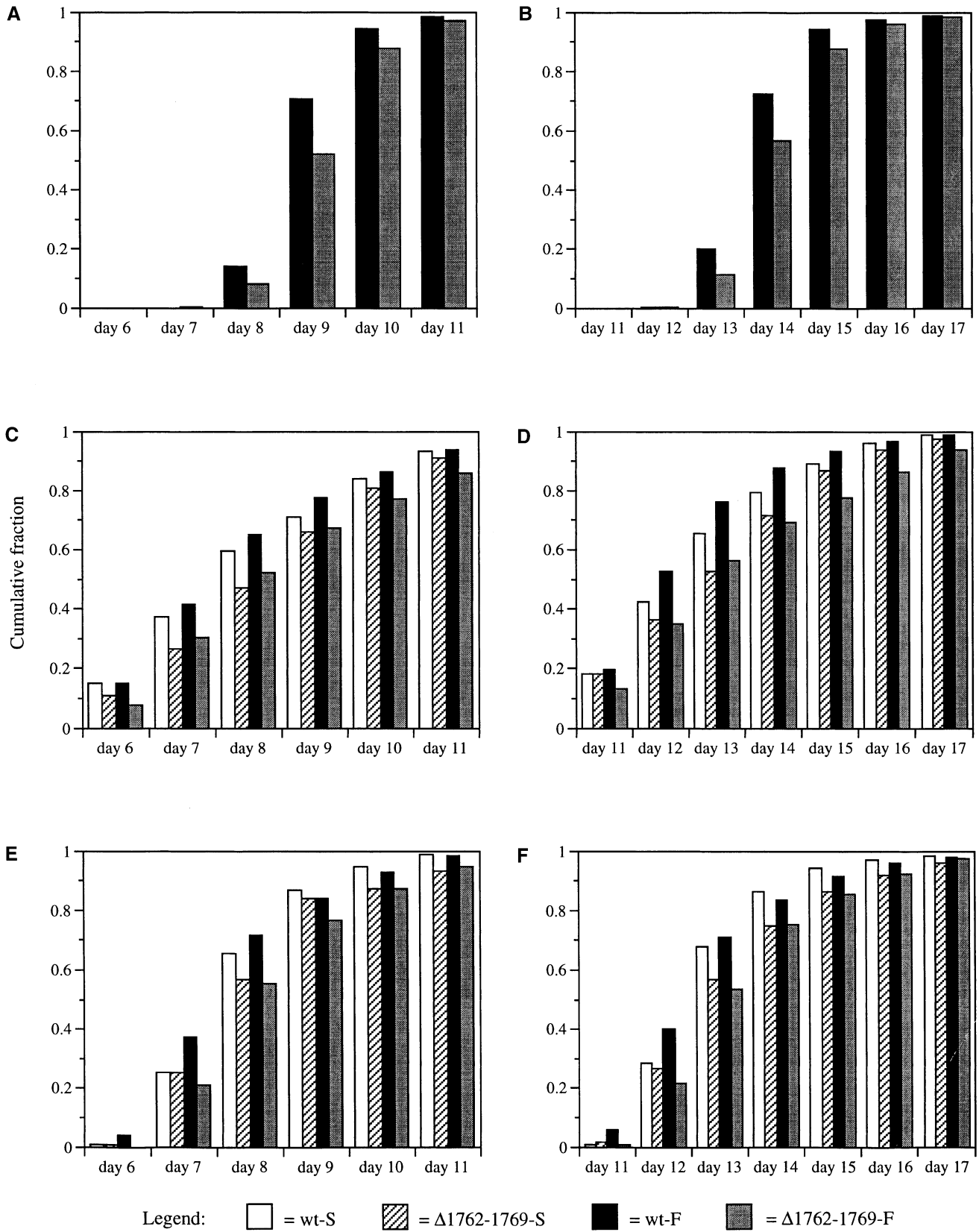


FIGURE 5.—Cumulative fraction of pupae and adults on each day of the developmental time-course experiments. (A) E1 pupae, (B) E1 adults, (C) E2 pupae, (D) E2 adults, (E) E3 pupae, (F) E3 adults.

1989). This is the time when we most expect to see a developmental slowdown in $\Delta 1762$ –1769 flies relative to wild type, and this is indeed where the slowdown occurs. We did not observe a difference in the length of pupation among any of our transformed lines. This is expected, however, because *Adh* is not expressed in pupae (CORBIN and MANIATIS 1989). The highest levels of *Adh* expression are seen in adult flies (Figure 2). As stated above, we have not observed any negative effects of increased *Adh* expression in adult flies. However, in experiments where females are placed in vials with males immediately after emerging, not aged for 2–5 days as in our experimental protocol, there is a significant reduction in the number of eggs laid by $\Delta 1762$ –1769-F females relative to wt-F females over the first 1–2 days of egg laying (J. PARSCH, unpublished results).

Our results indicate that the underlying mechanisms for increases in enzymatic activity need to be considered in models of enzyme evolution. For example, simple models based on metabolic control theory, such as that of HARTL *et al.* (1985), consider saturation kinetics in unbranched metabolic pathways in which fitness is proportional to flux through the pathway. In such a situation, the fitness of an organism is a function only of the enzymatic activity, whether the result of structural or regulatory mutations. But when deleterious pleiotropic effects associated with the metabolic cost of transcription/translation occur, these must also be taken into account. In this case, structural and regulatory changes are not equivalent. HARTL *et al.* (1985) also present rough estimates of selection coefficients (s) associated with changes in *Adh* activity. For a twofold reduction in activity, such as that observed in ADH-null heterozygotes, they estimate $s = 4.52 \times 10^{-3}$ against null heterozygotes relative to wild-type homozygotes. Due to the saturation curve associated with enzyme kinetics, a twofold increase in activity would result in a smaller, positive increase in the selection coefficient [$(s = 9.28 \times 10^{-4})$ under the model of HARTL *et al.* (1985)]. Since the *Adh* 3' UTR motif is conserved even among species, we conclude that selection pressure against increased levels of *Adh* expression through alteration in the 3' UTR motif must be even greater ($s > 9.28 \times 10^{-4}$). This is because the selective disadvantage of the twofold increase in *Adh* expression must outweigh any selective advantage associated with the twofold increase in ADH activity. Thus in the case of *Drosophila* ADH, expression levels may have a greater impact on fitness than do activity levels.

Another arena in which structural and regulatory changes are often regarded as functionally equivalent is in general discussions of the role of the two types of mutation in adaptive evolution. In bacteria, the importance of regulatory mutations in the adaptation to new carbon sources has been demonstrated experimentally (reviewed by WILSON 1977). Similarly, large interspecific differences in intestinal lysozyme concentration in

mice are determined by a single regulatory locus (HAMMER and WILSON 1987). Even in *Drosophila* *Adh*, the $\nabla 1$ intronic polymorphism is associated with an ~ 15 –20% difference in ADH activity (LAURIE and STAM 1994) and shows a geographical cline in frequency stronger than that of the ADH allozymes (BERRY and KREITMAN 1993). These examples demonstrate that adaptive changes in enzymatic activity may be the result of regulatory mutations. To account for the lack of correlation between molecular and morphological evolution in some lineages, WILSON (1977) proposed that regulatory mutations play a primary role in adaptation. More recently, HALL (1999) has argued that selective constraints are too poorly understood to predict which genes, or which mutations in those genes, are most likely to evolve a particular novel function. Our example of the 3' UTR motif in *Drosophila* *Adh* is a case in point: it is an example of a regulatory sequence that is more constrained in evolution than the coding sequence it regulates.

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