A Highly Conserved Sequence in the 3′-Untranslated Region of the Drosophila *Adh* Gene Plays a Functional Role in *Adh* Expression

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ABSTRACT

Phylogenetic analysis identified a highly conserved eight-base sequence (AAGGCTGA) within the 3′-untranslated region (UTR) of the Drosophila alcohol dehydrogenase gene, *Adh*. To examine the functional significance of this conserved motif, we performed *in vitro* deletion mutagenesis on the *D. melanogaster Adh* gene followed by *P*-element-mediated germline transformation. Deletion of all or part of the eight-base sequence leads to a twofold increase in *in vivo* ADH enzymatic activity. The increase in activity is temporally and spatially general and is the result of an underlying increase in *Adh* transcript. These results indicate that the conserved 3′-UTR motif plays a functional role in the negative regulation of *Adh* gene expression. The evolutionary significance of our results may be understood in the context of the amino acid change that produces the ADH-F allele and also leads to a twofold increase in ADH activity. While there is compelling evidence that the amino acid replacement has been a target of positive selection, the conservation of the 3′-UTR sequence suggests that it is under strong purifying selection. The selective difference between these two sequence changes, which have similar effects on ADH activity, may be explained by different metabolic costs associated with the increase in activity.

THE Drosophila alcohol dehydrogenase enzyme (ADH; EC 1.1.1.1) and the gene that encodes it, *Adh*, have been used as a model system for many studies of population genetics, molecular evolution, and molecular biology. ADH is responsible for the detoxification of environmental alcohols (David *et al.* 1976), and flies lacking ADH activity cannot survive in environments containing moderate levels of alcohol (Gibson and Oakeshott 1982; Van Delden 1982). ADH also plays an important role in the metabolism of ethanol into energy-storage lipids, particularly at the larval stage (Geer *et al.* 1986, 1991). In *D. melanogaster*, *Adh* produces two developmentally regulated transcripts that differ in their 5′-untranslated leaders but are identical in their protein-encoding regions and 3′-untranslated regions (UTRs) (Benyajati *et al.* 1983). Transcripts from a distal promoter are found predominantly in adult tissues, while those from a proximal promoter are found predominantly in larval tissues (Benyajati *et al.* 1983; Savakis *et al.* 1986). Experiments using *P*-element-mediated germline transformation have shown that all *cis*-acting sequence elements required for proper *Adh* expression are contained within an 8.6-kb *Sac*I-*Cla*I fragment, which contains the entire *Adh* mRNA-encoding region and ~5 kb of 5′ flanking sequence (upstream from the distal promoter) and 1 kb of 3′ flanking sequence (Goldberg *et al.* 1983; Laurie-Ahlberg and Stam 1987).

Two forms of the ADH protein, designated as Fast (ADH-F) and Slow (ADH-S), which differ by a single amino acid (Fletcher *et al.* 1978), have been found at high frequency in worldwide populations of *D. melanogaster* (Oakeshott *et al.* 1982). In addition, numerous polymorphisms have been detected at silent and noncoding sites within the *Adh* gene (Kreitman 1983; Laurie *et al.* 1991). ADH-F homozygotes have, on average, two- to threefold higher levels of enzymatic activity than ADH-S homozygotes. This difference is due to two factors, an increase in catalytic efficiency of the ADH enzyme and an increase in the total amount of enzyme in ADH-F flies (Choudhary and Laurie 1991). Experiments using site-directed mutagenesis and *P*-element-mediated germline transformation have shown that the Fast/Slow polymorphic site is responsible for the difference in ADH catalytic activity between ADH-F and ADH-S flies (Choudhary and Laurie 1991). This site, however, has no effect on *in vivo* concentration of ADH protein, which suggests that other, nonreplacement sites must also play an important role in determining *Adh* expression levels. For example, a complex nucleotide substitution within the first (adult) *Adh* intron has been shown to have a significant effect on ADH activity levels (Laurie and Stam 1994). Interestingly, this intronic sequence has no effect on the concentration of *Adh* mRNA, suggesting that there may be an interaction between this region of the first intron and other regions

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of the pre-mRNA during polyadenylation or other processing events that results in a change in the translational efficiency of the mature mRNA (Laurie and Stam 1994). Other nonreplacement sites that affect levels of *Adh* expression have been mapped to the protein-encoding region and the 3′-UTR, but have not yet been tested by mutational analysis (Stam and Laurie 1996).

Phylogenetic analysis has been used to identify noncoding regions of the *Adh* gene that may play a functional role in *Adh* expression. Kirby *et al.* (1995) used the phylogenetic-comparative method to predict secondary structures of the *Adh* pre-mRNA and suggest that the patterns of linkage disequilibrium found within the *Adh* gene of *D. pseudoobscura* (Schaeffer and Miller 1993) are the result of epistatic selection maintaining RNA pairing stems within intron sequences. Phylogenetic analysis has also identified several short-range RNA pairing stems within the *Adh* exon 2, as well as two long-range pairings between exon 2 and a highly conserved region of the 3′-UTR (Parsch *et al.* 1997). The functional significance of one of these long-range pairings was tested by mutational analysis in *D. melanogaster* (Parsch *et al.* 1997). A site-directed mutation at a silent third-codon position of exon 2, which disrupted a Watson-Crick base pair in the proposed long-range pairing, resulted in a significant reduction in adult ADH activity level. ADH activity was restored to wild-type levels by a second, "compensatory" mutation in the 3′-UTR. The 3′-UTR mutation by itself, however, had no measurable effect on ADH activity levels. Thus, these observations do not fit a simple model of compensatory evolution (Parsch *et al.* 1997).

To explore the possible cause of this discrepancy and further examine the role of phylogenetically conserved sequence elements on *Adh* expression, we investigated a highly conserved sequence located just downstream of the nucleotide site where the single 3′-UTR mutation was made. The high level of conservation within this region suggests that it may have a function that is distinct from that of the proposed RNA secondary structure. To test this hypothesis, *in vitro* deletion mutagenesis and *P*-element-mediated germline transformation were used to examine the effects of specific deletions within the conserved 3′-UTR sequence on ADH activity and *Adh* mRNA levels in *D. melanogaster*. The results indicate that deletion of the conserved 3′-UTR motif leads to a significant increase in *in vivo* ADH activity. Since the ADH protein-encoding sequence was not altered during mutagenesis, the observed increase in enzymatic activity must be the result of an increase in *Adh* gene expression. Thus, the conserved 3′-UTR sequence plays a functional role in the negative regulation of *Adh* expression.

MATERIALS AND METHODS

Plasmid construction and mutagenesis: Basic molecular techniques were carried out following the methods of Sambrook *et al.* (1989). All *Adh* constructs were derived from an 8.6-kb *Sac*I-*Cla*I fragment of the *D. melanogaster Wa-f* allele [originally described by Kreitman (1983)]. A pUC18 plasmid containing the entire *Adh* mRNA-encoding region within a 3.2-kb *Sal*I-*Cla*I fragment (Parsch *et al.* 1997) was used for mutagenesis following the QuikChange (Stratagene, La Jolla, CA) procedure. Following mutagenesis, a *Bam*HI-*Cla*I restriction fragment containing each mutant 3′-UTR was used to replace the corresponding fragment in the original 8.6-kb *Sac*I-*Cla*I clone. At this point, the entire *Bam*HI-*Cla*I region subjected to mutagenesis was sequenced using a cycle sequencing method (Life Technologies) to ensure that the desired mutation (and no other changes) was present.

***P*-element-mediated germline transformation:** The entire 8.6-kb *Adh Sac*I-*Cla*I fragment of each mutant construct was inserted into the polycloning region of the YES transformation vector, which contains the *D. melanogaster yellow* (*y*) gene as a selectable marker (Patton *et al.* 1992). Germline transformation of an ADH-null *D. melanogaster y w*; Adh^{fn6}Δ2-3,*Sb*/TM6 stock was achieved by embryo microinjection (Rubin and Spradling 1982; Spradling and Rubin 1982). At least four independent insertion lines were generated through embryo microinjection for each mutant construct. Additional transformed lines were then generated by mobilization of the YES vector constructs within the original lines through genetic crosses, using the Δ2-3 *P* element as a source of transposase (Robertson *et al.* 1988; Parsch *et al.* 1997). Transformants containing the wild-type 8.6-kb *Adh Wa-f* genomic fragment were used as a control (Parsch *et al.* 1997).

Previous experiments indicated that expression levels of *Adh* insertions on the *X* chromosome may be increased due to dosage compensation mechanisms in Drosophila (Laurie-Ahlberg and Stam 1987; Parsch *et al.* 1997). For this reason, only autosomal insertion lines were used for analysis. *X* chromosome insertion lines were identified as those that produced only y^+ female (and only y^- male) offspring when transformant males were mated to females of a *y w*, Adh^{fn6} stock. Furthermore, to ensure that the transformed lines contained only a single *Adh* insertion, a Southern blot was performed on genomic DNA prepared from each transformed line. The genomic DNA was digested with three six-cutter restriction enzymes (*Bgl*II, *Sal*I, and *Stu*I) and hybridized with a probe derived from the *Adh* 5′ flanking region (Parsch *et al.* 1997). The probe hybridizes to a fragment of constant size for the genomic *Adh* gene and a fragment of unique size for each *Adh* insertion. A total of 30 independent, autosomal single-insert lines were used for ADH assays, consisting of the wild-type (10), Δ1762–1765 (9), Δ1766–1769 (4), and Δ1762–1769 (7) transformant classes.

ADH assays: ADH enzymatic activity was measured following the procedure of Maroni (1978), using isopropanol as the substrate. For analysis of adult ADH activity, five 6- to 8-day-old males heterozygous with respect to the *Adh* insertion were used (Parsch *et al.* 1997). Units of ADH activity were measured as micromoles of NAD reduced per minute per milligram of total protein. Total protein was estimated using the method of Lowry *et al.* (1951). Differences in ADH activity between mutant and wild-type transformants were tested by analysis of variance (ANOVA) using a model that accounts for position effects within each transformant class (Laurie-Ahlberg and Stam 1987).

ADH activity levels were measured for different developmental stages and parts of the body using the above method, with the following modifications: For embryonic and larval assays, transformants homozygous for the appropriate *Adh* insertion were crossed to a *y w*; Adh^{fn6} stock to produce offspring heterozygous with respect to *Adh* insertion. Freshly laid eggs were collected ~1 hr after oviposition and incubated for

an additional 19 hr at room temperature. A total of 40 20-hr heterozygous embryos were used for each ADH assay. Larval assays were performed on preparations of 20 heterozygous larvae collected at each instar stage. For body part assays, 20 6- to 8-day-old heterozygous males were dissected and preparations from head, legs, thorax, and abdomen were used for separate ADH assays.

Analysis of *Adh* mRNA levels: Individual wild-type and Δ1762–1765 transformed lines exhibiting ADH activities typical of their respective transformant class were subjected to quantitative Northern blot analysis. Total RNA was prepared from 10, 6- to 8-day-old males heterozygous for the *Adh* insertion using TRIzol reagent (Life Technologies) and following the manufacturer's protocol. Northern blotting was performed as descibed in Sambrook *et al.* (1989), with ~0.5 μg of RNA loaded into each lane of the gel. The *Adh* probe was prepared from a PCR product spanning bases 739–1924 of the *Wa-f* allele (Kreitman 1983) and was labeled with [^{32}P] dATP using the RadPrime DNA labeling system (Life Technologies). As a control for equal sample loading, the blot was simultaneously hybridized with a probe prepared from a genomic clone of the *D. melanogaster Dras*2 gene (Bishop and Corces 1988). The *Adh* probe detects a transcript of 1.1 kb, while the *Dras*2 probe detects a transcript of 1.6 kb. Relative estimates of *Adh* mRNA levels were obtained by scanning densitometry using the Alpha Imager 2000 (Alpha Innotech Corporation). Because of the high concentration of *Adh* mRNA relative to *Dras*2 mRNA, two separate exposures of the same filter were used for mRNA quantitation. A short exposure (20 min) was used for *Adh*, while a longer (2-hr) exposure was used for *Dras*2. All exposures were carried out at −80° using BioMax (Eastman Kodak, Rochester, NY) film with the manufacturer's intensifying screen.

RESULTS

3′-UTR sequence organization: Previous phylogenetic analysis has identified a completely conserved sequence within the 3′-UTR of *Adh* sequences from 10 different Drosophila species spanning three subgenera (Parsch *et al.* 1997). The sequence consists of 8 bases (AAGGC TGA). Of the 8 bases (AAGTCTGA), 7 are conserved in the *Adh-2* sequence of the Mediterranean fruit fly, *Ceratitis capitata*. In all cases, the highly conserved sequence is located between the stop codon and the polyadenylation signal. The length of sequence separating these landmarks, however, varies among species. Species from the subgenus Sophophora, as well as *D. hydei* and *D. mulleri* from the subgenus Drosophila, show a similar sequence organization (Figure 1). In these species, the highly conserved 8-base sequence is located 74–115 bases downstream of the stop codon and 24–45 bases upstream of the polyadenylation signal. The two Hawaiian species from the subgenus Drosophila (*D. affinidisjuncta* and *D. silvestris*) have a very different organization of their 3′-UTRs. In these two species, the conserved 8-base motif is located much farther from the stop codon (236–240 bases) and much closer to the polyadenylation signal (6–7 bases). The *D. lebanonensis* 3′-UTR is similar to that of the Sophophora species, though the distance between the conserved 8-base sequence and the polyadenylation site is greater (82 bases). The medfly sequence resembles the Sophophora sequences in its distance between the stop codon and the conserved 8-base sequence (101 bases), but the distance between the conserved sequence and the polyadenylation signal is much shorter (4 bases) and is more similar to that found in the Hawaiian species.

Deletion analysis of the highly conserved 3′-UTR sequence: To test the functional significance of the highly conserved 3′-UTR sequence, *in vitro* deletion mutagenesis was performed on the wild-type *D. melanogaster Adh* gene. Three separate deletions were made within the conserved eight-base sequence: Δ1762–1765, which deleted the first four bases of the eight-base motif; Δ1766–1769, which deleted the last four bases; and Δ1762–1769, which deleted the entire eight-base sequence. The mutant constructs were introduced into the *D. melano-*

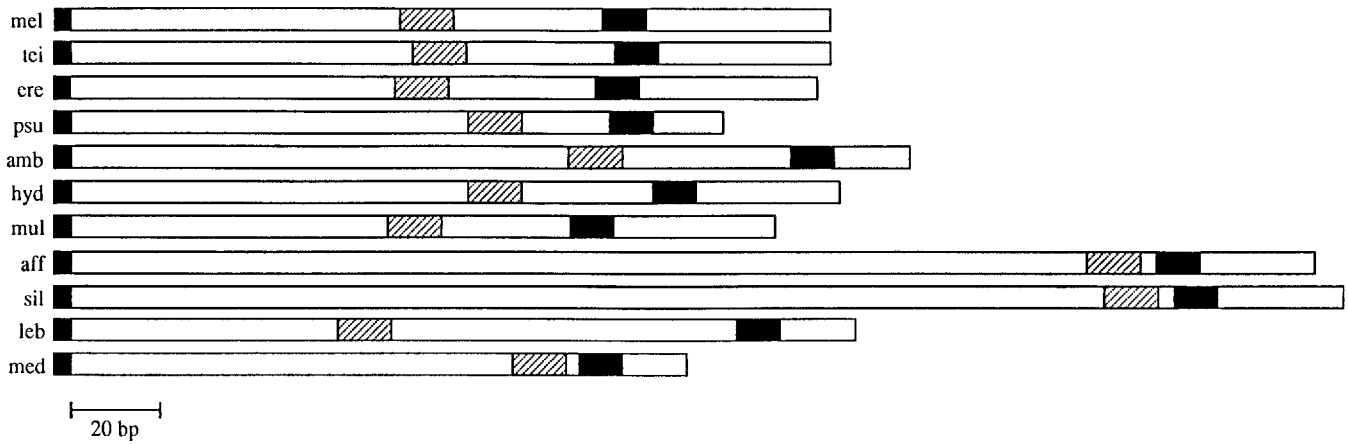

Figure 1.—Organization of the *Adh* 3′-UTR. The region depicted begins with the stop codon (leftmost solid box) and ends at the polyadenylation site. The highly conserved eight-base motif is shown as a hatched box and the polyadenylation signal is shown as a solid box. The separating sequences are shown as open boxes, drawn approximately to scale. mel, *D. melanogaster*; tei, *D. teisseri*; ere, *D. erecta*; psu, *D. pseudoobscura*; amb, *D. ambigua*; hyd, *D. hydei*; mul, *D. mulleri*; aff, *D. affinidisjuncta*; sil, *D. silvestris*; leb, *D. lebanonensis*; med, *Ceratitis capitata*.

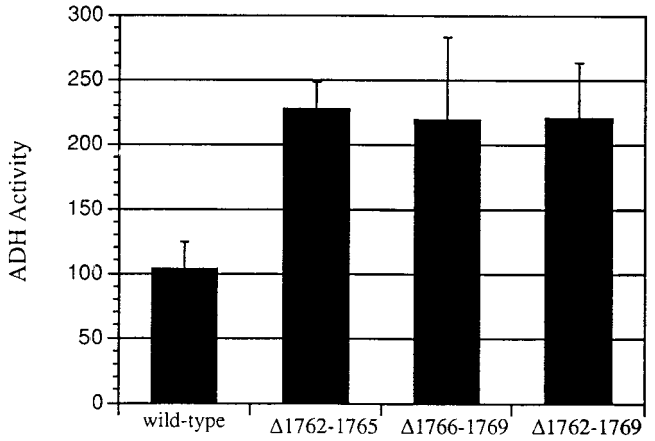

Figure 2.—Average ADH activity of wild-type, Δ1762–1765, Δ1766–1769, and Δ1762–1769 transformed lines. Activity is given in units of micromoles of NAD reduced per minute per milligram of total protein (multiplied by 100). Error bars represent the least significant difference at the 5% level. Least significant differences were calculated for individual comparisons, with the activity of each mutant line being compared to wild type.

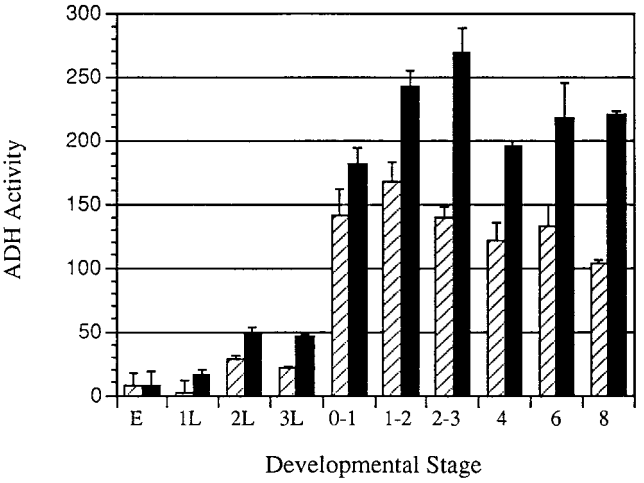

Figure 3.—Average ADH activity of wild-type (hatched boxes) and Δ1762–1765 (solid boxes) transformants at different developmental stages. Activity was measured for eggs (E), first instar larvae (1L), second instar larvae (2L), third instar larvae (3L), and adults of various days of age (0–1, 1–2, 2–3, 4, 6, 8). Units of ADH activity are the same as in Figure 2. Error bars represent the range of activities observed at each developmental stage.

gaster genome through *P*-element-mediated germline transformation, and the ADH activity of the transgenic flies was measured spectrophotometrically as micromoles of NAD reduced per minute per milligram of total protein (multiplied by 100). For all three deletion constructs, there was a greater than twofold increase in ADH activity relative to transformed lines containing the wild-type *Adh* gene (Figure 2). The average ADH activity of wild-type transformants was 103.2 units. The average activities of Δ1762–1765, Δ1766–1769, and Δ1762–1769 transformants were 226.9, 219.1, and 220.3 units, respectively. The difference in ADH activity level was highly significant ($P < 0.001$) for comparisons of all deletion lines with wild type. There were, however, no significant differences in activity levels among transformants containing the three deletion constructs. Thus, deletion of all or part of the highly conserved eight-base sequence has the same effect in increasing ADH activity. Under the assumption that the three deletions are functionally equivalent, we chose to focus on one of the deletions (Δ1762–1765, for which we had the greatest number of transformed lines) and selected a line that showed an average level of ADH activity for its transformant class for further characterization (see below).

Developmental stage and body-part ADH activity: Deletion of the highly conserved 3′-UTR sequence clearly leads to a significant increase in adult ADH activity (Figure 2). To determine whether or not this increase is present at different developmental stages, we performed additional ADH assays on eggs, larvae, and adults of wild-type and Δ1762–1765 transformants. The results are shown in Figure 3 and clearly indicate that there is an increase in ADH activity at all larval and adult stages. ADH activity could not be distinguished from background during the embryonic stage of either wild-type or Δ1762–1765 lines (Figure 3). The overall developmental pattern of ADH expression appears to be similar in both wild-type and Δ1762–1765 flies, with relatively low levels of ADH activity in larvae (slightly increased during second and third instar stages) and much higher levels in adults. Although the ADH activity of Δ1762–1765 transformants is greater than that of wild-type transformants at all adult stages, the difference in activity level is not constant throughout adult development (Figure 3). Within the first 3 days, both wild-type and Δ1762–1765 flies show the highest levels of ADH activity. The difference between wild-type and Δ1762–1765 activity is ~1.3-fold at these early adult stages. After day 3, the activity of both wild-type and Δ1762–1765 transformants appears to level off and the difference in ADH activity is approximately 2-fold (Figure 3). Such a developmental pattern in activity level may be indicative of a negative regulatory mechanism in which one or more components are not fully expressed until later in development (after day 2).

The ADH activity of various parts of the body was also examined in order to determine whether deletion of the highly conserved 3′-UTR sequence affected the spatial distribution of *Adh* expression in adult flies. Activity was measured for preparations of head, legs, thorax, and abdomen from both wild-type and Δ1762–1765 transformants (Figure 4). The results indicate that there is an increase in ADH activity in all Δ1762–1765 body part preparations relative to wild type (Figure 4). This difference in activity level is roughly twofold for all prep-

arations and is thus similar to the difference measured in whole adult flies (Figure 2).

Comparison of wild-type and mutant mRNA levels: Northern blot analysis was used to estimate the levels of *Adh* mRNA in wild-type and Δ1762–1765 transformants. Figure 5 shows the results of a Northern blot of two separate preparations of total RNA from adult flies of each transformant class. The blot was hybridized with an *Adh* probe, as well as a *Dras*2 probe, which served as a control for equal sample loading. Each probe detected only a single band corresponding to the mature transcript of the appropriate size. The relative amount of *Adh* mRNA in each lane was determined from band density (standardized by the density of the *Dras*2 band in the same lane). Figure 5 indicates that there is a clear increase in *Adh* mRNA levels in Δ1762–1765 transformants relative to wild type. The difference in *Adh* mRNA concentration was estimated to be 2.4-fold; thus the increase in ADH activity in the 3′-UTR deletion lines (Figure 2) can be accounted for by an underlying increase in *Adh* mRNA.

DISCUSSION

Noncoding regions of genes, such as introns and UTRs, typically show much higher levels of sequence divergence than do protein-encoding regions (Li and Graur 1991). Such a pattern is found for the Drosophila *Adh* gene. The *Adh* protein-encoding region is sufficiently conserved to allow for an unambiguous alignment of sequences from within the genus Drosophila (Sullivan *et al.* 1990; Parsch *et al.* 1997). The *Adh* noncoding regions, however, show too much divergence for unambiguous alignment across the entire genus (Sullivan *et al.* 1990; Parsch *et al.* 1997). In addition to the many nucleotide substitutions that have occurred within the 3′-UTR, there have also been numerous insertion/deletion events. The result is that the overall 3′-UTR length varies from 152 to 293 bases within the genus.

Given the high level of DNA sequence divergence within the 3′-UTR, it is remarkable that a perfectly conserved eight-base sequence can be found between the stop codon and the polyadenylation signal in every Drosophila species analyzed (Figure 1). The most distantly related Drosophila species are estimated to have diverged from a common ancestor 60 million years ago (Ayala *et al.* 1996; Kwiatowski *et al.* 1997). The sequence is also conserved (though not perfectly) in the *Adh-2* sequence of the Mediterranean fruit fly, which is estimated to have diverged from the Drosophila lineage 100 mya (Ayala *et al.* 1996; Kwiatowski *et al.* 1997). This high level of conservation suggests that the 3′-UTR sequence may be of functional significance, a hypothesis that was tested experimentally using the techniques of *in vitro* deletion mutagenesis and *P*-element-mediated germline transformation.

Deletion of the first four bases, the last four bases, or the entire eight-base sequence led to a significant increase in adult ADH activity relative to that of wild-type flies (Figure 2), indicating that this sequence does play a functional role in *Adh* gene expression. The activity increase was approximately twofold for each deletion construct and there was no significant difference in ADH activity among transformants containing different deletion constructs (Figure 2). These results, along with the high level of sequence conservation, suggest that

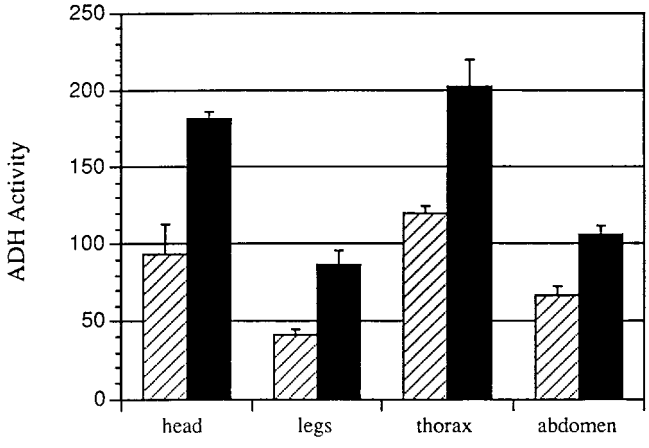

Figure 4.—Average ADH activity of various body part preparations of wild-type (hatched boxes) and Δ1762–1765 (solid boxes) transformants. Adult flies (6–8 days of age) from each line were dissected and activity was measured separately for preparations from head, legs, thorax, and abdomen. Units of ADH activity are the same as in Figure 2. Activity is scaled by the total amount of soluble protein isolated from each preparation. Error bars represent the range of activities observed for each preparation.

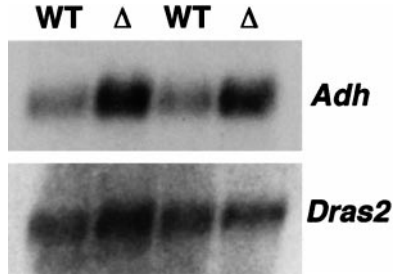

Figure 5.—*Adh* mRNA levels in wild-type (WT) and Δ1762–1765 (Δ) transformants. Two separate preparations of total RNA from adult flies (6–8 days of age) of each transformant class were used for quantitative Northern blot analysis. The blot was hybridized with an *Adh* probe that detects a mature transcript of 1.1 kb. As a control, the blot was simultaneously hybridized with a *Dras2* probe that detects a transcript of 1.6 kb. Relative amounts of *Adh* mRNA were estimated based on band density (corrected by the amount of total RNA in each lane). Because of the high concentration of *Adh* mRNA relative to *Dras*2 mRNA, two separate exposures of the same filter were used for mRNA quantitation (see materials and methods). The ratio of *Adh* mRNA in Δ1762–1765 to wild-type transformants is 2.4:1.

the entire eight-base motif must be intact for the sequence to function properly. It is possible, however, that certain substitutions within the eight-base consensus sequence do not disrupt function. This idea is supported by the observation that the eight-base motif is not perfectly conserved in the medfly, though its effect on *Adh* expression in the medfly is unknown.

What molecular mechanism lies behind the twofold increase in ADH activity caused by deletion of the conserved 3′-UTR sequence? Because deletion of the UTR sequence has no effect on the amino acid sequence of the ADH protein, the difference in activity must be the result of an increase in the amount of ADH protein. One possibility is that the 3′-UTR deletion mutations lead to a disruption of the *Adh* mRNA secondary structure. A portion of the conserved eight-base motif (bases 1762–1764) is involved in a phylogenetically predicted long-range pairing with bases 810–812 of exon 2 (Parsch *et al.* 1997). The 810–812/1762–1764 pairing, however, appears to be quite weak in *D. melanogaster*, consisting of two AU base pairs and one GU wobble pair. Disruption by a mutation at site 819 of a stronger long-range pairing (three GC pairs) between bases 817 and 819/1756 and 1758 resulted in a 15% reduction in *Adh* expression (Parsch *et al.* 1997). It thus seems unlikely that the disruption of the weaker pairing stem would result in the observed 100% increase in *Adh* expression. Furthermore, the Δ1766–1769 mutation does not disrupt the 810–812/1762–1764 pairing, yet results in a 100% increase in *Adh* expression (Figure 2). This suggests that, though it may be involved in a long-range RNA-RNA pairing, the eight-base 3′-UTR motif also has a function in *Adh* regulation that is unrelated to RNA secondary structure. The regulatory function may explain why we did not observe a reduction in ADH activity in our previous experiment in which we made a site-directed mutation at position 1756 (Parsch *et al.* 1997). It may be that this single nucleotide change adjacent to the conserved motif also leads to an increase in *Adh* expression that masks any reduction in expression caused by the disruption of the 817–819/1756–1758 pairing. In fact, our previous results indicate that there is a slight increase in ADH activity in transformed lines with the 1756 mutation, though this increase is not significant (Parsch *et al.* 1997).

A second possibility is that the 3′-UTR motif is involved in an RNA processing event that affects the translation rate of the mature mRNA. Laurie and Stam (1994) report that a noncoding sequence within the first *Adh* intron affects the amount of ADH protein in adult flies without affecting levels of *Adh* mRNA and suggest that this sequence may function in polyadenylation. The highly conserved 3′-UTR sequence, though it is located just upstream of the polyadenylation signal in all species analyzed, does not appear to function through the same mechanism as the intronic sequence. Results of Northern blot analysis indicate that there is a greater amount of *Adh* mRNA present in deletion lines than in wild-type lines (Figure 5). The difference in mRNA level appears to be equal to the difference in ADH activity between mutant and wild-type lines (Figures 2 and 5), and thus it is likely that the increase in the amount of ADH protein is the result of an underlying increase in mRNA levels.

The increased amount of *Adh* mRNA found in the deletion mutants could be the result of an increase in either *Adh* transcription rate or *Adh* mRNA stability. While the results of this study do not rule out the possibility of an increased level of *Adh* transcription in the mutant lines, this explanation seems unlikely for several reasons. All known regulatory elements that affect *Adh* transcription have been mapped to the 5′ flanking region of the gene, including the enhancer sequences required for proper larval and adult *Adh* expression. While transcription enhancing/silencing sequences have been identified within the 3′-UTR of other genes (Le Cam and Legraverend 1995; McDonough and Deneris 1997), such sequences appear to be exceedingly rare and so far have been reported only for highly regulated mammalian genes. The short length of the conserved sequence (eight bases) and the fact that its location appears to be constrained to a specific region of the 3′-UTR upstream of the polyadenylation signal also make it unlikely that it is involved in transcriptional control.

Two sequence motifs involved in post-transcriptional regulation of gene expression have been identified within the 3′-UTRs of *D. melanogaster* genes involved in the Notch signaling pathway (Lai and Posakony 1997; Leviten *et al.* 1997). These sequences, designated as the Brd box (AGCTTTA) and the GY box (GTCTTCC), have been shown to mediate negative regulation of both transcript and protein levels *in vivo* (Lai and Posakony 1997). The Brd box and the GY box of the *E(spl)m4* gene are both perfectly conserved between *D. melanogaster* and *D. hydei* and are located within a region of the 3′-UTR just upstream of the polyadenylation signal (Lai and Posakony 1997). While the highly conserved *Adh* 3′-UTR sequence (AAGGCTGA) does not match either of the above motifs, it is similar to the Brd box in its core sequence of GCT and in having an A at the first and last position of the motif. The medfly *Adh* 3′-UTR motif (AAGTCTGA) does not share the core sequence of GCT found in the Brd box, but does share four consecutive bases with the GY box (GTCT). The similarity of the *Adh* 3′-UTR sequence to these other conserved motifs, along with its role in the negative regulation of mRNA and protein levels, suggests that it may function through a similar mechanism. Though genes containing Brd box and GY box motifs are expressed in cells of the peripheral nervous system during development, the regulatory function of the Brd box has been shown to be both spatially and temporally general (Lai and Posakony 1997). This general effect on protein and

transcript levels is thus similar to that observed in the *Adh* 3′-UTR deletion experiments. The Brd box, however, differs from the *Adh* 3′-UTR sequence in that the former is often found in multiple copies (Lai and Posakony 1997), while the latter is found only as a single copy in all of the *Adh* sequences analyzed (Figure 1).

While the exact molecular mechanism responsible for the increase in *Adh* expression observed in the 3′-UTR deletion mutants remains obscure, the overall phenotypic effect of the deletions is clearly a large increase in *in vivo* ADH activity. This implies that the eight-base 3′-UTR sequence plays a negative regulatory role in *Adh* expression in wild-type flies. The finding that such a negative regulatory element is highly conserved is unexpected in light of previous population genetic and molecular evolutionary studies of *Adh*. For example, the amino acid replacement that distinguishes the Fast and Slow ADH allozymes in *D. melanogaster* results in an approximatly twofold increase in ADH activity in Fast homozygotes, yet there does not appear to be purifying selection against this replacement. On the contrary, it appears that the Fast allele has risen to high frequency relatively recently and may be favored by selection, at least in some environments (Oakeshott *et al.* 1982; Kreitman 1983; Aquadro *et al.* 1986; Mercot *et al.* 1994). Similarly, an intronic polymorphism that is typically associated with *Adh-f* alleles has been shown to increase ADH activity levels and also appears to be a target of positive selection (Laurie *et al.* 1991; Berry and Kreitman 1993; Laurie and Stam 1994). These observations raise the question of why nucleotide changes within the highly conserved 3′-UTR sequence are strongly selected against, while changes at other sites that have the same phenotypic effect on ADH activity appear to be selectively favorable.

A clear difference between the effect on ADH activity caused by deletion of the conserved 3′-UTR sequence and that caused by the Fast/Slow amino acid replacement is that the 3′-UTR deletion results in an increase in mRNA levels, while the amino acid replacement affects only the catalytic efficiency of the ADH enzyme, not the amount of *Adh* mRNA. Thus, even though these two sequence changes may have nearly identical phenotypic effects when measured as *in vivo* ADH activity, the phenotypes may differ greatly (twofold) when measured as *in vivo Adh* mRNA levels. Similarly, other naturally occurring polymorphisms within the *D. melanogaster Adh* gene have been shown to affect the total amount of ADH protein in adult flies, resulting in an increase in *in vivo* ADH activity, without affecting levels of *Adh* mRNA (Laurie and Stam 1988, 1994; Stam and Laurie 1996). It is thus likely that any potential benefit of increased ADH activity caused by disruption of the 3′-UTR sequence is outweighed by the cost of maintaining such high concentrations of *Adh* mRNA. The translation of an amount of mRNA greater by twofold may have a large energetic cost and may occupy a substantial fraction of the available ribosomes within the cytoplasm, leaving them unavailable for the translation of other cellular proteins. ADH may be particularly sensitive to such effects because it is expressed at very high levels, accounting for an estimated 1–2% of the total translational activity in wild-type adult flies (Benyajati *et al.* 1980).

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