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# Translation Initiation in *Drosophila melanogaster* Is Reduced by Mutations Upstream of the AUG Initiator Codon

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**The importance to in vivo translation of sequences immediately upstream of the *Drosophila* alcohol dehydrogenase (*Adh*) start codon was examined at two developmental stages. Mutations were introduced into the *Adh* gene in vitro, and the mutant gene was inserted into the genome via germ line transformation. An A-to-T substitution at the −3 position did not affect relative translation rates of the ADH protein at the second-instar larval stage but resulted in a 2.4-fold drop in translation of ADH at the adult stage. A second mutant gene, containing five mutations in the region −1 to −9, was designed to completely block translation initiation. However, transformant lines bearing these mutations still exhibit detectable ADH, albeit at substantially reduced levels. The average fold reduction at the second-instar larval stage was 5.9, while at the adult stage a 12.5-fold reduction was observed.**

Inspection of the mRNA sequence immediately flanking the initiator codon of eukaryotic mRNAs has led to the construction of consensus sequences for vertebrates, *Drosophila* species, plants, and *Saccharomyces cerevisiae* (3, 7, 11, 13, 19, 20). All eukaryotic groups examined to date have a strong preference for a purine at the −3 position upstream of the AUG codon and prefer A or C at positions −1, −2, −4, and −5. In general, the degree of A or C bias among the eukaryotic groups can be explained by the normal base composition bias of each species as opposed to mechanistic differences between species (3a). Mutagenesis experiments have demonstrated the importance of these sequences to relative translation rates of the rat preproinsulin mRNA in COS cells (12). As predicted from the consensus sequence, substituting T for A at the −3 position had the most deleterious effect on translation of any of the single mutations. In addition, strong interactions were observed between certain nucleotides at the −3 position and those at the −1, −2, and +4 positions. In particular, if T was at the −3 position, nonconsensus sequences at the −2, −1, and +4 positions greatly depressed translation. Indeed, translation was virtually undetectable if T's were present at all four of these positions. Consistent with this observation, no eukaryotic gene has been reported which contains T's at all four of these positions flanking a start codon for a major coding region, and less than 1% of reported eukaryotic mRNAs contain T's at both positions −3 and +4 (unpublished analysis of GenBank release 63). A further extension of these mutational studies (14) led Kozak to propose GCCG CCA/GCCAUGG as the optimal sequence for initiation of translation.

Despite the strong concordance between the mutational analysis of the preproinsulin gene and the statistical analysis of the mRNA data base, the generality of these studies to other mRNAs and to other eukaryotes has been questioned. First, considerable variation in the consensus sequences exists among various eukaryotic groups (3). Second, a severalfold-smaller effect of substituting nonconsensus sequences in *S. cerevisiae* was observed (1), and substitutions at the −3 position had no effect on translation in a cell-free wheat germ translation system (20). However, Kozak (16) has shown recently that the −3 position has a strong influence on the wheat germ in vitro translation system in the presence of higher concentrations of magnesium (which also increases the fidelity of start codon usage). This result suggests that measurements of in vivo translation of cultured cells may also be affected by culture conditions.

The in situ effects of mutations on start codon flanking sequences in a multicellular eukaryote have not been investigated previously, and as a consequence, potential developmental differences have not been examined. We report herein the in situ effects of such mutations on the sequences flanking the start codon of the alcohol dehydrogenase (ADH) gene in *Drosophila melanogaster*. In vitro-synthesized point mutations of the *Adh* gene were transduced into the genome, and the relative translation rates of the resulting transformant lines were assessed in situ at two developmental stages. Two mutant *Adh* genes were analyzed, one containing an A-to-T mutation at the critical −3 position and the other, containing five point mutations, designed to give the poorest predicted sequence context.

## MATERIALS AND METHODS

**In vitro mutagenesis.** Oligonucleotide-directed, site-specific mutagenesis of the *Adh* gene was performed by the methods of Sarkar and coworkers (25). Two mutant genes were constructed from the following oligonucleotides: CAA AGTAAACGACATAAAGAGTTGTTTTTTGCTTTAGCA (designated A) and CGACATGGAGACTTCTT (designated B). The sequences of these oligonucleotides correspond to the minus strand of the *Adh* start codon region, with the exception of the underlined nucleotides designed to introduce substitutions. A clone containing a 3.2-kb *Xba*I insert of the *D. melanogaster* $Adh^F$ gene in the Bluescript vector (Stratagene, Inc.) was used for the mutagenesis experiments. After the in vitro synthesis of the mutations, the plasmid DNAs were transfected into *Escherichia coli* cells. Plasmids bearing the mutations were screened by differential colony hybridization by using the appropriate $^{32}$P-end-labeled oligonucleotides (described above). Hybridizations

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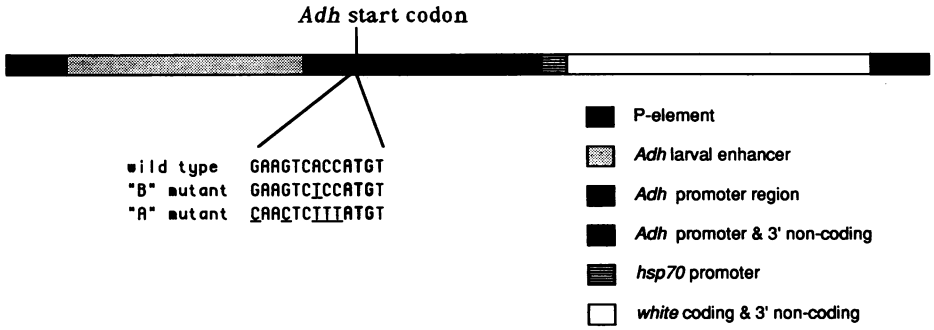


FIG. 1. Map of the P-element transformation vector bearing the *Adh* translation initiation mutations. A 3.9-kb *Sal*I-*Xba*I fragment containing the *Adh* larval enhancer and a 3.2-kb *Xba*I fragment containing the *Adh* promoter–ADH-coding–3′ noncoding region were inserted into the pW5 P-element transformation vector. This vector contains the *white* gene, under the regulation of the *hsp70* promoter, as the selectable marker for *Drosophila* germ line transformation.

were conducted at 42°C without formamide. The filters were first washed at room temperature for 10 min in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.2% sodium dodecyl sulfate (SDS). The filters bearing the A mutant plasmids were further washed at 58 to 60°C for 15 min in 0.2× SSC–0.2% SDS. The filters bearing the B mutant plasmid DNA were further washed at 52°C in 2× SSC–0.2% SDS for 10 min. Positive colonies were picked, plated, and subjected to a second round of colony hybridization to isolate colonies containing only *Adh* mutant gene-bearing plasmids. The sequence of the mutations was confirmed by dideoxy-chain termination with double-stranded plasmid DNA and an oligonucleotide primer (AGACCAATGCCTC CCAGACC) corresponding to the minus strand of the *Adh* gene downstream of the start codon.

**Germ line transformation.** The 3.2-kb *Xba*I fragments containing the A or B *Adh* mutations and an *Xba*I-*Sal*I 3.9-kb fragment containing the larval *Adh* enhancer (4) were ligated into the pW5 P-element germ line transformation vector (10). The *Adh* gene in this construct contains the wild-type transcriptional units for the proximal and distal transcripts. Although most of the larval enhancer as described by Corbin and Maniatis (4) is present in these constructs, we do not obtain full quantitative expression at the larval stages of our constructs in this particular vector. The *Drosophila* transformation vector carries an ampicillin resistance gene for selection in *E. coli* and a wild-type *white* ($w^+$) gene (under the transcriptional regulation of the *Hsp70* promoter) for selection in *D. melanogaster*. Plasmids were injected into embryos of the *Drosophila* ES3 strain, which has constitutive P-element transposase functions (24) and is a *w* mutant. Adult survivors were crossed singly to ES3 flies, and their progeny were screened for red eyes. Genetic crosses were done to determine which chromosomes contained the transduced mutant *Adh* genes and to replace the endogenous *Adh* wild-type gene with the $Adh^{fn6}$ null mutation. The Oregon-R control strain was homozygous for the same *Adh* allele (i.e., $Adh^F$) as that used in the mutagenesis experiments.

**RNase protection.** Total nucleic acid was isolated from late-second-instar (20 h) or 4- to 7-day-old adults (using an equal number of males and females) and subjected to quantitative RNase protection analysis as described by Murtha and Cavener (23). $^{32}$P-radiolabeled cRNA probes for the *Adh* and α*1-tubulin* mRNAs were synthesized. The *Adh* probe corresponded to a 604-nt fragment of the *Adh* gene containing portions of the second and third exons. The α*1-tubulin* probe corresponded to a 85-nt fragment of the α*1-tubulin* gene (4). A total of $10^5$ to $10^6$ cpm of each probe was hybridized to the same RNA sample (6 μg per sample for larvae and 10 μg per sample for adults). Duplicate samples of the RNase protection products were fractionated on 6% acrylamide–8 M urea gels and exposed first on XAR-5 film and then on a phosphor screen. The phosphor screen exposures were quantitatively analyzed by using a PhosphorImager (Molecular Dynamics, Inc.). Only the 320-nt protected fragment (ADH D+P) corresponding to a common region of both the proximal and distal *Adh* mRNAs was used in the determination of the abundance of *Adh* mRNA. The sum of the four α*1-tubulin* bands (80 to 90 nt) was used to estimate the relative abundance.

**ADH enzyme activity.** ADH enzyme assays were performed on larvae or adults homogenized in 50 mM $Na_2CO_3$ (pH 9.5) (50 μl of $Na_2CO_3$ per adult and 10 μl of $Na_2CO_3$ per larva). ADH activity reaction mixtures contained 10 to 45 μl of cleared supernatants from the whole fly homogenates in 50 mM $Na_2CO_3$–2 mM $NAD^+$–0.12% (vol/vol) 2-butanol. The reduction of the $NAD^+$ was monitored spectrophotometrically for 5 min at 340 nm. Under these conditions, the amount of ADH protein is directly proportional to ADH activity (21). The soluble protein concentration of each fly homogenate was estimated by the method of Bradford (2). The same group of flies reared at the same time was used to determine the levels of ADH activity and *Adh* mRNA. Flies for both experiments were homogenized on the same day.

## RESULTS

**Isolation of *Adh* mutant germ line transformants.** Two *Adh* mutants were generated in vitro (Fig. 1). The B mutant contains a single A-to-T mutation at the −3 position upstream of the *Adh* start codon. The A mutant contains five point mutations: C to T at −1, C to T at −2, A to T at −3, G to C at −6, and G to C at −9. The identities of these mutations were confirmed by DNA sequence analysis. Three independent transformant lines were obtained for both the A and B series. The transformant-bearing chromosomes for each line were crossed into an *Adh* null background for further study. All transformant strains were homozygous for their transduced *Adh* mutant gene and were reared on a standard cornmeal-molasses diet containing proprionic acid to inhibit fungal growth.

**Relative translation rates.** In order to estimate relative

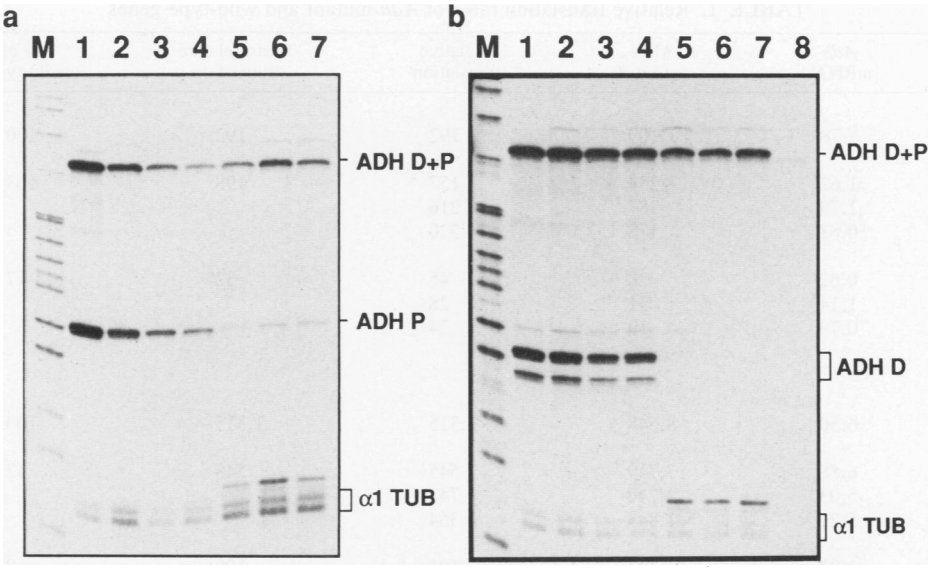


FIG. 2. RNase protection of *Adh* and *α1-tubulin* mRNAs. (a) Larval samples; (b) adult samples. Lanes: 1, wild-type; 2, B18; 3, B21; 4, B27; 5, A11; 6, A43; 7, A51; 8, $Adh^{fn6}$ negative control, which lacks the *Adh* protected fragments described above. In lanes 5 to 7, an extra band of approximately 100 nt is due to a partial RNase digestion of ADH P (a) or ADH D (b) caused by the mismatched bases at the site of the five substitution mutations in the A series transformant lines. However, the quantitation of *Adh* mRNA was determined from the ADH D+P band only. This band corresponds to a region downstream of the alternative transcription start sites and downstream of the A and B mutations. The quantitation of the *α1-tubulin* mRNA was determined from the sum of the four bracketed bands labeled α1 TUB. M, Molecular weight standards; ADH D+P, 320-nt protected fragment common to both distal (D) and proximal (P) *Adh* transcripts; ADH P, 168-nt protected fragment from the proximal *Adh* transcript; ADH D, 134-nt protected fragments from the distal *Adh* transcript; α1 TUB, four protected fragments approximately 80 to 90 nt from the *α1-tubulin* mRNA.

translation rates, the level of *Adh* mRNA was determined by quantitative RNase protection experiments (Fig. 2). Since the genetic background has some influence on the transcription of *Adh* transformants (6), the amount of *Adh* mRNA was normalized to the level of *α1-tubulin* mRNA. *α1-tubulin* is constitutively expressed throughout development and serves as a useful standard for mRNA expression (4, 8). A Molecular Dynamics PhophorImager, which provides an accurate linear quantitation of radioactivity over several orders of magnitude, was used to estimate the relative abundance of the mRNAs. ADH enzyme activity was measured in triplicate for each of the transformant lines. Each relative translation rate was estimated as the ratio of ADH activity to the normalized level of *Adh* mRNA.

The relative translation rates of the B series transformant lines, which bear a single A-to-T transversion at the −3 position upstream of the *Adh* start codon, are not significantly different from that of the wild-type Oregon-R control strain at the second instar larval stage (Table 1). At the adult stage, this mutation effects a modest 42% (2.4-fold) decrease from wild-type levels. A more severe reduction of relative translation rates was observed for the A series transformant lines, which contain mutations at five positions upstream of the start codon. For the second larval instar, relative translation was reduced to 17% of the wild-type level, and at the adult stage the level averaged only 8% of the wild-type level (a 5.9- and a 12.5-fold reduction, respectively). Although the parameters estimated in these calculations are expected to exhibit a considerable amount of strain variation, the relative translation rates among each of the three independent strains per mutant gene display good agreement within each developmental stage.

## DISCUSSION

We assert that the reduction of ADH expression in the *Adh* mutants is due to a reduction in the relative rates of translational initiation. The *Adh* mutations generated in this study all exist in the 5′ untranslated region of the *Adh* mRNA. Therefore, the decrease in the ratio of ADH activity to the level of *Adh* mRNA is not due to differences in the ADH enzyme, nor are they due to differences in translational elongation rates. Although these mutations might effect mRNA synthesis or stability, such changes should not effect the ratio of ADH activity to the level of *Adh* mRNA. An alternative possibility is that the relative translation efficiencies of the mutations may affect the steady-state levels of the mRNA. Although we see a reduced level of mRNA in larvae bearing the mutants compared with that of the Oregon-R wild-type control, it is unlikely that this difference is due to the effect of the relative efficiencies of translation, since the mRNA levels of the two mutants are similar but exhibit large differences in the levels of ADH protein. Moreover, the lower mRNA levels in these transformant lines at the larval stage are most likely due to a lack of full wild-type transcription from the proximal (larval) promoter characteristic of this recombinant *Adh* gene in the presence of the P-element vector.

Certain conditions and assumptions regarding the accuracy of the ratio of ADH activity to *Adh* mRNA must be considered. First, the steady-state level of the mRNA must be measured quantitatively. RNase protection experiments have proven to be the most quantitative method for estimating *Adh* mRNA (18). We have increased the accuracy of these estimates by (i) using a method to measure radioactivity in a gel fractionated band that provides linear detection

TABLE 1. Relative translation rates of *Adh* mutant and wild-type genes

| Stage and strain reduction | *Adh* mRNA[a] | ADH activity[b] | Relative translation[c] | Mean relative translation | % of wild type[d] | Fold decrease |
|---|---|---|---|---|---|---|
| Larval | | | | | | |
| Oregon-R | 5.78 | 1,109 | 192 | 192 | 100 | |
| B18 | 1.62 | 254 | 157 | 198[e] | 103 | 0 |
| B21 | 1.79 | 387 | 216 | | | |
| B27 | 0.81 | 178 | 220 | | | |
| A11 | 0.62 | 28 | 45 | 32[f] | 17 | 5.9 |
| A43 | 1.14 | 31 | 28 | | | |
| A51 | 0.79 | 19 | 24 | | | |
| Adult | | | | | | |
| Oregon-R | 6.50 | 8,548 | 1,315 | 1,315 | 100 | |
| B18 | 6.88 | 3,749 | 545 | 548 | 42 | 2.4 |
| B21 | 5.03 | 3,744 | 744 | | | |
| B27 | 5.21 | 1,843 | 354 | | | |
| A11 | 3.21 | 323 | 101 | 100 | 8 | 12.5 |
| A43 | 2.95 | 301 | 102 | | | |
| A51 | 3.32 | 323 | 97 | | | |

[a] *Adh* mRNA normalized to α*l-tubulin* mRNA (average of two values).
[b] ADH activity is expressed in micromoles of $NAD^+$ reduced per milliliter per minute per milligram of soluble protein × 100 (average of three independent samples).
[c] Relative translation is calculated as the ratio of ADH activity to level of *Adh* mRNA.
[d] Normalized to Oregon-R wild type.
[e] Mean of values for three B strains.
[f] Mean of values for three A strains.

over several orders of magnitude and (ii) normalizing the amount of *Adh* mRNA to the constitutively expressed α*l-tubulin* mRNA. The second assumption that we have made is that ADH activity is directly proportional to ADH protein levels. Detailed quantitative genetic studies have been conducted on the molecular expression of the *Adh* gene (21). These studies indicate that under the appropriate reaction conditions (used in this study), the level of ADH activity is directly proportional to the level of ADH protein.

We predicted from the analysis of the *Drosophila* sequence data base (3) and Kozak's (12) mutational analysis of the rat preproinsulin gene that the single most influential nucleotide change in the sequences flanking the *Adh* start codon would be an A-to-T substitution at the −3 position. Since the *Adh* gene already contains a T at the +4 position, this single mutation results in T at both −3 and +4. Kozak has previously noted the rarity of this combination in eukaryotes (11, 13). Indeed, less than 2% of the *Drosophila* start codons contain T at both these positions (reference 3 and unpublished data). It should be noted that the rest of the start codon context of the B mutant *Adh* gene agrees well with the consensus sequence. Specifically, at the −2 and −1 positions C is present. Kozak (12) has shown that C in these positions can partially compensate for the repression of translation caused by the presence of T at −3 and +4. Nonetheless, a four- to fivefold reduction in relative translation in the preproinsulin gene with the sequence TCC ATGT, compared with the sequence ACCATGT, is still seen. For the *Drosophila Adh* gene, no difference between these same two sequences (B mutant and wild type) at the second-instar larval stage is observed, and only a 2.4-fold reduction for adults is observed.

The A mutant was designed to create the worst predicted sequence context by substituting T at −3, −2, and −1. This combination, along with T at +4, leads to at least a 20-fold reduction in the relative translation of the preproinsulin gene (12). No *Drosophila* gene with T at all four of these positions has been reported. In addition, we mutated G to C at positions −9 and −6, since a recent report by Kozak (14) has indicated that G at these positions has a modest positive effect upon translation initiation. Thus, extrapolation from previous studies predicts that these mutations should completely block translation. Contrary to this prediction, significant translation of the A mutant is observed, albeit at a reduced level. When compared with wild-type levels, a 5.9-fold and a 12.5-fold reduction is observed for second-instar larvae and adults, respectively. It is interesting to note that for both of the *Adh* mutants, the effects in adults are approximately twofold more severe than they are in second-instar larvae. No other studies in eukaryotes are available to compare with these developmental differences.

Kozak (17) has recently shown that an mRNA secondary structure downstream of a start codon can compensate for a poor context. We searched the entire *Adh* mRNA for potential secondary structures and did not find structures which would form stable intramolecular duplexes with sequences near the 3′ side of the start codon. Thus, it is unlikely that the less dramatic reduction in translation observed in these experiments was due to compensation by secondary structures.

In general, we believe that this study confirms the importance of the sequences immediately flanking the start codon to translation initiation in eukaryotes, as originally proposed by Kozak (11–13). Specifically, the prediction that T at −3, −2, −1, and +4 should have a negative influence on translation was confirmed. Quantitatively, the influence of these

mutations on the *Adh* gene is less severe than it is on the rat preproinsulin gene in a mammalian cell but more severe than is suggested by in vivo studies in yeast (1). These interspecific differences are also reflected in the relative preferences for T at these four positions: in vertebrates, 3, 12, 7, and 13%; in *D. melanogaster*, 8, 18, 8, and 20%; and in yeast, 8, 23, 21, and 34% preference for the −3, −2, −1, and +4 positions, respectively (3a). Recent studies have suggested that the abundance and/or phosphorylation state of the translation initiation factor eIF2 may influence the degree of start codon selectivity (5, 9). Perhaps such differences in eIF2 may be partly responsible for the differences between species and between developmental stages in the degree of influence of the sequences flanking start codons.

The relative severity of these mutations is also relevant to the potential translation of upstream open reading frames (URFs). Approximately 9% of all eukaryotic genes contain URFs (13). The potential importance of URFs to translational regulation has been underscored by the demonstration that four URFs in the leader sequence of the yeast GCN4 mRNA regulate the translation of the GCN4 transcription factor (22). Kozak (15) has observed that translation of the preproinsulin gene is depressed by the insertion of an URF. However, the depression of translation by this URF is modulated by the sequence context surrounding the URF's start codon. A poor context represses downstream translation less than a good context. This observation lead Kozak (15) to propose the leaky scanning model for translation initiation. This model asserts that when the preinitiation complex encounters a start codon with a poor sequence context, it has a high probability of simply ignoring it and continuing downstream to the major start codon. Indeed, vertebrate (13) and *D. melanogaster* (3a) URF start codons typically have poor contexts as defined by the consensus sequences. Thus, under this scenario the URF paradox is resolved by the failure of the ribosome to recognize the URF start codon as such. However, our data for the *Drosophila Adh* mutants suggest that the preinitiation complex may frequently engage URF start codons even if they have poor sequence contexts. This is particularly germane to *D. melanogaster* development inasmuch as a large fraction of the mRNA encoding regulators of development contain multiple URFs. For example, an *antennapedia* mRNA contains 17 URFs (26). How the *Drosophila* translation machinery copes with the presence of multiple URFs remains a mystery.

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