Effects of a Transposable Element Insertion on Alcohol Dehydrogenase Expression in Drosophila melanogaster

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ABSTRACT

Variation in the DNA sequence and level of alcohol dehydrogenase (Adh) gene expression in Drosophila melanogaster have been studied to determine what types of DNA polymorphisms contribute to phenotypic variation in natural populations. The Adh gene, like many others, shows a high level of variability in both DNA sequence and quantitative level of expression. A number of transposable element insertions occur in the Adh region and one of these, a copia insertion in the 5′ flanking region, is associated with unusually low Adh expression. To determine whether this insertion (called R142) causes the low expression level, the insertion was excised from the cloned R142 Adh gene and the effect was assessed by P1 element transformation. Removal of this insertion causes a threefold increase in the level of ADH, clearly showing that it contributes to the naturally occurring variation in expression at this locus. Removal of all but one LTR also causes a threefold increase, indicating that the mechanism is not a simple sequence disruption. Furthermore, this copia insertion, which is located between the two Adh promoters and their upstream enhancer sequences, has differential effects on the levels of proximal and distal transcripts. Finally, a test for the possible modifying effects of two suppressor loci, su(w)/ and su(f), on this insertional mutation was negative, in contrast to a previous report in the literature.

Many studies have shown high levels of DNA sequence variation within the structural and regulatory regions of specific genes in natural populations of Drosophila (reviewed by Aquadro 1990). In addition, many other studies have used quantitative genetic methods to demonstrate high levels of genetic variation affecting the levels of expression of many of the same genes (reviewed by Laurie-Ahlberg 1985). However, there is still little connection between these two types of data. To determine what fraction of the DNA polymorphisms contribute to phenotypic variation in gene expression and whether certain types of polymorphism (base substitutions, unique sequence insertions/deletions or transposable element insertions) have greater (or qualitatively different) effects than others, individual sequence variants must be isolated and analyzed for their phenotypic effects.

One of the earliest investigations of restriction fragment length polymorphism (RFLP) in natural populations was the survey by Aquadro et al. (1986) of variation in the Alcohol dehydrogenase (Adh) gene region among 50 second chromosomes sampled from four North American populations. From this study, it was estimated that between any two randomly chosen chromosomes, one in 156 nucleotide sites differ by a base substitution. In addition, 80% of the chromosomes had at least one insertion or deletion relative to a consensus restriction map for the 13-kb region analyzed. Most of these length changes involve unique sequences up to ~200 bp in length, but there were also several transposable element insertions ranging in size from 0.34 to 10.2 kb. Although there is some variation in the levels and relative abundance of different types of polymorphisms, the Adh results provide a fairly typical picture of the extensive sequence variation found in other genes such as Ddc (Aquadro et al. 1992), Amy (Langley et al. 1988a), rosy (Aquadro et al. 1988), G6pd (Eanes et al. 1989), and white (Miyashita and Langley 1988).

The 50 chromosome substitution lines analyzed for RFLPs in the Adh region were also surveyed for variation in ADH expression using quantitative genetic methods (Aquadro et al. 1986). Much of the ADH activity variation among these lines is associated with a thr/lys substitution that produces two allozymes, Fast and Slow. There is an approximately continuous distribution of ADH activity within each allozymic class, but there is a clear break between the two distributions. In addition, several lines that appeared to be outliers in the initial survey, consistently show ADH activities that are not typical of their allozymic class (Laurie and Stam 1988; Laurie et al. 1991). This pattern of quantitative variation in enzymatic activity within each allozymic class (approximately continuous, but with a few apparent outliers) is typical of that for many other enzymes (Laurie-Ahlberg et al. 1980, 1982).

Associations between DNA sequence polymorphisms in the Adh region and quantitative genetic variation in...
ADH levels have suggested some hypotheses about which sequence variants may have causal effects on Adh expression (AQUADRO et al. 1986; LAURIE et al. 1991). Clearly, such hypotheses require direct experimental tests because associations for naturally occurring alleles may be spurious due to high levels of sequence polymorphism and linkage disequilibrium. We have used in vitro mutagenesis and P-element transformation for testing some of these associations. For example, we have shown that a 2.5–3.0-fold difference in ADH activity associated with the allozyme polymorphism is due to separate effects on the level of ADH protein and on catalytic efficiency of the enzyme. The thr/lys substitution affects catalytic efficiency but does not affect the level of ADH protein (CHOUDHARY and LAURIE 1991), while a unique sequence length polymorphism (V1), as well as other unidentified polymorphisms, affect the ADH protein level (LAURIE and STAM 1994). The V1 polymorphism accounts for some of the outliers in ADH activity level in the AQUADRO et al. (1986) survey.

Another outlier line in the AQUADRO et al. (1986) survey, RI42, will be the focus of this paper. It is the only line out of 50 analyzed that contains a transposable element in the 5' flanking region of Adh. It has a complete, 5.2-kb copia retrotransposon at position −243 bp upstream of the distal transcriptional start site. We have previously reported that this line shows an unusually low level of ADH activity, protein and RNA compared with other Slow lines (LAURIE and STAM 1988).

A substantial fraction of the Drosophila melanogaster genome (~10%) consists of transposable elements, including many different types of retrotransposons (MANNING et al. 1975; YOUNG 1979). Numerous studies have shown that natural populations of this species are highly polymorphic for transposable element insertions (CHARLESWORTH and LANGLEY 1991). An interesting feature of the observed pattern of variation is that the frequency of occupancy of a given site by a transposable element is typically very low. It is still not clear what process(es) account for this pattern, but two possibilities are selection against insertions due to their deleterious effects on fitness and ectopic exchange between elements at different locations that generate lethal chromosome rearrangements (MONTGOMERY et al. 1987; LANGLEY et al. 1988b). Further understanding of the population dynamics of transposable elements will require specific knowledge of the phenotypic effects of these insertions.

Although a large fraction of spontaneous visible mutations in the laboratory are caused by transposable element insertions (GREEN 1988), there is very little information about how they contribute to phenotypic variation in natural populations. The distinction between laboratory isolates of transposable element insertions and those that occur in wild-caught individuals is important. Analysis of the former demonstrates the mutagenic potential of insertions, whereas the latter represent a selected population. For example, it may be the case that the majority of insertions found in natural populations have very little or no phenotypic effect. Occasionally, associations between transposable element insertions and phenotypic variation have been observed in nature (e.g., bristle number variation and insertions in ac/sc) (MACKAY and LANGLEY 1987), but such associations have seldom, if ever, been tested directly. The copia insertion in RI42 provides a good opportunity for such a test and here we report the results of an in vitro mutagenesis experiment showing that, indeed, it does have a marked phenotypic effect on Adh expression.

In D. melanogaster, ADH is encoded by a single gene that produces two distinct transcripts from alternative promoters (Figure 1A). These transcripts are developmentally regulated such that the proximal transcript is predominant in larvae until mid to late third instar (BENVJAJATI et al. 1983; SAVAKIS et al. 1986) when there is a promoter switch. The distal transcript accumulates until the pupal stages at which point transcription of Adh is turned off until the adult stage when the distal transcript is the predominant form. Deletion mutagenesis and P-element transformation have shown that transcription of Adh is regulated by sequences immediately upstream of each promoter in conjunction with more distant enhancer elements (POSAKONY et al. 1985; CORBIN and MANIATIS 1989a, 1990). In RI42, the copia element has inserted between the enhancer sequences and the promoters for Adh (Figure 1A).

The copia element is 5.2 kb in length and, like other retrotransposons, has two direct long terminal repeats (LTRs) that flank each end of the element (Figure 1A). The LTRs are each 276 bp in length and each contains transcription initiation and polyadenylation sites (MOUNT and RUBIN 1985). In the case of the RI42 insertion, the transcriptional orientation is the same as the Adh gene. Copia transcripts are present throughout development, but reach their highest levels in second and third instar larvae. (PARKHURST and CORCES 1987).

In laboratory stocks, there are many examples of spontaneous mutations where the insertion of a transposable element has changed the expression of a gene by altering gene regulation, but the mechanism is different in each case depending on the type of transposable element and the location of the insertion (see SMITH and CORCES 1991, for review). A few examples are yellow2 (GEYER et al. 1986), cut3III (DORSETT 1993), forked1 (HOOVER et al. 1992), vermilion6 (FRIDELL et al. 1990) and white3prox (LEVIS et al. 1984; ZACHAR et al. 1985).

In this study of the RI42 allele, we have considered possible mechanisms by which a reduction in Adh expression could be caused by the copia insertion and some preliminary experiments were done to investigate these possibilities. In addition, we have tested for possible modifying effects of two suppressor loci, suppressor
of white<sup>pro</sup> [su(zea)] and suppressor of forked [su(f)]. Previous studies have shown that mutants at these loci modify the effects of the white<sup>pro</sup> mutant, which is caused by a copia insertion (Levis et al. 1984; Zacchar et al. 1985).

MATERIALS AND METHODS

Plasmid constructions: Three of the plasmids used for P-element transformation (pRH2, pLTR and pΔcopia) all have the same basic structure in which a 14.3 kb SacI/XhoI fragment of the RH42 allele (obtained from a λ clone provided by C. F. Aquadro) was inserted into the vector pPLΔI (Laurei-Ahlberg and Stam 1987) and then modified by the deletion of copia sequences in the case of pLTR and pΔcopia (Figure 1B). pRH2 contains the complete 5.2 kb copia element, pLTR contains just one 276-bp LTR and pΔcopia contains no copia sequence. The p WR-S construct (from Laurei-Ahlberg and Stam 1987) is similar except that it has an 8.0 kb SacI/Clal fragment containing an Adh allele originating from the Wr-S wild-type allele of Kreitman (1983), which has an Adh expression level typical of Slow alleles (Laurei et al. 1991). All four constructs have an 8.1 kb SacI fragment containing a wild-type rosy gene inserted at the XhoI site.

The pLTR construct was made by ligating an 11.3 kb SacI/BamHI fragment from the RH42 Adh allele into a pBS(-) vector (Stratagene, Inc.). This construct was digested with BalI, which removed all but the 276-bp LTR from the copia element, leaving the rest of the fragment intact. The new 6.3-kb SacI/BamHI fragment was religated into the original vector creating a 9.5-kb SacI/XhoI fragment with just the LTR at the copia insertion site.

To construct pΔcopia, pLTR was partially digested with XmaI, cutting out a 672-bp fragment containing the LTR. Then a replacement fragment was generated by polymerase chain reaction (PCR). The 5' PCR primer (located at position −248 bp from the distal transcription initiation site) has a single substitution creating an XmaI site and the 3' primer was 413 bp downstream of an XmaI site (Figure 1A). The PCR product was digested with XmaI and religated back into the XmaI partial digest of pLTR. This process precisely deleted the entire copia element including the 5-bp insertion site duplication (confirmed by DNA sequencing).

Production and sampling of transgenic fly stocks: The following procedures were described previously in detail (Ghoudary and Laurei 1991). Transformant stocks were produced by microinjection of embryos (from the ADH-null host stock Adh<sub>0</sub>-<sub>encry</sub>) and chromosome extraction. In addition, two stocks of the pRH2 transformant type were created by mobilizing a pRH2 insertion with the Δ2–3 chromosome (Robertson et al. 1988). Southern blot analysis was used to select isochromosomal stocks with a single insert and to check whether transformed constructs were still intact. The 41 single-insert stocks and nine control stocks were sampled in a randomized block design with two blocks and two replicates per block.

Comparison of transformants with wild-type stocks: Each Adh fragment used in these experiments consists of ≥5.9 kb of 5' flanking DNA, the transcriptional unit, and >500 bp of...
3' flanking DNA. Previously, this fragment size was shown to be sufficient for an approximately normal pattern and level of ADH expression in transformed flies (GOLDBERG et al. 1985). Additional quantitative comparisons between transformed and wild-type flies were made here. Transformants with pH42 insertions were compared with the original RI42 isochromosomal line and with a line in which the RH2 allele was introgressed into the transformation host stock by 10 generations of backcrossing. There was no significant difference in ADH activity levels among the three types of lines. In addition, the pWa-S transormant stocks did not differ significantly from the Wa-S isochromosomal stock.

**Protein assays:** ADH activity was measured by the spectrophotometric method (MARONI 1978) using isopropanol as the substrate. ADH units are nanomoles NAD+ reduced per minute per milligram total protein. Total protein was determined by folin phenol (LOWRY et al. 1951) and by the biocinchonic acid procedure (SMITH et al. 1985). ADH activity is expressed as the ratio of activity units to total protein per fly. ADH protein or cross-reacting material (CRM) was estimated by radial immunodiffusion (MANCEMI et al. 1965); units are the number of standard (Hochi-R inbred) fly equivalents per milligram total protein.

**RNase protection assays:** Total nucleic acids were prepared by minor modifications of the method of FISCHER and MANNLIS (1985). The modifications for adults (sets of 50–100 males aged 7–10 days) and for larvae are described by LAURIE and STAM (1988 and 1994, respectively). RNA transcription, hybridization, digestion, and acrylamide gel analysis were performed according to MELTON et al. (1984) with modifications described in LAURIE and STAM (1994). Radiolabeled RNA fragments were quantified from electrophoretic gels using a Phosphorimagerr and ImageQuant software (Molecular Dynamics, Inc.).

**Sequencing:** Both strands of the RH2 Adh allele were sequenced from a plasmid clone with a series of oligonucleotide primers and standard dideoxy chain termination procedures. This sequence will appear in the GenBank Nucleotide Sequence Database under accession number U20765.

**Measuring the effects of suppressors:** Six lines were produced by combining wild-type, su(f) or su(w°) X chromosomes with the RH2 and WI09 second chromosomes using a CyO/In(2LR)bow balancer stock. The WI09 second chromosome contains a typical Slow Adh allele. The six lines were each crossed to the ADH-null deletion mutant Adh²k² and heterozygous progeny were sampled in two blocks with two replicates per block. Adult males (7–9 days old) were collected to measure ADH activity, ADH protein and ADH RNA. RNase protection assays with the BD probe (Figure 1A) were used to quantitate RNA levels in heterozygotes from each line (as described by LAURIE and STAM 1988). The BD probe spans the fn23 deletion point, which results in protected fragments of different sizes for fn23 RNA compared with wild-type RNA. Heterozygotes produce both sets of protected fragments, which allows quantitation of the amount of wild-type RNA relative to the amount of fn23, which serves as an internal control for comparing different wild-type alleles.

**Statistical analysis:** The data were analyzed using the GLM procedure of the SAS software package (SAS Institute, Inc.). For the transformation experiment, each of the three data sets (males, females and larvae) was analyzed separately using an analysis of variance (ANOVA) model consisting of the following main effects and their interactions: block, transposon type, line nested within transposon type and replicate nested within the block by line interaction term. Transposon type was considered fixed while all other effects were random. The standard error of a mean was calculated using the mean square term used as the denominator in the F test of that

![Figure 2](image-url) — The average ADH activity levels (and their SE) in late third instar larvae and in adults are shown for four transposon types. The number of single insert transformant lines analyzed for each transposon type (n) is given to the left and, to the right, the structure of the Adh fragments contained within each transposon is shown. Units are defined in MATERIALS AND METHODS.

**RESULTS**

The *copia* insertion affects ADH activity level: To test whether the *copia* insertion is the cause of the reduced level of ADH activity observed in the RI42 line, *in vitro* mutagenesis was used to precisely delete the insertion in the cloned *RH2* gene, and the effects were assessed by *p*element transformation. ADH activity was measured in single insert transformants of the two constructs, *pRH2* and *pΔcopia*, which are identical except for the presence or absence of the *copia* element (and its 5-bp insertion site duplication). The results clearly show that the *copia* insertion has a large effect on ADH activity (Figure 2 and Table 1). Least significant difference tests from the analysis of variance show that the *pΔcopia* stocks had significantly more ADH activity than *pRH2* (*P* < 0.00001 for males, females and larvae). The ratio of ADH activity in the *pRH2* to *pΔcopia* lines is 0.57, 0.37 and 0.28 in adult males, females and larvae, respectively. Therefore, the *copia* insertion causes approximately a threefold decrease in level of *Adh* expression.

A solo LTR has no detectable effect in adults but a small effect in larvae: The *pLTR* and *pRH2* constructs both contain insertions at the same position, D-248 bp (upstream from the distal transcription start site), but *pLTR* has only the 276-bp LTR while *pRH2* has the entire 5.2-kb element. For males, females and larvae, the ADH activity in *pLTR* was significantly higher than the *pRH2* stocks (*P* < 0.0001; see Figure 2 and Table 1) showing that the whole *copia* element has a much greater effect than the solo LTR at both stages. In adults, the ADH
TABLE 1

Least significant difference comparisons among the four transformant types for ADH activity

<table>
<thead>
<tr>
<th>Comparisons</th>
<th>Females</th>
<th>Males</th>
<th>Larvae</th>
</tr>
</thead>
<tbody>
<tr>
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<td>&lt;0.00001</td>
<td>&lt;0.00001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>pLTR and pΔcopia</td>
<td>NS</td>
<td>NS</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>pΔcopia and pWa-S</td>
<td>NS</td>
<td>&lt;0.01</td>
<td>&lt;0.04</td>
</tr>
</tbody>
</table>

Least significant difference tests were performed using the least square means and the mean square of single insert stocks within a transformant type. NS, not significant (P > 0.05). activity levels in the pLTR stocks were not significantly different from the pΔcopia stocks, indicating that a solo LTR at this position has no effect. These results indicate that the effect of the copia insertion in adults is not due to disruption of important Adh regulatory sequences at the site of insertion, the creation of novel junction sequences, or the regulatory action of copia sequences contained entirely within the LTR. In larvae, the stocks carrying the pLTR transgene had only 75% of the activity of those carrying the pΔcopia transgene, which is statistically significant (P < 0.02). This result shows that the LTR insertion does have a small effect on larval activity levels, which means that part of the reduction in larval ADH expression could be explained by one of the three mechanisms mentioned above, but that most of the difference between pRH42 and pΔcopia in larvae is due to other mechanisms. The larval results are similar to those obtained for the wΔ copia insertion, in which case a solo LTR has much less of an effect than the entire element, but is not completely wild-type (CARBONARE and GEHRING 1985; ZACHAR et al. 1985; MOUNT et al. 1988).

The copia insertion affects the relative levels of proximal and distal transcripts: The fact that ADH is reduced in both larvae and adults suggests that the copia insertion causes a general reduction in Adh expression, but the proximal and distal promoters might be affected differently. Therefore, the proportion of distal vs. proximal transcripts was examined quantitatively. Late third instar larvae were chosen for this analysis for the following reasons. A promoter switch occurs in mid-third instar, in which distal gradually replace proximal the following reasons. A promoter switch occurs in mid-third instar, in which distal gradually replace proximal us. (PARKHURST and CORCES 1987), so any effects on Adh due to copia transcription should be detectable. Late third instar larvae (wandering stage) are convenient to identify and collect. The proportion of distal vs. proximal RNAs was measured using an RNase protection assay. The DP probe (Figure 1A) protects fragments of different sizes from distal (142 nucleotides) and proximal (175 nucleotides) transcripts as well as a 160-nucleotide fragment common to both transcripts (see Figure 3). The transformation host strain Adh<sup>poe</sup>, which is null for ADH protein, produces low levels of aberrant transcripts (BENVAJATI et al. 1982). In this protection assay, larval jn6 RNA produces small amounts of a fragment that comigrates with the normal proximal fragment, but only trace amounts of a distal-sized fragment and no detectable common fragment. Therefore, the proportion of distal versus proximal RNAs in larvae was estimated as the ratio of the distal to the common fragment amounts. This ratio was estimated from four independent larval RNA samples from each of four or five transformant lines of each type. The results for just one of these 16–20 samples per transformant type are shown in Figure 3. This experiment and the data in Figure 3 were designed for making comparisons among genotypes with respect to distal/proximal ratios and not with respect to absolute amounts of message. The RI42, pRH42 and jn6 lanes contain four times as much total RNA as the other lanes.

Figure 4 shows results for the quantitative analysis of larval samples. In pLTR, pΔcopia and pWa-S transformants, <80% of the ADH-RNA is distal whereas that fraction is only ~45% for pRH42. Analysis of variance showed that there is no significant difference among pLTR, pΔcopia and pWa-S in the distal proportion. (Some variation among lanes representing these genotypes is apparent in Figure 3, but such variation among individual samples has several causes in addition to genotype, such as position effects of different transposon insertions and random variations in developmental timing.) However, the difference between this group of three transposon types and pRH42 is highly significant (P < 0.00001).

The significantly lower fraction of distal RNA in RI42 could be caused by effects on the absolute levels of one or both transcript types. As mentioned previously, this experiment was not designed to make absolute comparisons, but the quantitative data on proportions of distal transcript and total ADH activity level can be combined to obtain a rough estimate of the amounts of proximal and distal RNAs in pRH42 relative to pΔcopia. This estimation assumes that ADH level is proportional to total RNA level regardless of the proximal/distal ratio. This assumption appears to be true in a comparison of RI42 and Wa-S adults for which the degree of reduction in ADH level is the same as the degree of reduction in total RNA even though the proximal/distal ratios differ (LAURIE and STAM 1988 and see below). The pRH42 lines have 0.28 the activity level of pΔcopia, of which 0.55 is due to proximal transcripts, giving a product of 0.15 to be compared with 0.20 for the proximal level in pΔcopia. The corresponding figures for distal RNA are 0.13 for pRH42 vs. 0.80 for pΔcopia. Thus, it appears that the copia insertion has relatively little, if any, effect on the absolute amount of proximal transcript in late third
instar larvae, but it significantly decreases the amount of distal transcript.

The larval results suggested that the proximal/distal ratio in adults might also be affected by the copia insertion. A complete quantitative analysis was not performed in adults, but a small number of samples of each type were analyzed (four replicates each of RI42 and pRH42 and two replicates each of the other types, from just one line per transformant type). Representative results are shown in Figure 3. Approximately 50% of the transcripts in RI42 and pRH42 adults are distal whereas nearly all of the transcripts are distal in the other lines. Although there is clearly a large effect of the copia element on the level of distal transcript in adults, there is insufficient data to determine whether it also affects the level of proximal transcript in adults. Accurate quantitative data would be required for this determination because the proximal transcript occurs at very low levels in adults with or without the copia insertion.

**Possible additional effects of RH42 sequence variants:** Removing the copia element from the RH42 allele showed that copia has a large affect on ADH activity, but the copia insertion may not be the only sequence variant in the RH42 allele that has an affect on ADH activity. The analysis of variance of ADH activity in transformant stocks also revealed a small but significant difference between pΔcopia and pWa-S. This difference is significant for males ($P < 0.01$) and larvae ($P < 0.04$) but not for females, although females show a difference in the same direction. This difference between the pΔcopia and pWa-S stocks could be caused by two differences in transposon construction: the rosy marker gene is downstream of Adh in pWa-S but upstream in pΔcopia and the pWa-S clone has 575

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**FIGURE 4.—The average proportions (and their SE) of proximal and distal transcripts in Adh RNA from late third instar larvae are shown for four transposon types. n, number of single insert transformant lines analyzed for each transposon type.**

**FIGURE 3.—Representative results of an RNase protection assay using the DP probe (Figure 1A). Larval samples are for late (wandering) third instars unless indicated otherwise and adult samples are aged 6–8 days posteclosion. The sample labeled fn6 is from the transformation host strain; RI42 and Wa-S are isochromosomal lines; and those samples beginning with p are transformants. The pRH42, RI42 and fn6 lanes (1, 2, 7, 9, 10 and 15) each contain four times as much total RNA as the other lanes. Fragment sizes (nucleotides) are shown on the right.
fewer bases from the 3' region of the Adh allele. However, neither of these possibilities seems likely, because transgenic flies with both types of construction are similar in ADH level to normal flies with the corresponding Adh allele (see MATERIALS AND METHODS). Therefore, the RH42 Adh allele was sequenced and compared to the Wa-S allele (KREITMAN 1983) to determine what sequence variants the RH42 allele has in addition to the copia insertion (Table 2).

In a 2.0-kb fragment covering the entire transcriptional unit, 25 differences between RH42 and Wa-S were found (Table 2). Among these, 13 were silent substitutions in the coding region. Five sequence variants (all in noncoding regions) were unique to the RH42 allele and not found in other representative slow alleles (KREITMAN 1983; LAURIE et al. 1991 and Table 2). These results indicate that the expression level difference between RH42 (without copia) and Wa is not due to activity or stability of the Adh protein, but is due to some regulatory effect of the noncoding substitutions in Table 2 or perhaps to one or more substitutions in the flanking regions outside of the sequenced fragment.

**Lack of suppressor effects on RH42:** To test for modifying effects of su(f) and su(w*) on the RH42 copia insertion, each suppressor and a wild-type control X chromosome were combined with second chromosomes containing either the copia insertion mutant from RH42 or a typical wild-type slow Adh allele from W109. Each of the six genotypes was analyzed for ADH activity, protein and RNA levels. The results in Figure 5 show that neither suppressor has an effect on Adh expression. ANOVAs of the three RH42 lines show no significant difference among the three genotypes for any variable, which was also the case for the W109 lines. However, a separate ANOVA shows there is a highly significant difference (about fourfold in magnitude) between the sets of RH42 and W109 stocks for all three variables \( P < 0.00001 \), which confirms our previous result that the low level of ADH protein in RH42 is due to a low level of transcripts (LAURIE and STAM 1988).

**DISCUSSION**

The results of the transformation experiment clearly show that the RH42 copia insertion causes a reduction in the level of Adh gene expression and that it has differential effects on the levels of proximal and distal transcripts. The total level of reduction is approximately

<table>
<thead>
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<td>INS' copia element</td>
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<td>1698</td>
<td>INS AAA</td>
<td></td>
</tr>
</tbody>
</table>

*Sequence numbering begins at the distal transcription start site.
*Sequence of the Wa-S allele is from KREITMAN (1983).
*INS, insertion.
*Differences unique to RH42. All others are found in other slow alleles ja-S, Fr-S, AfS, F12-S, and a fast allele ja-F (KREITMAN 1983).
threefold in late third instar larvae and adults, which is primarily due to reduction in the level of distal transcript. There appears to be little, if any, effect on the proximal transcript, but quantitative differences would be difficult to detect because the absolute level of proximal transcript is low at these stages. Further work is needed to establish whether proximal transcription is affected at earlier larval stages when nearly all of the transcripts in wild type are of the proximal type.

Although the precise mechanism by which the R142 insertion causes the reduction in distal transcript level is not understood yet, it is likely that the rate of transcription is affected. Data presented here and by Laurie and Stam (1988) show that the reduction in ADH activity in R142 compared with typical wild-type Slow strains is accounted for by quantitatively similar reductions in steady state levels of ADH protein and ADH RNA. In addition, it is very unlikely that this insertion affects ADH protein structure, translation rate or RNA stability, because the insertion is not contained within the Adh transcriptional unit.

There are at least four possible mechanisms for how the copia insertion could reduce the Adh distal transcription rate: sequence disruption, transcriptional interference, enhancer displacement or enhancer inactivation through boundary regions. As discussed below, present evidence only allows for rejection of the sequence disruption hypothesis as it applies to adult transcription.

As we argued earlier, if the copia element has inserted into a critical regulatory element, then the pLTR transgene, which contains a 276-bp insertion at the same location as the complete copia insertion, should exhibit a significant reduction in Adh expression. However, in adults there is no reduction of ADH level in pLTR compared with pΔcopia transformants, which have the entire element precisely deleted. In larvae, there is a small reduction of pLTR compared with pΔcopia. Thus, we conclude that the insertion does not disrupt a regulatory sequence important for adult transcription but may disrupt a sequence that has a small effect in larvae. These results also indicate that the effect of the complete copia insertion is not due to novel junction sequences or to the regulatory action of copia sequences contained entirely within the LTR.

Transcriptional interference could explain a decrease in Adh transcription rate, because the copia element is upstream of and in the same transcriptional orientation as the Adh gene. Run-through transcripts from either the 5' or 3' LTR in copia could interfere with the downstream Adh distal promoter just as transcription from the distal Adh promoter appears to interfere with transcription from the proximal promoter (Corbin and Maniatis 1989a,b). The pLTR transformants have a solo LTR, which contains a transcription initiation site, yet these flies have essentially wild-type Adh expression. If transcription is initiated from the solo LTR at the same rate as the complete element, the transcriptional interference hypothesis could be rejected. We have no direct evidence concerning transcription initiation from the Adh solo LTR, but this is a distinct possibility, because a partial revertant of w* produces transcripts initiated in its solo LTR at similar abundance to the complete copia in w* (Zachar et al. 1985; Rabionow et al. 1993).

The last two possible mechanisms involve interference with the normal functions of Adh enhancer sequences. Corbin and Maniatis (1989a,b) have defined two enhancer regions, the ALE (Adh Larval Enhancer), which primarily enhances proximal transcription in the larval stages, and the AAE (Adh Adult Enhancer), which
primarily enhances distal transcription in late third instar larvae and adults. The copia element is 5.2 kb long, which introduces a large distance between the promoters and the enhancers, which may prevent normal interactions between them (Figure 1A). Although enhancers typically work at relatively long distances from the promoter, there may be a limit to the distance at which an enhancer can act on a specific promoter (Wasilik et al. 1984). If enhancer displacement is the cause of reduced distal transcription, one might also expect proximal transcription to be affected, because the ALE is displaced even further upstream than the AAE. Again, more work is needed to determine whether proximal transcription is affected in early larval stages.

The copia element may be inactivating the AAE by forming a boundary region similar to that found in another retrotransposon, gypsy (Roseman et al. 1993). In the 
\( y^2 \) mutant, a gypsy has inserted between enhancer elements and the yellow promoter, causing phenotypic changes in the coloration of the wing and body cuticle (Geyer et al. 1986). Further experimentation showed that a 430-bp gypsy sequence containing binding sites for the suppressor of Hairy-wing [su(Hw)] protein is sufficient to cause the mutant effect in a wild-type background, but not in a su(Hw) null background (Geyer and Corces 1992). Thus, when the su(Hw) protein binds to the gypsy element located between the enhancers and promoter, it prevents enhancer function (Smith and Corces 1992; Geyer and Corces 1992). The copia element, like the gypsy element in \( y^2 \), has inserted between the Adh enhancers and promoters. Although copia does not have a su(Hw) binding site based on sequence similarity (Mount and Rubin 1985; Smith and Corces 1992), it may have a similar boundary region involving the binding of other proteins. The boundary mechanism is also expected to affect interactions between the ALE and the proximal promoter.

Quantitative data presented here provide strong evidence that the mutations su(w') and su(f) have no modifying effects on the RI42 copia insertion. This result is in contrast to the report of Strand and McDonald (1989), which suggests that both mutations suppress the effects of this insertion on Adh. The Strand and McDonald study involves the same RI42 strain (supplied by us), but did not provide a quantitative analysis of levels of Adh expression. Furthermore, their results are not easy to explain on the basis of current understanding of functions of su(w') and su(f).

The su(w')- mutation modifies expression of a copia insertion in the second intron of the white locus that causes w' (Zachar et al. 1985). In w', a large fraction of transcripts are initiated properly, but terminate in the 3' LTR of the copia insertion. A small fraction of run-through transcripts are produced and some of these are spliced properly to produce wild-type mRNA, giving a leaky phenotype. The su(w')- mutation leads to an increase in the level of wild-type mRNA. The su(w') gene has been cloned and its wild-type function analyzed (Chou et al. 1987; Zachar et al. 1987). This gene autoregulates its own expression by controlling the splicing of its primary transcript. A functional protein product represses splicing of two introns, leading to the production of apparently nonfunctional mRNAs. It appears likely that the su(w') protein also represses splicing of the w' second intron, and that elimination of that repression through mutation allows more run-through transcripts to be spliced properly to give wild-type white mRNA (Zachar et al. 1987). One would not expect a splicing mutation to affect the expression of the RI42 Adh gene, because the copia insertion in this case is located in the 5' flanking region rather than an intron.

The su(f) gene has also been cloned and its product is homologous with yeast RNA14 (Mitchelson et al. 1993; Minvielle-Sebastia et al. 1994) and with a subunit of human CSF (Takagaki and Manley 1994), both of which have demonstrated roles in mRNA 3' end processing. The su(f') mutation suppresses the f' mutation (a gypsy insertion within an intron) (Parkhurst and Corces 1986), but enhances the w' mutation (Zachar et al. 1985). These are apparently contradictory effects, but Takagaki and Manley (1994) suggest that this could be explained if changes in the levels of su(f) product results in differential usage of poly(A) sites, thereby affecting the fraction of transcripts that terminate prematurely within a transposable element contained within an intron. It is not clear how such a mechanism would apply to the RI42 case.

In conclusion, although the su(w') and su(f) suppressors may have additional, as yet unidentified functions, current understanding of their roles is consistent with our observation that they do not modify the copia mutation in RI42.

Here we have demonstrated a clear phenotypic effect of a transposable element insertion sampled from a natural population. Although this effect has been described in terms of enzymatic activity, it is likely that fitness related characters are also affected in this instance because ADH plays important roles in the detoxification of environmental alcohols and in energy metabolism (David et al. 1976; Geer et al. 1985). Furthermore, variation in ADH levels is frequently associated with variation in alcohol tolerance and/or utilization (Gibson and Cakeshott 1982; Van Delden 1982). Unfortunately, it is difficult to generalize from one specific example like this, but the RI42 case suggests that at least some of the transposable element insertion polymorphisms found so frequently in natural populations of Drosophila may be held in low frequency by their deleterious phenotypic effects.

The RI42 results presented here also contribute to our understanding of the types of DNA sequence polymorphisms that cause the quantitative variation of protein expression found in natural populations. Our studies of variation in ADH levels in D. melanogaster have
revealed that all three major types of sequence variants make important contributions at different levels of gene regulation: a nucleotide substitution causes an amino acid replacement that affects the catalytic efficiency of the ADH molecule (CHAUDHARY and LAURIE 1991), a unique sequence length polymorphism within an intron (V1) affects the steady state level of ADH protein without affecting ADH RNA level (LAURIE and STAM 1994) and this study shows that a transposable element insertion (Ri42) affects ADH expression through altered transcript levels.

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LITERATURE CITED


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