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 RI42) causes the low expression level, the insertion was excised from the cloned RI42 *Adh* gene and the effect was assessed by *P*-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^a)* 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

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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 ($\nabla 1$), as well as other unidentified polymorphisms, affect the ADH protein level (LAURIE and STAM 1994). The $\nabla 1$ 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 (BENYAJATI *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 *yellow*^{2} (GEYER *et al.* 1986), *cut*^{83H} (DORSETT 1993), *forked*^{1} (HOOVER *et al.* 1992), *vermilion*^{k} (FRIDELL *et al.* 1990) and *white*^{apricot} (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*

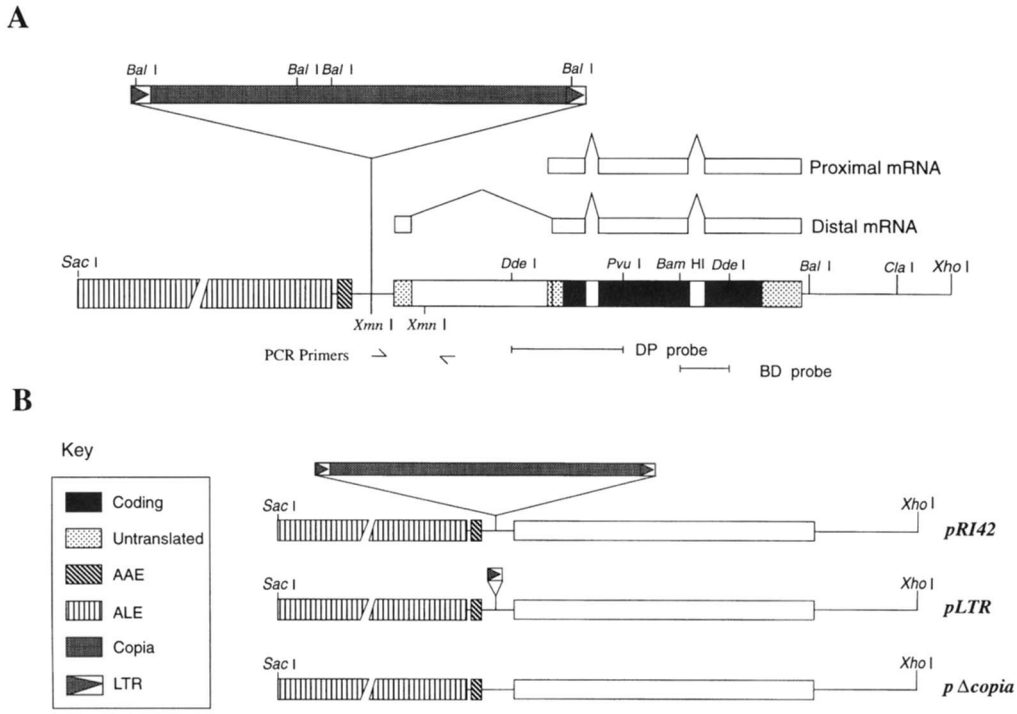

FIGURE 1.—The *Adh* gene. (A) The *Adh* transcriptional unit (1.9 kb in length) lies within a *Sac*I/*Xho*I fragment used in the transformation experiments. The 5′ flanking region of *Adh* contains two enhancer regions, one acting primarily in adults (AAE) and one acting primarily in larvae (ALE). Proximal and distal transcript splicing patterns are shown. The proximal and distal promoters (not shown) are located 32 and 31 bp, respectively, upstream of the transcriptional start sites (BENYAJATI *et al.* 1983). The *copia* element in *RI42* is inserted 243 bp upstream of the start site of the distal transcript, between the enhancers and the promoters. Locations of the DP (a *Dde*I/*Pvu*I fragment) and BD (a *Bam*HI/*Dde*I fragment) probes used in RNase protection assays and the primers used for PCR are shown. (B) Structures of the *Adh* fragments derived from *RI42*. *pRI42* is the unaltered fragment containing a 5.2-kb *copia* element, *pLTR* has all but one LTR of the element removed and *pDcopia* has the entire element precisely deleted.

of white^{apricot} [*su(wa)*] and *suppressor of forked* [*su(f)*]. Previous studies have shown that mutants at these loci modify the effects of the *white*^{apricot} 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 (*pRI42, pLTR* and *pΔcopia)* all have the same basic structure in which a 14.3 kb *Sac*I/*Xho*I fragment of the *RI42* allele (obtained from a λ clone provided by C. F. AQUADRO) was inserted into the vector *pPLΔ1* (LAURIE-AHLBERG and STAM 1987) and then modified by the deletion of *copia* sequences in the case of *pLTR* and *pΔcopia* (Figure 1B). *pRI42* contains the complete 5.2-kb *copia* element, *pLTR* contains just one 276-bp LTR and *pΔcopia* contains no *copia* sequence. The *pWa-S* construct (from LAURIE-AHLBERG and STAM 1987) is similar except that it has an 8.0-kb *Sac*I/*Cla*I fragment containing an *Adh* allele originating from the *Wa-S* wild-type allele of KREITMAN (1983), which has an *Adh* expression level typical of Slow alleles (LAURIE *et al.* 1991). All four constructs have an 8.1-kb *Sal*I fragment containing a wild-type *rosy* gene inserted at the *Xho*I site.

The *pLTR* construct was made by ligating an 11.3-kb *Sac*I/*Bam*HI fragment from the *RI42 Adh* allele into a pBS(−) vector (Stratagene, Inc.). This construct was digested with *Bal*I, which removed all but the 276-bp LTR from the *copia* element, leaving the rest of the fragment intact. The new 6.3-kb *Sac*I/*Bam*HI fragment was religated into the original vector creating a 9.5-kb *Sac*I/*Xho*I fragment with just the LTR at the *copia* insertion site.

To construct *pΔcopia, pLTR* was partially digested with *Xmn*I, 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 *Xmn*I site and the 3′ primer was 413 bp downstream of an *Xmn*I site (Figure 1A). The PCR product was digested with *Xmn*I and religated back into the *Xmn*I 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 (CHOUDHARY and LAURIE 1991). Transformant stocks were produced by microinjection of embryos (from the ADH-null host stock *Adh*^{fn6}*cn;ry*^{506}) and chromosome extraction. In addition, two stocks of the *pRI42* transformant type were created by mobilizing a *pRI42* 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.* 1983). Additional quantitative comparisons between transformed and wild-type flies were made here. Transformants with *pRI42* insertions were compared with the original RI42 isochromosomal line and with a line in which the *RI42* 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* transformant 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 bicinchoninic 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 (MANCINI *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 MANIATIS (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 Phosphorimager and ImageQuant software (Molecular Dynamics, Inc.).

Sequencing: Both strands of the *RI42 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^a)* *X* chromosomes with the RI42 and WI09 second chromosomes using a *CyO/In(2LR)bw^V* balancer stock. The WI09 second chromosome contains a typical Slow *Adh* allele. The six lines were each crossed to the ADH-null deletion mutant *Adh^{fn23}* 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 mean effect. Least significant difference tests were used to compare the means of the different transformant types. The mean square error term for line within transposon type from the ANOVA was used as the error term in these tests.

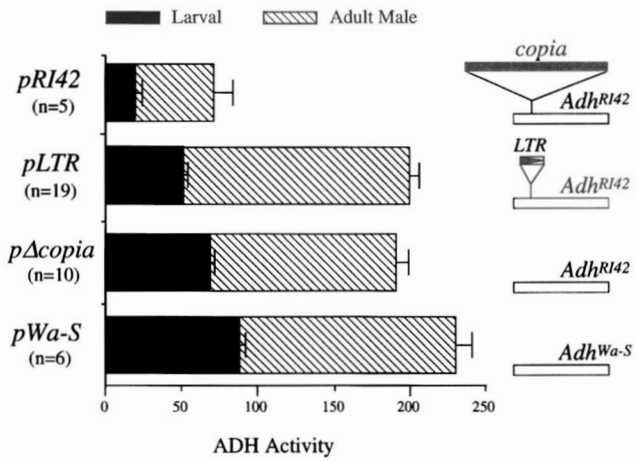

FIGURE 2.—The average ADH activity levels (and their SE) in late third instar larvae and in adults are shown for four transposon types. The number of single insert transformant lines analyzed for each transposon type (*n*) is given to the left and, to the right, the structure of the *Adh* fragments contained 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 *RI42* gene, and the effects were assessed by *P*-element transformation. ADH activity was measured in single insert transformants of the two constructs, *pRI42* 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 *pRI42* ($P < 0.00001$ for males, females and larvae). The ratio of ADH activity in the *pRI42* to *pΔcopia* lines is 0.37, 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 *pRI42* 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 *pRI42* has the entire 5.2-kb element. For males, females and larvae, the ADH activity in *pLTR* was significantly higher than the *pRI42* 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

Comparisons	Probability		
	Females	Males	Larvae
pRI42 and *pLTR*	<0.00001	<0.00001	<0.0001
pLTR and *pΔcopia*	NS	NS	<0.02
pΔcopia and *pWa-S*	NS	<0.01	<0.04

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 *pRI42* and *pΔcopia* in larvae is due to other mechanisms. The larval results are similar to those obtained for the w^a *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 transcripts (BENYAJATI *et al.* 1983); both transcripts are detectable in late third instar larvae. *Copia* transcripts are very abundant in the third instar (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^{fn6}, which is null for ADH protein, produces low levels of aberrant transcripts (BENYAJATI *et al.* 1982). In this protection assay, larval *fn6* 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, *pRI42* and *fn6* 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 *pRI42*. 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 *pRI42* 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 *pRI42* 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 *pRI42* 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 *pRI42 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

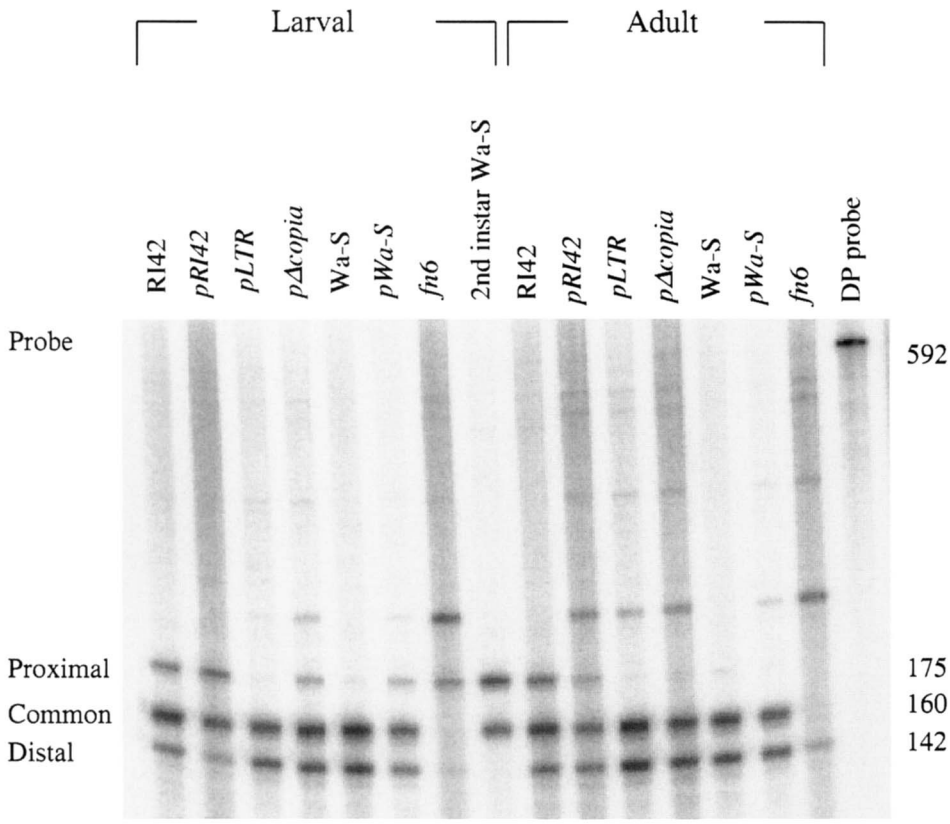

FIGURE 3.—Representative results of an RNase protection assay using the DP probe (Figure 1A). Larval samples are for late (wandering) third instars unless indicated otherwise and adult samples are aged 6–8 days posteclosion. The sample labeled *fn6* is from the transformation host strain; RI42 and Wa-S are isochromosomal lines; and those samples beginning with p are transformants. The *pRI42*, 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.

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 *pRI42* 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 *pRI42* 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.

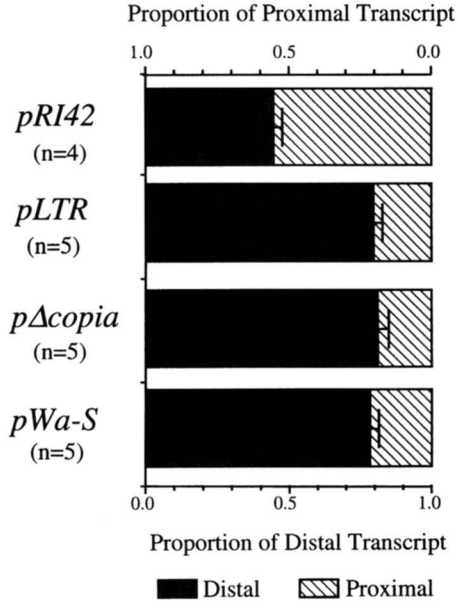

FIGURE 4.—The average proportions (and their SE) of proximal and distal transcripts in *Adh* RNA from late third instar larvae are shown for four transposon types. *n*, number of single insert transformant lines analyzed for each transposon type.

Possible additional effects of *RI42* sequence variants: Removing the copia element from the *RI42* allele showed that *copia* has a large affect on ADH activity, but the *copia* insertion may not be the only sequence variant in the *RI42* 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

TABLE 2

Sequence comparison between the *RI42* and *Wa-S Adh* alleles

Location within *Adh*	Position[a]	*Wa-S*[b]	*RI42*
5′ regulatory region	−243		INS[c] *copia* element[d]
	−231	T	A[d]
	−228	T	A[d]
	−53		INS CT[d]
Adult Intron	107	C	A
	113	A	G
	143	A	G
	169	T	G
	173	A	G
	183	TATT	ATAGTAAAATTGAA[d]
	287	T	G
Exon 2	1068	T	C
	1229	T	C
	1283	A	C
Intron 2	1354	C	G
	1362	A	G
	1400	T	A
	1405	A	T
Exon 3	1425	A	C
	1431	C	T
	1452	C	T
	1518	C	T
	1527	T	C
	1557	A	C
3′ untranslated region	1693	A	C
	1698		INS AAA

[a] Sequence numbering begins at the distal transcription start site.
[b] Sequence of the Wa-S allele is from KREITMAN (1983).
[c] INS, insertion.
[d] Differences unique to *RI42*. All others are found in other slow alleles *Ja-S, Fr-S, Af-S, FI2-S,* and a fast allele *Ja-F* (KREITMAN 1983).

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 *RI42 Adh* allele was sequenced and compared to the *Wa-S* allele (KREITMAN 1983) to determine what sequence variants the *RI42* allele has in addition to the *copia* insertion (Table 2).

In a 2.0-kb fragment covering the entire transcriptional unit, 25 differences between *RI42* 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 *RI42* 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 *RI42* (without *copia*) and *Was* 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 *RI42*: To test for modifying effects of *su(f)* and *su(w^a)* on the *RI42 copia* insertion, each suppressor and a wild-type control *X* chromosome were combined with second chromosomes containing either the *copia* insertion mutant from RI42 or a typical wild-type Slow *Adh* allele from WI09. 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 RI42 lines show no significant difference among the three genotypes for any variable, which was also the case for the WI09 lines. However, a separate ANOVA shows there is a highly significant difference (about fourfold in magnitude) between the sets of RI42 and WI09 stocks for all three variables ($P < 0.00001$), which confirms our previous result that the low level of ADH protein in RI42 is due to a low level of transcripts (LAURIE and STAM 1988).

DISCUSSION

The results of the transformation experiment clearly show that the RI42 *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

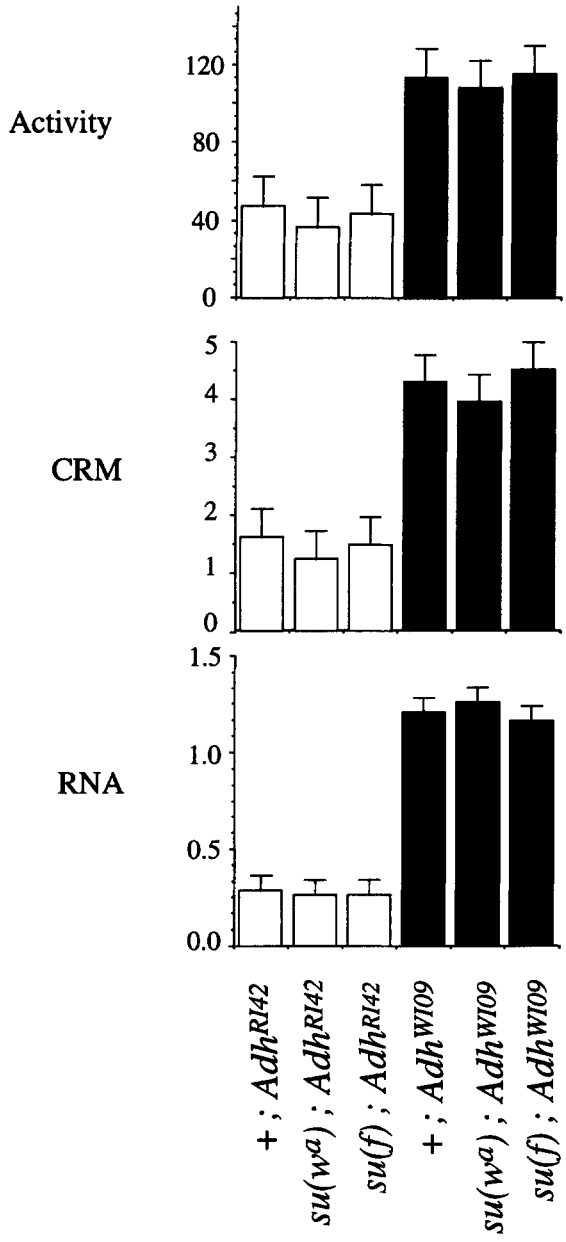

FIGURE 5.—The average ADH activity, CRM and RNA levels (and their SE) are given for six genotypes that differ in their X and second chromosomes. Units are defined in MATERIALS AND METHODS.

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 estabish 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 RI42 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 RI42 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^a produces transcripts initiated in its solo LTR at similar abundance to the complete *copia* in w^a (ZACHAR *et al.* 1985; RABINOW *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 (WASYLYK *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^a)* 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^a)* and *su(f)*.

The *su(w^a)$^-$* mutation modifies expression of a *copia* insertion in the second intron of the *white* locus that causes w^a (ZACHAR *et al.* 1985). In w^a, 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^a)$^-$* mutation leads to an increase in the level of wild-type mRNA. The *su(w^a)* 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^a)* protein also represses splicing of the w^a 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 CstF (TAKAGAKI and MANLEY 1994), both of which have demonstrated roles in mRNA 3′ end processing. The *su(f)$^-$* mutation suppresses the f^1 mutation (a *gypsy* insertion within an intron) (PARKHURST and CORCES 1986), but enhances the w^a 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^a)* 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 OAKESHOTT 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 (CHOUDHARY and LAURIE 1991), a unique sequence length polymorphism within an intron (∇1) 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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